GDAŃSK UNIVERSITY OF TECHNOLOGY

The author of the doctoral dissertation: Antoni Taraszkiewicz

Scientific discipline: Chemical sciences

DOCTORAL DISSERTATION

Title of doctoral dissertation: Characterization of bioactive peptides from chicken feather keratin and products of their transformations occurring during the Maillard reaction

Title of doctoral dissertation (in Polish): Charakterystyka bioaktywnych peptydów z keratyny z piór drobiowych oraz produktów ich przemian zachodzących w trakcie reakcji Maillarda

Supervisor
Hanna Staroszczyk, PhD, DSc, Associate Professor

Gdańsk, 2025

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DESCRIPTION OF DOCTORAL DISSERTATION

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Language of doctoral dissertation: English

Supervisor: Hanna Staroszczyk, PhD, DSc, Associate Professor

Date of doctoral defence: ……

Keywords of doctoral dissertation in English: bioactive peptides; biological activity; chicken feathers; keratin hydrolysate; keratin isolate; Maillard reaction; physicochemical properties

Keywords of doctoral dissertation in Polish: aktywność biologiczna; bioaktywne peptydy; hydrolizat keratyny; izolat keratyny; pióra kurze; reakcja Maillarda; właściwości fizykochemiczne

Summary of doctoral dissertation in English

The dissertation focuses on the valorisation of chicken feathers into peptides for food applications, combining *in silico* peptide release prediction with experimental analysis of keratin composition, structure, thermal stability, antioxidant activity and nutritional safety, pre- and post-processing. The prediction showed keratin as a source of dipeptidyl peptidase IV, angiotensin-converting enzyme and prolyl oligopeptidase inhibitory, and antioxidant peptides, among others. The experimental analysis revealed that L-Cys keratin extraction decreases the crystallinity and α-helix content of native protein in favour of β-sheet, resulting in its slower but more gradual thermal decomposition. The isolate obtained showed high purity, good water solubility, retained backbone and favourable gelling, oil-binding and foaming. Isolate hydrolysis with trypsin, chymotrypsin and pepsin only slightly modified protein secondary structure but altered techno-functional properties in a degree of hydrolysis- and pH-dependent way. Subtilisin hydrolysate showed the greatest changes in protein structure, resulting in the best solubility, antiradical, Fe^{2+} chelating and reducing properties. The glycation products of this hydrolysate with Xyl had enhanced antioxidant activity post simulated gastrointestinal digestion. Neither processing nor digestion of keratin induced cytotoxic or mutagenic effects.

Summary of doctoral dissertation in Polish

Rozprawa dotyczy waloryzacji piór kurzych do peptydów o zastosowaniach spożywczych, łącząc symulację *in silico* uwalniania peptydów z doświadczalną analizą składu, struktury, stabilności termicznej, aktywności przeciwutleniającej i bezpieczeństwa żywieniowego keratyny przed i po przetworzeniu. Symulacja wykazała, że keratyna jest m.in. źródłem inhibitorów dipeptydylopeptydazy IV, konwertazy angiotensyny i oligopeptydazy prolinowej oraz peptydów przeciwutleniających. Doświadczenia wykazały, że ekstrakcja keratyny z L-Cys obniża krystaliczność i zawartość α-helis natywnego białka na rzecz β-kartek, co skutkuje jego wolniejszym, stopniowym rozkładem termicznym. Otrzymany izolat miał dużą czystość i rozpuszczalność w wodzie, zachowany szkielet i korzystne właściwości żelujące, wiążące olej i pianotwórcze. Hydroliza izolatu trypsyną, chymotrypsyną i pepsyną nieznacznie zmodyfikowała II-rzędową strukturę białka, ale zmieniła właściwości technologiczne w stopniu zależnym od stopnia hydrolizy i pH. Hydrolizat subtylizynowy wykazał największe zmiany w strukturze białka, co skutkowało najlepszą rozpuszczalnością i aktywnością przeciwrodnikową, chelatacji Fe^{2+} oraz redukcyjną. Produkty glikacji tego hydrolizatu z Xyl miały zwiększoną aktywność przeciwutleniającą po symulowanym trawieniu żołądkowo-jelitowym. Przetwarzanie i trawienie keratyny nie wywołały cytotoksyczności ani mutagenności.

Doctoral dissertation carried out within the framework of the project “Characterization of bioactive peptides from chicken feather keratin and products of their transformations occurring during the Maillard reaction” (project number DEC-2021/41/N/NZ9/04466) funded by the National Science Centre, Poland through the Preludium 20 grant programme.

Key words: bioactive peptides, chicken feather keratin, Maillard reaction products, metabolic syndrome

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List of papers constituting the doctoral dissertation

Original papers

A1. **Taraszkiewicz, A.**, Sinkiewicz, I., Sommer, A., Dąbrowska, M., & Staroszczyk, H. (2022). Prediction of bioactive peptides from chicken feather and pig hair keratins using *in silico* analysis based on fragmentomic approach. *Current Pharmaceutical Design*, 28(10), 841–851. https://doi.org/10.2174/1381612828999220114150201

A2. **Taraszkiewicz, A.**, Sinkiewicz, I., Sommer, A., Kusznierewicz, B., Giblin, L., & Staroszczyk, H. (2025). Chemical composition and techno-functional properties of high-purity water-soluble keratein and its enzymatic hydrolysates. *Food Chemistry*, 472, 142641. https://doi.org/10.1016/j.foodchem.2024.142641

A3. Staroszczyk, H., Sommer, A., **Taraszkiewicz, A.**, & Michalec M. Changes in the structure and thermal properties of feather keratin induced by L-Cys extraction followed by enzymatic hydrolysis. https://doi.org/10.2139/ssrn.5382858 *(under review)*

A4. **Taraszkiewicz, A.**, Sommer, A., Sinkiewicz, I., Koss-Mikołajczyk, I., Kusznierewicz, B., Giblin, L., & Staroszczyk, H. (2025). Valorising feather keratin: Antioxidant activity, cytotoxicity and mutagenicity during processing and gastrointestinal digestion. *Food Chemistry*, *493P2*, 145820. https://doi.org/10.1016/j.foodchem.2025.145820

Review paper

A5. **Taraszkiewicz, A.,** Sinkiewicz, I., Sommer, A., & Staroszczyk, H. (2024). The biological role of prolyl oligopeptidase and the procognitive potential of its peptidic inhibitors from food proteins. *Critical Reviews in Food Science and Nutrition*, 64(19), 6567–6580. https://doi.org/10.1080/10408398.2023.2170973

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List of abbreviations

ABTS – 2,2′-**a**zino-**b**is(3-ethylbenzo**t**hiazoline-6-**s**ulfonic acid)
ACE – **a**ngiotensin-**c**onverting **e**nzyme
ACE2 – **a**ngiotensin-**c**onverting **e**nzyme **2**
AChE – **a**cetyl**ch**olin**e**sterase
AngI – **ang**iotensin **I**
AngII – **ang**iotensin **II**
ATR FT-IR – **a**ttenuated **t**otal **r**eflectance **F**ourier **t**ransform **i**nfra**r**ed
BDNF – **b**rain-**d**erived **n**eurotrophic **f**actor
CrI – **cr**ystallinity **i**ndex
DG – **d**egree of **g**lycation
DH – **d**egree of **h**ydrolysis
DPP-IV – **dip**eptidyl **p**eptidase **IV**
DSC – **d**ifferential **s**canning **c**alorimetry
DTG – **d**erivative **t**hermo**g**ravimetry
E/S ratio – **e**nzyme/**s**ubstrate ratio
EE – **e**thylenediaminetetraacetic acid **e**quivalent
FAO – **F**ood and **A**griculture **O**rganization of the United Nations
FCR – **F**olin-**C**iocâlteu **r**eagent
GAE – **g**allic **a**cid **e**quivalent
GLP-1 – **g**lucagon-**l**ike **p**eptide **1**
GRAS – **G**enerally **R**ecognized **a**s **S**afe
HAT – **h**ydrogen **a**tom **t**ransfer
HP-SEC – **h**igh **p**erformance **s**ize **e**xclusion **c**hromatography
IC_{50} – half-maximal **i**nhibitory **c**oncentration
KI – **k**eratin **i**solate
KI-C – **k**eratin **i**solate hydrolysate by **c**hymotrypsin
KI-P – **k**eratin **i**solate hydrolysate by **p**epsin
KI-S – **k**eratin **i**solate hydrolysate by **s**ubtilisin
KI-S-G – mixture of **k**eratin **i**solate hydrolysate by **s**ubtilisin and **G**lc *(see Table 1, **A4**)*
KI-S-X – mixture of **k**eratin **i**solate hydrolysate by **s**ubtilisin and **X**yl *(see Table 1, **A4**)*
KI-T – **k**eratin **i**solate hydrolysate by **t**rypsin
MasR – **Mas r**eceptor
MRPs – **M**aillard **r**eaction **p**roducts
MTT – 3-(4,5-di**m**ethyl**t**hiazol-2-yl)-2,5-diphenyl**t**etrazolium bromide
MW – **m**olecular **w**eight
OPA – **o**-**p**hthaldi**a**ldehyde
POP – **p**rolyl **o**ligo**p**eptidase
RAS – **r**enin-**a**ngiotensin **s**ystem
SET – **s**ingle **e**lectron **t**ransfer
SGID – **s**imulated **g**astro**i**ntestinal **d**igestion
TE – **T**rolox **e**quivalent
TGA – **t**hermo**g**ravimetric **a**nalysis
XRD – **X**-**r**ay **d**iffraction

THEORETICAL PART

1. Bioactive peptides

1.1. Introduction

Peptides are a highly diverse class of chemical compounds whose unique physicochemical properties and numerous biological activities render them highly attractive for food, pharmaceutical and biotechnological applications. The medical and nutritional relevance of peptides first came to light with the introduction of insulin in 1922, marking the dawn of peptide-based therapeutics and initiating decades of research and development in this field (Xiao et al., 2025). Currently, hundreds of peptide-based drugs are being evaluated in early-phase clinical trials, 38 have reached phase III, and nearly 100 have already been approved. The surge in scientific and clinical research has been mirrored by impressive commercial performance, with the global peptide market growing from $41.44 billion in 2023 to $45.66 billion in 2024 and projected to reach $68.83 billion by 2028 (Xiao et al., 2025). In light of peptides' remarkable potential and the momentum of current research, the present era may be regarded as "The Age of Peptides" (Cabral, 2021).

In recent decades, increasing attention has been paid to a specific group of peptides, i.e., those derived from food proteins. Although the conventional assessment of food proteins' nutritional value has focused mainly on their capacity to provide amino acids, necessary for cellular growth and metabolic functions, it is now widely acknowledged that the understanding of the proteins' biological quality is incomplete without considering their potential to serve as precursors of bioactive peptides (Singh et al., 2023; Sun et al., 2024). These so-called "encrypted" peptides can become bioactive after their release from precursor proteins, which is achieved through enzymatic, microbial or chemical hydrolysis (Iwaniak et al., 2018). Typically 2-30 amino acid long, such peptides not only serve as nutrients but also display medically relevant bioactivities similar to those of active pharmaceutical ingredients, such as antidiabetic, antihypertensive, anti-inflammatory, antimicrobial, antioxidant, immunomodulatory or neuroprotective. Although synthetic drugs tend to be more potent, peptides released from food proteins are usually better tolerated and rarely cause adverse effects (Hayes, 2021; Iwaniak et al., 2018; Sun et al., 2024). The earliest reported example of food protein-derived bioactive peptides was phosphopeptides originating from casein, which facilitated bone mineralization in infants independently of vitamin D (Mellander, 1950). Since then, a very broad spectrum of bioactive peptides has been identified. BIOPEP-UWM database of bioactive peptides of food origin contains data regarding a total of 5,456 sequences with experimentally confirmed bioactivities (accession date: 22.09.2025) (Iwaniak et al., 2024).

1.2. Selected activities

1.2.1. Antihypertensive activity

Hypertension is one of the leading risk factors for cardiovascular diseases, with over 1.28 billion people affected worldwide (Jiang et al., 2025). Its pathophysiology is largely governed by the renin-angiotensin system (RAS), a hormonal cascade that controls blood pressure and fluid homeostasis (Figure 1). The RAS begins with the conversion of angiotensinogen to angiotensin I (AngI) by renin, followed by angiotensin-converting enzyme (ACE)-mediated transformation of AngI into angiotensin II (AngII), a potent vasoconstrictor that exerts hypertensive effects via AngII type 1 receptors (AT1R). A counter-regulatory mechanism involves angiotensin-converting enzyme 2 (ACE2), which converts AngII into Ang(1-7), a heptapeptide that activates Mas receptors (MasR), promoting vasodilation. Disruption of the balance in favour of the ACE/AngII/AT1R axis leads to chronic elevation of blood pressure (Jiang et al., 2025; Yao et al., 2025). The suppression of key enzymatic and receptor-mediated mechanisms within the RAS is one of the most common pharmacological strategies in the management of clinical hypertension. However, the synthetic antihypertensive agents, although effective, are frequently associated with adverse side effects such as dry cough, dizziness, palpitations and impaired renal function (Yao et al., 2025).

Most of the identified antihypertensive peptides act through the inhibition of ACE, and the peptidic ACE inhibitors are the most extensively characterized class of food-derived bioactive peptides. Structure-activity relationship studies indicate that low MW (typically < 2.5 kDa) and high hydrophobicity favour ACE inhibition, as small hydrophobic peptides more effectively interact with the enzyme's active site (Xiang et al., 2023). Higher ACE inhibitory activity is typically observed in peptides with specific C-terminal motifs – especially when the penultimate residue is Val, Ile, Ala, Pro, Trp or Phe, and the terminal residue is Tyr, Trp, Phe, Arg, Leu, Ile, Ala or Met (Yao et al., 2025). Pro holds particular importance, as it not only contributes to strong ACE-peptide binding affinity but also enhances peptides' bioavailability by increasing resistance to proteolytic degradation in the gastrointestinal tract (Okagu et al., 2022; Xiang et al., 2023). Additionally, N-terminal Gly, Ala, Ile, Leu and Val enhance the interaction with ACE, with Leu occurring most commonly in highly active sequences (Yao et al., 2025). Among the food-originating ACE inhibitors, the casein-derived lactotripeptides (Leu-Pro-Pro, Val-Pro-Pro and Ile-Pro-Pro) are one of the most promising and well-studied examples, with respective half-maximal inhibitory concentration (IC_{50}) values of 10, 9 and 5 μM (Maruyama et al., 1989; Nakamura et al., 1995). Their clinically confirmed antihypertensive efficacy has led to their use as active ingredients in functional beverages, including Calpis® (Japan), Evolus® and Valio® (Finland) (Iwaniak et al., 2018).

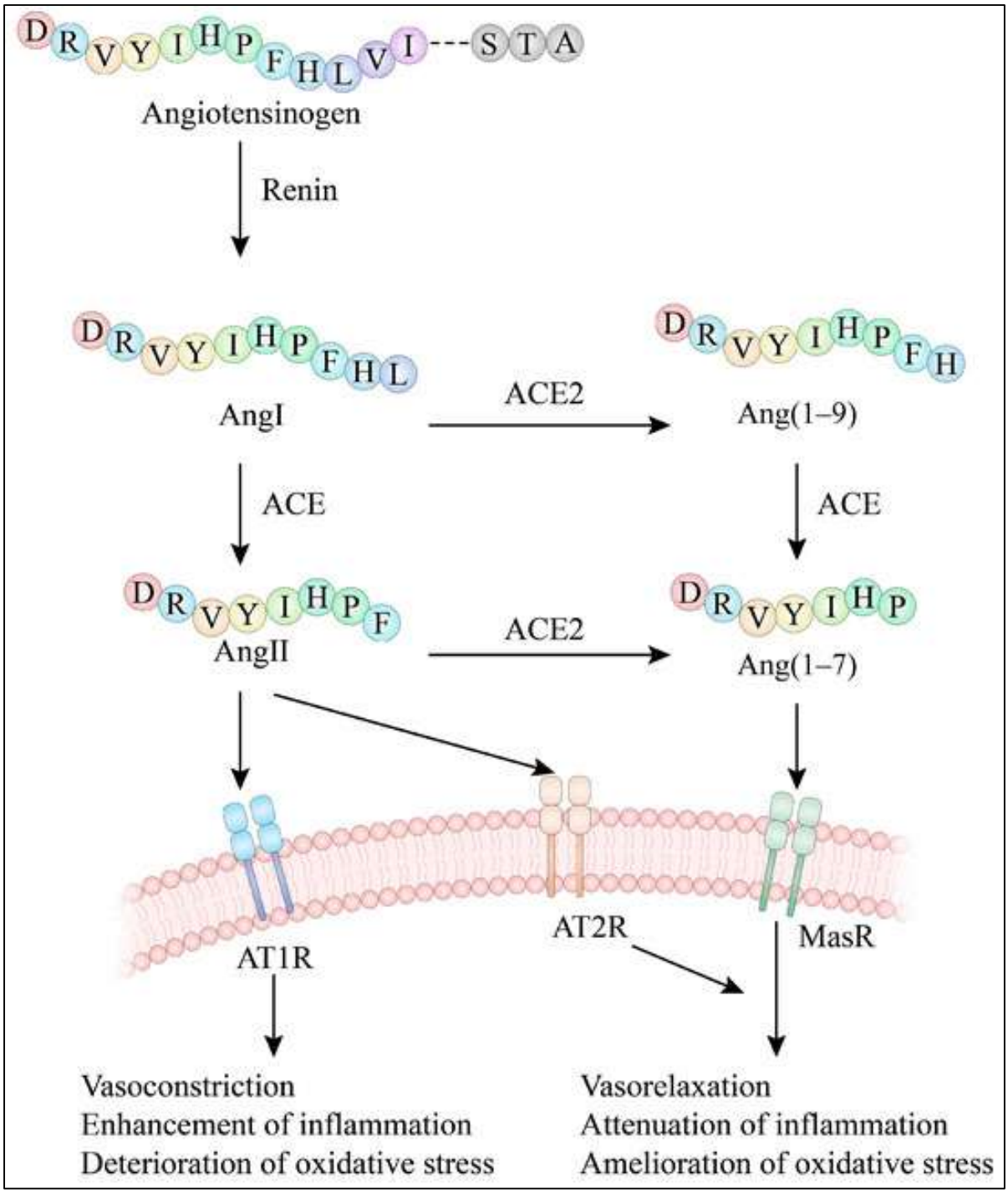

Figure 1. The renin-angiotensin system (RAS) with opposing regulatory effects of ACE/AngII/AT1R (left side) and ACE2/Ang(1-7)/MasR (right side) pathways. AngI – angiotensin I, ACE – angiotensin I-converting enzyme, ACE2 – angiotensin-converting enzyme 2, AngII – angiotensin II, AT1R – angiotensin II type 1 receptor, AT2R – angiotensin II type 2 receptor, MasR – Mas receptor. Reproduced from Yao et al. (2025), CC BY 4.0.

Although ACE inhibition remains the primary antihypertensive mechanism, it may lead to AngI accumulation and AngII formation through ACE-independent pathways. Consequently, dual inhibition of both ACE and renin is considered a more effective antihypertensive treatment. Renin-inhibitory peptides typically exhibit low MW, hydrophobic N-terminal residues, and bulky C-terminal residues that favour enzyme binding (Yao et al., 2025). Moreover, recent evidence suggests that certain peptides exert hypotensive effects via RAS-independent mechanisms, including stimulation of endothelial nitric oxide synthase, blockade of L-type calcium channels, and activation of cyclooxygenase and prostaglandin pathways (Okagu et al., 2022).

1.2.2. Antidiabetic activity

Diabetes affects more than 828 million people globally and remains a leading contributor to mortality (Zhou et al., 2024). The mechanism of action of bioactive peptides in regulating blood glucose levels involves primarily inhibition of dipeptidyl peptidase IV (DPP-IV) (Figure 2), α-amylase and α-glucosidase (Figure 3) (Acquah et al., 2022). DPP-IV is a plasmatic membrane enzyme responsible for the inactivation of incretin hormones, i.e., glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic peptide, that lower plasma glucose levels and promote the growth of insulin-producing pancreatic β cells. As a result, DPP-IV inhibition prolongs incretins' antihyperglycemic effect (Iwaniak et al., 2018). α-Amylase, produced by salivary glands and the pancreas, catalyses the hydrolysis of α-(1→4)-glycosidic bonds of polysaccharides like starch and glycogen into oligosaccharides, which are subsequently hydrolysed by α-glucosidase, secreted in the brush border enterocytes of the small intestine, into absorbable monosaccharides. The inhibition of both enzymes delays saccharides' digestion, reducing the rate of glucose absorption and moderating postprandial blood glucose and insulin levels (Iwaniak et al., 2018). Although synthetic antidiabetic agents, including DPP-IV and α-glucosidase inhibitors, are widely used in clinical practice, they may cause side effects such as gastrointestinal disturbances, infections, skin reactions and increased risk of pancreatitis (Acquah et al., 2022; Iwaniak et al., 2018).

The inhibitory effect on DPP-IV has so far received the greatest research focus in the context of antidiabetic activities of peptides (Iwaniak et al., 2018; Minkiewicz et al., 2019). The most potent DPP-IV inhibitors are typically low MW peptides (below 1 kDa), rich in hydrophobic residues at the N-terminus, particularly Trp, and containing Pro or Ala in the penultimate position (Kadam et al., 2024; Rivero-Pino et al., 2020). A well-documented example of a potent DPP-IV inhibitory peptide is Ile-Pro-Ile (Diprotin A) with an IC_{50} of 5.0 μM (Kadam et al., 2024). Peptides inhibiting α-amylase are usually short (below 3 kDa), with sequences between 3 and 23 amino acid residues, and contain branched-chain or cationic amino acids that facilitate binding to the enzyme's active site (Iwaniak et al., 2018; Kadam et al., 2024; Yap & Gan, 2020).

In the case of α-glucosidase inhibitors, sequence characteristics include hydroxyl- or basic-side-chain residues at the N-terminal end, Pro within the sequence, and small aliphatic residues, such as Ala, Met or Leu, at the C-terminal. Representative of strong α-glucosidase inhibitory motifs are Tyr-Pro-Leu and Ser-Thr-Tyr-Val (Iwaniak et al., 2018; Kadam et al., 2024).

Beyond enzyme inhibition, some antidiabetic peptides display multifunctional biological effects, including antioxidant, anti-inflammatory and appetite-regulating activities. For example, some peptides act as GLP-1 receptor agonists, thereby mimicking endogenous incretin hormones to promote insulin secretion in a glucose-dependent manner (Acquah et al., 2022). Such multifunctionality may contribute to overall metabolic benefits and disease prevention, extending beyond glycaemic control (Yap & Gan, 2020). However, relatively few peptides with antidiabetic potential have been verified *in vivo*. Examples include peptides derived from salmon and tilapia skin collagen, systematic supplementation of which reduced blood glucose levels and increased secretion of incretin hormones and insulin in diabetic rats (Liu et al., 2019). The antidiabetic efficacy of peptides derived from salmon proteins was also confirmed in a clinical trial, although only in patients without insulin resistance (Takahashi et al., 2021).

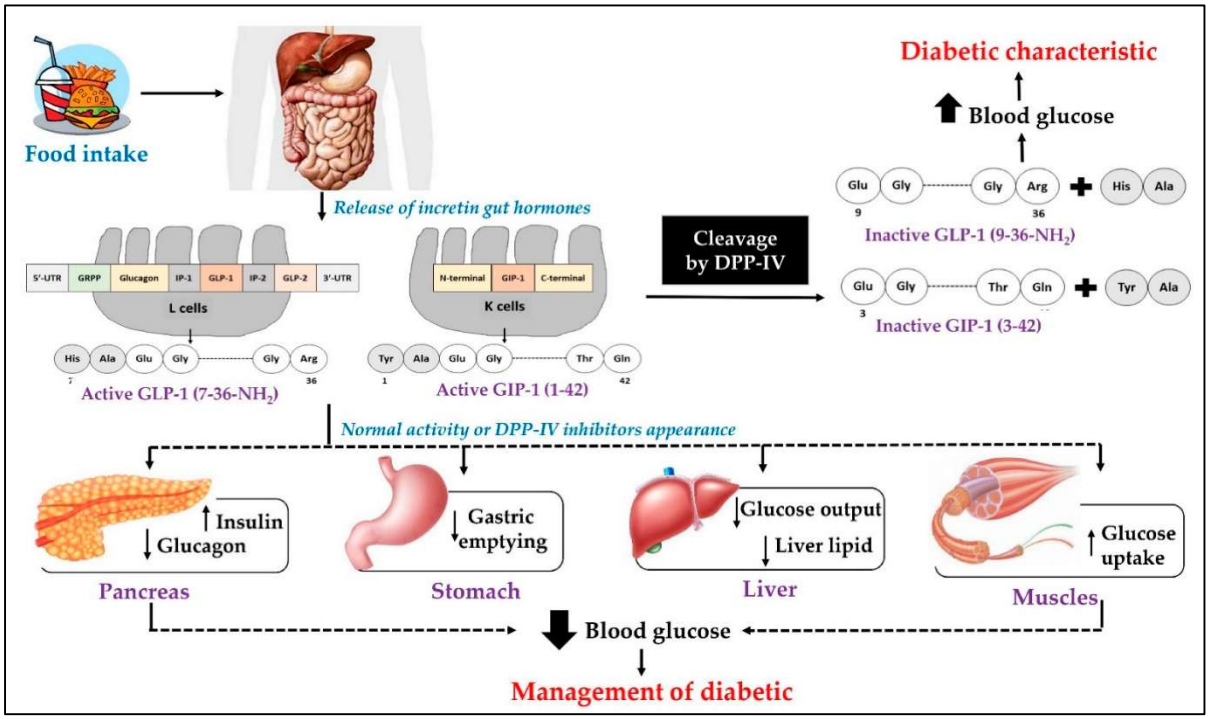

Figure 2. Schematic representation of the antidiabetic mechanism of dipeptidyl peptidase IV (DPP-IV) inhibitors. GIP – glucose-dependent insulinotropic polypeptide, GLP-1 – glucagon-like peptide 1. Reproduced from Nong & Hsu (2021), CC BY 4.0.

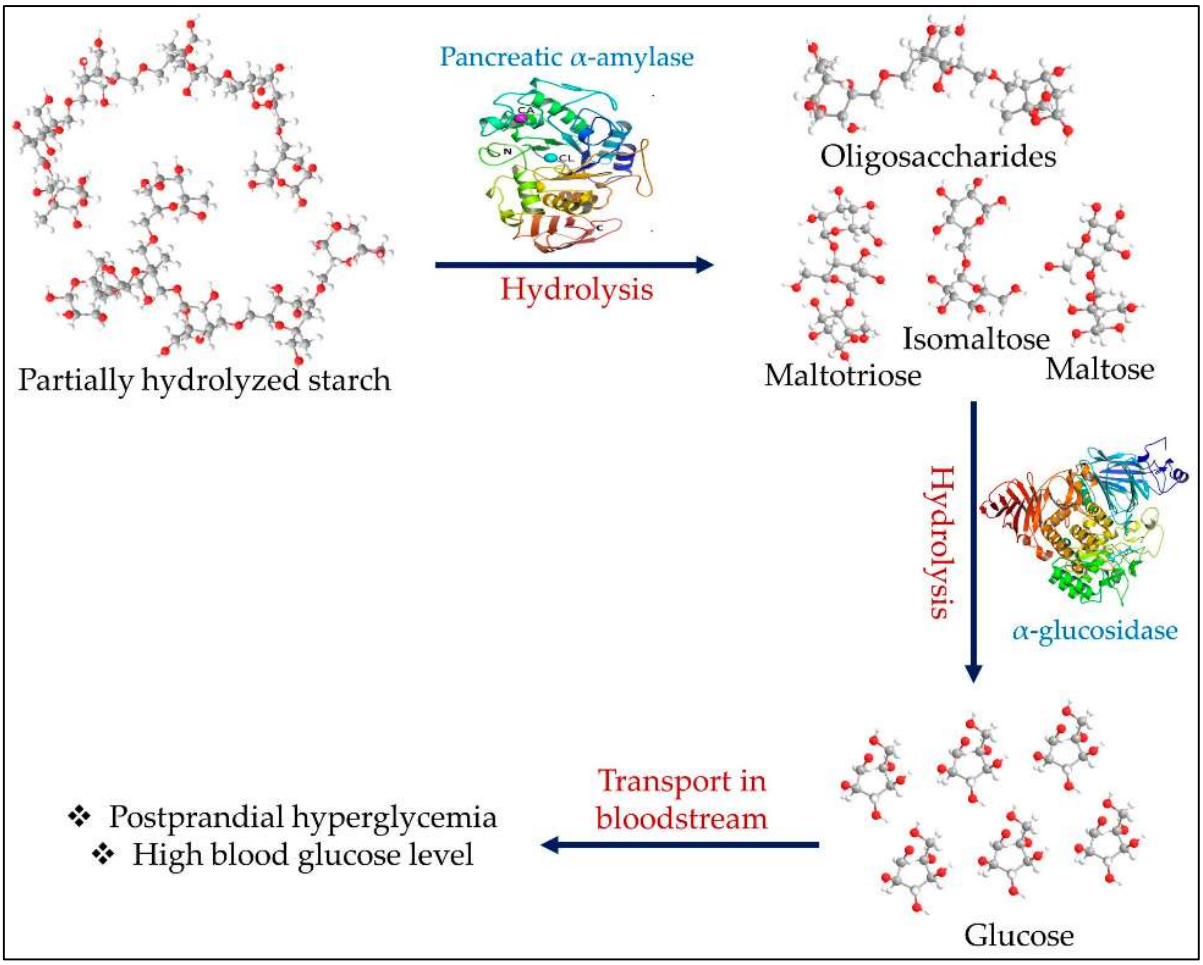

Figure 3. Scheme of pancreatic α-amylase and α-glucosidase action during starch digestion and absorption. Reproduced from Kashtoh & Baek (2023), CC BY 4.0.

1.2.3. Antioxidant activity

Oxidative stress arises when the balance between reactive oxygen and nitrogen species production and the body's antioxidant defence mechanisms is disrupted, favouring oxidative processes. This results in damage to important biomolecules (e.g. DNA, proteins and lipids) and is linked to the onset and progression of various diseases, including cancer, diabetes, hypertension and neurodegenerative disorders (Zhu, Wang et al., 2024). In recent years, significant attention has been given to antioxidants – agents capable of reestablishing redox equilibrium. Peptides with antioxidant properties act through several complementary mechanisms (López-García et al., 2022; Lv et al., 2022). Directly (Figure 4), they can scavenge free radicals, chelate prooxidative ions and inhibit lipid peroxidation by stabilizing the oil-water interface and limiting the access of prooxidative species (Xiong, 2010). Indirectly (Figure 5), they can upregulate endogenous antioxidant pathways and contribute to glutathione biosynthesis (Corrochano et al., 2018).

Current research findings point to MW as the main determinant of peptide antioxidant activity, with primary structure (sequence) ranking next and secondary structure contributing a lesser, yet still significant extent, and most reports identify 3-6-residue peptides as the most active (Nwachukwu & Aluko, 2019; Zou et al., 2016). The most thoroughly studied mechanism of peptides' antioxidant action is the scavenging of free radicals, such as superoxide anion (O_2^{-}•), hydroxyl (•OH) and peroxyl radicals (ROO•). It can occur through either hydrogen atom transfer (HAT) (Figure 4A), single electron transfer (SET) (Figure 4B), or through both of these modes (Mardani et al., 2023; Zhu, Xu et al., 2024). The antiradical activity of peptides is influenced by their amino acid composition. For example, aromatic residues such as Trp, Tyr and Phe can act as electron donors and contribute to radical stabilization via resonance delocalization (Zhu, Xu et al., 2024), Cys residues can donate both electrons and hydrogen atoms, while the negatively charged side chains of Glu and Asp residues serve as electron donors. Hydrophobic residues, like Leu, Val, Ile and Pro, enhance peptide-radical interactions and facilitate electron transfer, especially in a lipid-rich environment (Nwachukwu & Aluko, 2019; Zhu, Xu et al., 2024). Some peptides exert their antioxidant activity by chelating prooxidative transition metals, particularly Fe^{2+} and Cu^{+} ions (Figure 4C). These metals catalyse Fenton-type reactions, in which H_2O_2 is decomposed into •OH – highly reactive species that contribute significantly to oxidative cellular damage. By binding these ions, peptides reduce their catalytic availability and thus prevent the initiation of metal-driven oxidative cascades (Xu et al., 2024). Effective chelators often contain amino acid residues with nucleophilic or charged side chains, such as Cys, Glu, Asp and especially His. These residues coordinate metal ions through specific functional groups: the imidazole ring in His, the thiol group in Cys, and the carboxyl groups in Glu and Asp. Additionally, acidic residues can contribute to chelation by donating lone-pair electrons and stabilising metal-peptide complexes (Du et al., 2022; Jiang et al., 2022; Zou et al., 2016). Moreover, certain hydrophobic peptides act by shielding lipids in physical membranes that hinder their interactions with prooxidative species (Figure 4D) (López-García et al., 2022).

Some peptides exert their effects indirectly by activating endogenous antioxidant systems (Xu et al., 2024). One well-characterised mechanism involves activation of the Keap1-Nrf2-ARE pathway (Figure 5, left side), which regulates the expression of antioxidant enzymes such as heme oxygenase 1, superoxide dismutase, catalase and glutathione peroxidase. Under oxidative stress conditions, Nrf2 dissociates from Keap1, translocates to the nucleus and promotes transcription of antioxidant enzymes, thereby enhancing cellular resilience (Lv et al., 2022; Xu et al., 2024). Some peptides, for example, milk-derived Val-Leu-Pro-Val-Pro-Gln-Lys, have been shown to upregulate antioxidant enzyme expression through this pathway (Lv et al., 2022). In parallel, several peptides, such as rice-derived Ala-Ala-Gly-Ala-Leu-Pro-

Ser, also suppress the NF-κB signalling pathway (Figure 5, right side), reducing oxidative damage by limiting pro-inflammatory responses (Lv et al., 2022).

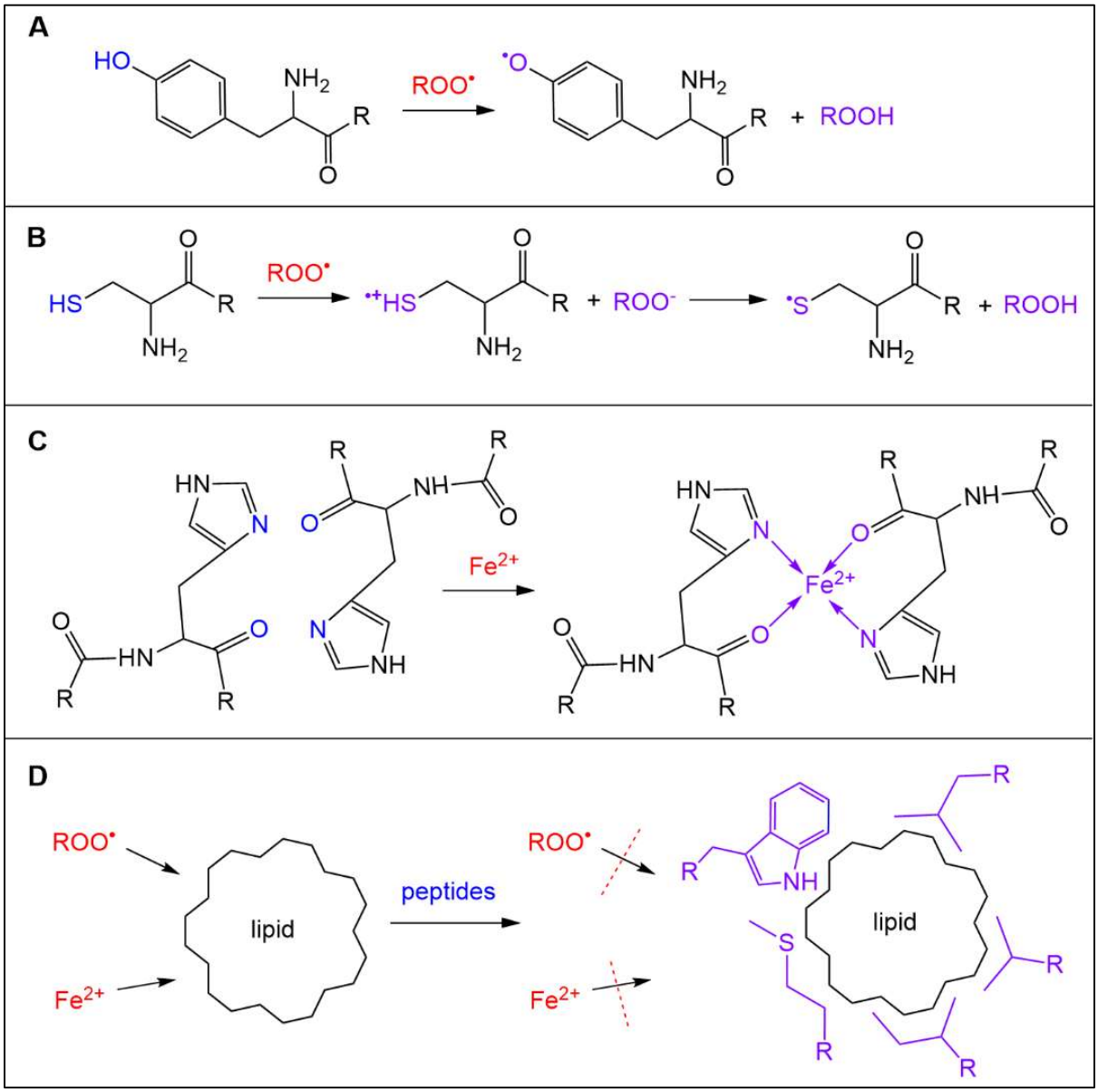

Figure 4. Overview of direct antioxidant mechanisms of peptides involving: **A)** hydrogen atom transfer (HAT), **B)** single electron transfer (SET), **C)** chelation of prooxidative metals, **D)** physical shielding of lipids. Coloured labels indicate: red – prooxidative species, blue – peptide functional groups involved in antioxidant activity, purple – stabilized intermediates and products. ROO• - peroxyl radical. Based on Xiong (2010) and Esfandi et al. (2019).

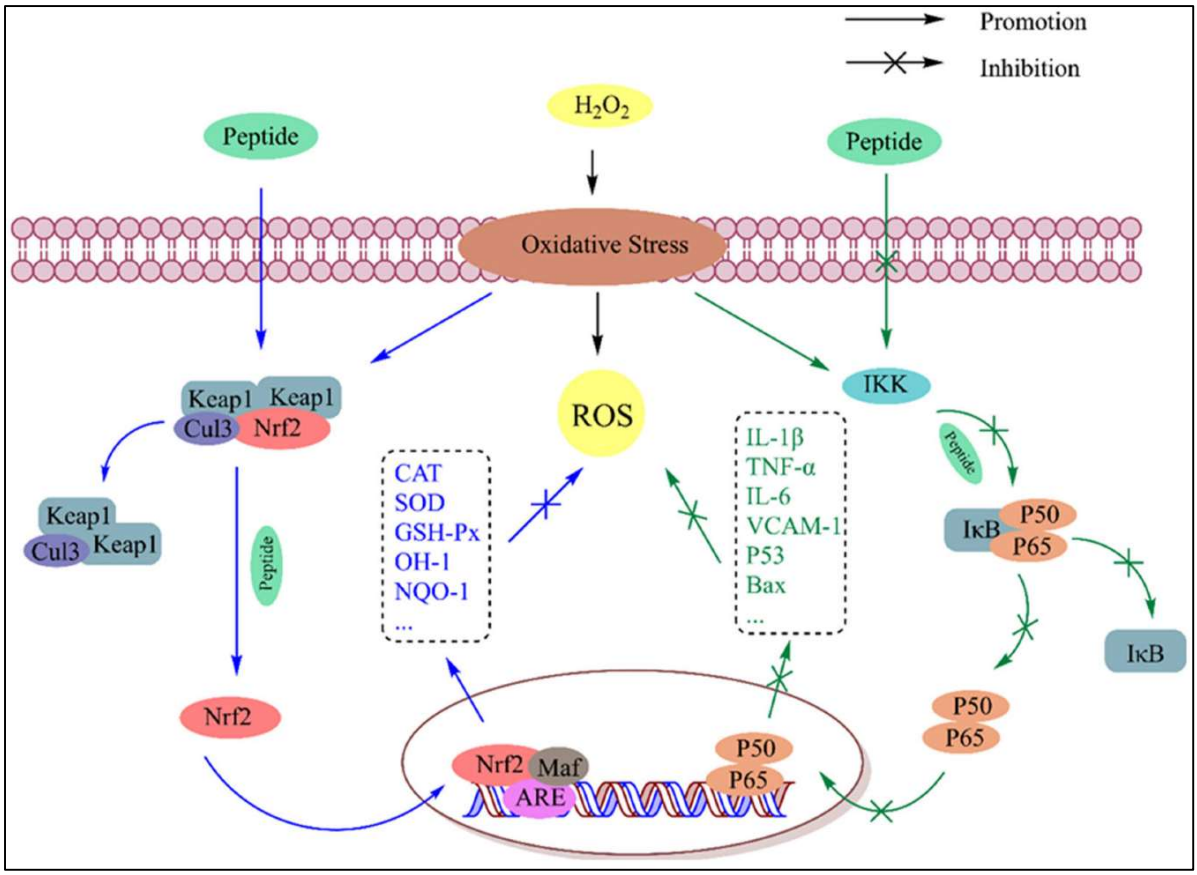

Figure 5. Overview of indirect antioxidant mechanisms of peptides involving regulation of the Keap1-Nrf2-ARE (left side) and NF-κB (right side) signalling pathways. ROS – reactive oxygen species; SOD – superoxide dismutase; CAT – catalase; GSH-Px – glutathione peroxidase; OH-1 – heme oxygenase 1; Prx – peroxiredoxin; Nrf2 – nuclear factor erythroid 2-related factor 2; Keap1 – Kelch-like ECH-associated protein 1; Cul3 – cullin 3; Maf – musculoaponeurotic fibrosarcoma oncogene; ARE – antioxidant response element; IL-1β – interleukin-1β; TNF-α – tumour necrosis factor α; IL-6 – interleukin-6; VCAM-1 – vascular cell adhesion molecule-1; P53 – tumour protein p53; Bax – Bcl-2-associated X protein; NF-κB – nuclear factor κB; IKK – IκB kinase; IκB – inhibitor of κB; P50/P65 – NF-κB subunits. Reproduced from Xu et al. (2024), CC BY 4.0.

1.2.4. Neuroprotective and procognitive activity

Neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and age-related cognitive impairment constitute an escalating health concern in ageing populations. Recently, neuroprotective and procognitive peptides have emerged as promising agents with the potential to support various brain functions, enhance memory and mitigate neurodegeneration. Some peptides inhibit acetylcholinesterase (AChE), the enzyme responsible for degrading acetylcholine, which results in its prolonged availability in synaptic clefts, thereby improving the learning process and memory (Lee & Hur, 2019). Anti-inflammatory peptides reduce the secretion of pro-inflammatory cytokines and inhibit the activation of microglia, contributing to the preservation of neuronal integrity (Katayama &

Nakamura, 2019). Anti-amyloidogenic peptides interfere with the aggregation of amyloid-β into toxic oligomers and plaques, mitigating one of the central pathological hallmarks of Alzheimer's disease (Zhao et al., 2022). Some peptides enhance the expression of brain-derived neurotrophic factor (BDNF) and related growth factors, thereby supporting synaptic plasticity and neurogenesis (Katayama & Nakamura, 2019). The neuroprotective activity of certain peptides has also been linked to their antioxidant properties, which protect neurons against oxidative stress (Lee & Hur, 2019). Procognitive effects have also been attributed to prolyl oligopeptidase (POP) inhibitory peptides, described comprehensively in paper **A5** of the dissertation.

The pea-derived Gln-Ser-Gln-Ser, Leu-Gln-His-Asn-Ala and Ser-Gln-Ser-Arg-Ser are examples of peptides with potent AChE inhibitory properties *in vitro*, characterised by IC_{50} values of 4.25, 3.10 and 3.26 μM, respectively. These peptides show activity comparable to the pharmaceutical standard galantamine, with an IC_{50} of 5.57 μM (Asen et al., 2022). Peptides with 4-7 amino acid residues, particularly hydrophobic, with Gln at the penultimate N-terminal, tend to have superior AChE inhibitory activity (Asen et al., 2022; Malomo & Aluko, 2019). Peptides that attenuate neuroinflammation also contribute to neuroprotection. For instance, Leu-Pro-Phe, Gly-Val-Tyr-Tyr and Ala-Pro-Thr-Leu-Trp, originating from walnut protein, downregulate several pro-inflammatory mediators (nitric oxide, prostaglandin E_2, tumour necrosis factor-α, interleukin-1β), while improving memory in mice (Wang et al., 2020). Trp, Gly and Leu residues were considered particularly important for the anti-neuroinflammatory activity (Wang et al., 2020). An example of an anti-amyloidogenic peptide is walnut-derived Pro-Pro-Lys-Asn-Trp, which exhibited procognitive effects in mice (Wang et al., 2019). Peptides upregulating the expression of BDNF are represented by soybean-derived Gly-Arg and rice-derived His-Ser-Met-Asn-Pro-Ser-Thr-Asn-Pro-Trp-His-Ser-Thr-Val-His-Thr, which were reported to enhance neurogenesis and attenuate memory impairment in mice (Corpuz et al., 2019; Shimizu et al., 2018). According to Katayama et al. (2021), Arg, Lys and His are particularly important for the BDNF upregulating activity.

Generally, neuroprotective peptides are characterized by low MW (below 1 kDa) and a high proportion of hydrophobic amino acids. Their sequences often include acidic residues such as Asp or Glu at the N-terminal position, which enhance interaction with target receptors or signalling proteins. Many of these peptides are obtained by enzymatic hydrolysis using broad-specificity proteases such as papain and subtilisin, which favour the release of small, bioactive fragments. Neuroprotective peptides are closely associated with the modulation of cell survival and apoptosis-related signalling pathways (Katayama et al., 2021; Lee & Hur, 2019).

1.3. Methods for the release of bioactive peptides

1.3.1. Introduction

The production of bioactive peptides from protein substrates involves the controlled cleavage of peptide bonds to release encrypted sequences with specific physiological effects. Several methodological approaches have been developed to achieve this, each characterized by distinct selectivity, operational requirements, and potential relevance to biotechnological, cosmetic, food or pharmaceutical applications (Table 1). Enzymatic hydrolysis (with commercial protease preparations), microbial hydrolysis (fermentation involving proteolytic bacteria or fungi), and chemical hydrolysis (under acidic or alkaline conditions) are the principal *in vitro* methods used to generate bioactive peptides (Mardani et al., 2023). The release of such peptides also occurs *in vivo* in the gastrointestinal tract during the digestion of dietary proteins (Du et al., 2024). Each of these processes yields peptides with distinct molecular structures, which directly affect their functional and bioactive properties. Key reaction parameters, such as pH, temperature, reaction time, type and concentration of reagents, play a crucial role in determining the composition, activity and bioavailability of the resulting peptides. Thus, the choice of hydrolysis approach must be tailored to the intended application (Nongonierma & FitzGerald, 2017).

1.3.2. Enzymatic hydrolysis

Enzymatic hydrolysis is the most widely employed method for generating bioactive peptides from protein substrates. It is a highly specific process in which proteolytic enzymes, either of microbial (e.g. subtilisin, thermolysin, proteinase K), animal (e.g. pepsin, trypsin, chymotrypsin) or plant origin (e.g. papain, bromelain, ficin), cleave peptide bonds at defined recognition sites. The reaction proceeds under mild conditions and with precise regio- and stereo-selectivity, resulting in mixtures of free amino acids and peptides of defined size and sequence (Cruz-Casas et al., 2021; Iwaniak et al., 2020; Sun et al., 2024). Typical enzymatic hydrolysis procedure involves initial isolation and solubilization of the protein substrate, followed by enzymatic reaction and is finalized by inactivation of the enzyme. Each of these steps can be independently optimized and each exerts a considerable influence on the physicochemical and biological properties of the resulting hydrolysates, including MW distribution, solubility, surface activity and bioactivity profile (Brodkorb et al., 2019; Le Maux et al., 2018; Mardani et al., 2023; Nongonierma & FitzGerald, 2017).

Isolation of proteins from raw materials ensures a high purity of the substrate for enzymatic hydrolysis, reducing the content of non-protein contaminants that may interfere with enzymatic specificity or final peptide characterization. Purified protein substrates coupled with

Table 1. Comparison of hydrolysis approaches for bioactive peptide release *in vitro.*

Parameter	Hydrolysis approach			
	Enzymatic	**Microbial**	**Chemical**	**Gastrointestinal**
Specificity	High – selective cleavage at specific amino acid residues	Medium to high – depends on microbial strain, secreted enzymes	Low – non-specific, random bond cleavage	Medium to high – varies with digestion and life stage
Peptide bioactivity control	Excellent – tailored peptide size and sequence	Moderate – some control through strain selection and growth conditions optimization	Poor – limited control, often degraded or inactive products	Moderate – bioactivity affected by susceptibility to proteolysis
Reproducibility	High – purified enzymes ensure batch-to-batch consistency	Moderate – dependent on microbial growth and environmental conditions	Low to moderate – simple process but harsh conditions	Moderate – high inter-individual variability, simplifications in models
Cost	High – enzyme cost is significant	Low to medium – cheap fermentation media and microbial strains	Low – cheap and common acids/alkalis	Variable – cheap static models, costly dynamic models
Reaction time	Short – several minutes to several hours	Long – days to weeks	Moderate – several hours to several days	Short – several hours
Control over reaction	Excellent – easily adjustable reaction parameters	Limited – microbial metabolism is complex and dynamic	Poor – harsh, less controllable conditions	Limited – constrained by biological or model physiology
Product purity	High – well-characterized peptide mixtures	Medium – peptides mixed with microbial metabolites	Low – heterogeneous hydrolysates, possible by-products	Medium — complex mixtures, even in static models
Suitability for food/pharma applications	Good – meets regulatory and purity demands	Moderate to good – probiotic synergy possible but requires further purification	Limited – potential toxic by-products and lack of peptide specificity	High relevance in functional evaluation, not a production method per se
Technical complexity	Moderate – requires careful optimization and enzyme handling	Moderate – microbial culture maintenance and fermentation control	Low – technically simple but involves hazardous chemicals	Variable – from simple static to highly complex dynamic systems
Compatibility with *in silico* tools	Available – predictive tools support cleavage site and activity prediction	No – enzyme identity and activity too complex	No – cleavage patterns too random	Available – predictive tools support cleavage site and activity prediction
Main advantages	High specificity and reproducibility, high product quality and purity, mild conditions, compatibility with *in silico* tools	Low cost, mild conditions, possible synergistic metabolites	Cheap, simple, effective for resistant proteins	Mimics physiological digestion, predicts bioaccessibility and bioavailability
Main drawbacks	High cost, technical complexity	Slow process, moderate purity and reproducibility, low yield	Harsh conditions, potential formation of toxic by-products, impaired bioactivity	Multi-phase enzymatic digestion with complex kinetics

standardized enzymatic protocols enable consistent control of hydrolysate composition and reproducibility of their bio-functional properties (Echave et al., 2021; Leduc et al., 2020; Mora & Toldrá, 2023). Solubilization of the protein substrate enhances the accessibility of peptide bonds to enzymatic attack and increases hydrolysis yield (Feng et al., 2025). Methods used for solubilization can be physical (e.g. pressurisation, ultrasonication, thermal treatment) or chemical (e.g. acids, alkalis, chaotropic and reducing agents), and their selection depends on the nature of the protein (Mora & Toldrá, 2023; Shavandi et al., 2017). Once the protein substrate is solubilised and the initial hydrolysis parameters are adjusted, the enzyme preparation is added to initiate proteolysis. Hydrolysis reaction conditions, such as temperature, pH, enzyme/substrate (E/S) ratio and duration, must be carefully optimized for each protease-protein system. These parameters affect the degree of hydrolysis (DH), MW distribution, biological and techno-functional properties of the hydrolysate produced. DH threshold is sometimes used to differentiate limited (DH < 10%) from extensive (DH ≥ 10%) hydrolysis; however, this distinction is somewhat arbitrary and does not necessarily reflect a functional cutoff in terms of peptide activity or yield (Gouseti et al., 2023; Mardani et al., 2023). Controlled hydrolysis aims to produce peptides of specific structure and activity without over-degradation, which may lead to loss of peptide function. When the desired DH is achieved, the reaction is terminated by inactivating the enzyme, either via thermal treatment, pH shift or addition of enzyme inhibitors (Brodkorb et al., 2019; Le Maux et al., 2018).

Enzymatic hydrolysis has a number of advantages that make it a reliable approach for producing bioactive peptides. The reaction is characterised by excellent selectivity, allowing for the generation of well-defined peptide mixtures with high process reproducibility. Additionally, rapid reaction speed (usually several minutes to several hours) and its mild conditions (typically below 100 °C and near-neutral pH) favour energy savings and minimize the risk of undesired side reactions or degradation of labile amino acids, which is particularly advantageous in high-value applications such as food and pharmaceutical (Cruz-Casas et al., 2021; Hayes, 2021; Lorenzo et al., 2018). Moreover, enzymatic hydrolysis is compatible with *in silico* tools that reduce experimental workload and facilitate data analysis. These tools enable prediction of peptide cleavage sites, identification of potentially bioactive sequences or selection of suitable enzymes for targeted hydrolysis (Iwaniak et al., 2019). Such predictive modelling is impossible in non-specific methods like chemical hydrolysis or microbial hydrolysis, where the identity and specificity of proteolytic enzymes remain undefined or too complex to simulate. A key limitation, however, is the relatively high cost of commercial enzyme preparations, which may hinder industrial scalability unless compensated by the use of inexpensive protein-rich by-products or high-value target applications (Hayes, 2021; Sun et al., 2024).

1.3.3. Microbial hydrolysis (fermentation)

Microbial hydrolysis occurring during fermentation is a biologically driven process in which living microorganisms secrete a broad spectrum of endogenous proteases. In contrast to enzymatic hydrolysis (with purified enzymatic preparations), microbial hydrolysis yields not only peptides and amino acids but also secondary microbial metabolites, some of which may have beneficial or harmful effects. The commonly used microbes include bacteria such as *Lactobacillus helveticus*, *Bacillus subtilis* and *Lactobacillus plantarum*, filamentous fungi (moulds) such as *Aspergillus oryzae*, and yeasts including *Saccharomyces cerevisiae* and *Pichia fermentans* (Cruz-Casas et al., 2021; Zhao et al., 2024). The general microbial hydrolysis procedure comprises preparation of the protein substrate, inoculation with the selected microorganism and incubation, allowing proteolytic activity. These steps are dependent on microbial strain properties, fermentation mode (solid or liquid state), and control of environmental parameters (temperature, pH, aeration and duration) (Barati et al., 2020; Stiborova et al., 2016; Zhao et al., 2024).

As in enzymatic hydrolysis, the preparation of the protein substrate for microbial hydrolysis involves solubilization or mechanical disruption to improve accessibility for microbial enzymes. In this case, however, substrate preparation also aims to enhance microbial adhesion and activity. Protein denaturation via mild thermal or chemical pre-treatment may help expose cleavage sites for microbial proteases (Mardani et al., 2023; Zhao et al., 2024). The inoculation involves the use of pure cultures or starter consortia, with microbial density and viability being crucial for efficient colonization and enzymatic activity. During fermentation, proteases secreted by microbes hydrolyse peptide bonds in the substrate. This proteolysis is usually slower than in enzymatic hydrolysis and can span several hours to days, but it occurs under mild conditions, typically 25-37 °C and near-neutral pH (Cruz-Casas et al., 2021; Zhao et al., 2024). In solid-state fermentation, limited water activity favours fungal strains and results in highly concentrated peptide extracts. In liquid-state fermentation, liquid media allow better control and scalability but may dilute the hydrolysate (Barati et al., 2020; Zhao et al., 2024).

Microbial hydrolysis offers several advantages that make it the second most commonly used method for producing bioactive peptides. This method relies on the endogenous enzymatic activity of microorganisms, thereby lowering production costs compared to methods utilizing costly purified enzymes. The process proceeds under mild conditions, avoiding the use of harsh reagents, which enhances its environmental sustainability. Additionally, the metabolic activity of certain microorganisms may contribute to the generation of secondary metabolites with potential synergistic health-benefiting effects (Cruz-Casas et al., 2021; Zhao et al., 2024). However, the proteolytic activity of microorganisms is less specific and efficient

than that of purified enzymes. Moreover, during fermentation, part of the protein substrate is inherently utilized as a source of carbon, nitrogen and energy for microbial growth rather than for peptide release alone (Lasekan et al., 2013; Stiborova et al., 2016). Although cheaper than enzymatic hydrolysis, microbial hydrolysis often delivers lower overall peptide concentrations and requires additional processing steps to achieve comparable purity (Barati et al., 2020; Sun et al., 2024). Furthermore, microbial hydrolysis is intrinsically more susceptible to reaction variability and poor batch-to-batch reproducibility, compared to enzymatic hydrolysis which relies on well-characterized commercial enzymes. Deviations in microbial growth, enzyme production and environmental conditions can lead to inconsistencies in the composition and bioactivity of the resulting peptides (Cruz-Casas et al., 2021).

1.3.4. Chemical hydrolysis

The cleavage of peptide bonds in proteins during chemical hydrolysis occurs due to strong acid or base solutions, usually at high temperatures and sometimes under high pressure. Under acidic conditions, peptide bonds are cleaved via protonation of the carbonyl oxygen, facilitating nucleophilic attack by water molecules. Under alkaline conditions, hydrolysis is driven by direct nucleophilic attack of hydroxide ions on the carbonyl carbon (Pan & Ricci, 2010; Sun et al., 2020). Unlike enzymatic or microbial hydrolysis, this method relies entirely on chemical reactivity rather than biological catalysis, resulting in more random cleavage patterns. Chemical hydrolysis, although it lacks the specificity, has been historically important in peptide production due to its simplicity, high efficiency and low cost of reagents. The most commonly used acids in this process include HCl and H_2SO_4, while the most prevalent alkalis are NaOH and KOH (Mardani et al., 2023; Sinkiewicz et al., 2018; Stiborova et al., 2016). The typical chemical hydrolysis procedure, apart from preparation of the protein substrate as in the case of enzymatic and microbial hydrolysis, includes chemical treatment under controlled pH and temperature conditions, finalized by neutralization. Though harsh, this method allows for rapid breakdown of resistant proteins and has been successfully used for processing protein-rich wastes in industrial settings (Mardani et al., 2023).

Pre-treatment of raw material intended for chemical hydrolysis often involves mechanical grinding or shredding to increase the surface area and reagent accessibility to peptide bonds. The protein substrate is then exposed to a strong acid or alkali solution, at temperatures ranging from room temperature to above 120 °C, for a period ranging from a few to several dozen hours. Unlike the selective cleavage achieved through specific enzymes, chemical hydrolysis induces non-specific scission of peptide bonds, generating a heterogeneous mixture of free amino acids, peptides and their derivatives. The reaction is terminated by neutralization, typically with a strong base (for acid hydrolysis) or strong acid (for

alkaline hydrolysis), generating significant amounts of salts as by-products (Marcet et al., 2016; Mardani et al., 2023). Controlling the sequence of peptides obtained through chemical hydrolysis is difficult due to the severity and non-selectivity of the process. When the desired outcome is the full release of constituent amino acids, the process can be extended to complete hydrolysis (Marcet et al., 2016; Mardani et al., 2023). Normative procedure to ensure full cleavage of peptide bonds involves hydrolysing proteins in 6 M HCl at 110 °C for 24 h (ISO 13903:2005).

Chemical hydrolysis offers several practical advantages. It is a low-cost and relatively fast method suitable for large-scale processing, particularly when dealing with recalcitrant proteinaceous biomass. It enables the solubilization of structurally resistant proteins and is effective in recovering peptides from industrial by-products, which are difficult to process enzymatically. Additionally, it requires no specialized facilities and is very operationally simple (Marcet et al., 2016; Mardani et al., 2023; Talha et al., 2024). On the other hand, the method suffers from critical limitations. Chief among these is its lack of specificity, which results in heterogeneous peptide profiles and worse peptide biofunctionality. Furthermore, chemical hydrolysis risks racemization and destruction of certain amino acids, as well as formation of potentially toxic and non-nutritive derivatives such as lysinoalanine, lanthionine, β-aminoalanine, H_2S, diketopiperazines and acrylamide, especially under long time, high temperature and high pressure (Friedman et al., 1984; Moktip et al., 2025; Schirone et al., 2022; Sinkiewicz et al., 2018).

1.3.5. Gastrointestinal digestion

Within the context of bioactive peptides, gastrointestinal digestion plays a dual role. Not only is it a key physiological process for liberating functional peptides from intact dietary proteins following their consumption, but it is also a transformative stage in which pre-released peptides may undergo further modifications. Peptides generated before ingestion (through enzymatic, microbial or chemical hydrolysis) do not necessarily retain their structure or function through gastrointestinal transit. Due to exposure to digestive enzymes, these peptides are susceptible to further enzymatic cleavage. Depending on their amino acid sequence, this process can either potentiate their biological activity by releasing shorter, more active fragments or abolish it entirely by degrading them into non-functional free amino acids or oligopeptides (Bohn et al., 2018; Du et al., 2024). Hence, the stability of peptides under digestive conditions is a crucial determinant of their bioefficacy *in vivo.* Although thousands of bioactive peptide sequences have been identified, the fate of most of these peptides post-digestion remains unknown.

The protein/peptide digestion process begins in the stomach and continues through the small intestine, involving a sequence of proteolytic enzymes (proteases and peptidases) with

distinct pH optima and substrate specificities. Pepsin is the main enzyme involved in protein digestion in the gastric phase. It is activated at low pH, with optimal activity around pH 2. Pepsin is a nonspecific protease with broad substrate specificity. It is most effective in cleaving peptide bonds adjacent to hydrophobic and aromatic amino acid residues, particularly Trp, Phe, Tyr and Leu. Still, it does not hydrolyse bonds containing Ala, Gly and Val (Yang et al., 2025). Following the gastric digestion phase, the protein breakdown continues in the small intestine due to the action of pancreatic endopeptidases and exopeptidases that are active at a pH close to 7. The principal endopeptidases involved in this digestion stage are trypsin, very specific to bonds consisting of Lys and Arg residues, chymotrypsin, preferably cleaving the bonds adjacent to Tyr, Phe and Trp residues, and elastase, which is active particularly towards the bonds consisting of Ala, Gly and Ser residues. The action of these endopeptidases is complemented by exopeptidases, particularly carboxypeptidases A and B, which hydrolyse C-terminal amino acids (Gurina & Mohiuddin, 2022). In the final stage of intestinal digestion, brush border membrane peptidases (aminopeptidases, carboxypeptidases, dipeptidases and endopeptidases), located on the apical membrane of enterocytes, release readily absorbable dipeptides, tripeptides and free amino acids (Di Stasio et al., 2024; Martineau-Côté et al., 2024).

To evaluate the digestive fate of bioactive peptides and other dietary components, simulated gastrointestinal digestion (SGID) models have been developed. These models support the investigation of food components' bioaccessibility – defined as the fraction of a compound released from the food matrix during digestion and available for absorption, and bioavailability – referring to the fraction that is absorbed and reaches systemic circulation in an active form (Grundy et al., 2024, 2025; Minekus et al., 2014). SGID methodologies encompass *in silico* and *in vitro* protocols, the latter including static, semi-dynamic and fully dynamic digestion systems (Iwaniak et al., 2019; Kondrashina et al., 2024). *In silico* tools provide a rapid and cost-effective means for simulating the enzymatic digestion of proteins, enabling the prediction of specific peptide fragments that may be released during gastrointestinal transit. These predicted peptides can then be screened for potential bioactivity using computational models based on structural motifs, amino acid sequences and known biological activity databases. Although *in silico* methods do not replicate the full physiological complexity of the human gastrointestinal tract, such as enzyme kinetics, pH gradients, transit times or interactions with the food matrix, they remain valuable for high-throughput screening of peptide sequences and for facilitating *in vitro* SGID experiments (Iwaniak et al., 2019, 2025).

Among *in vitro* SGID protocols, static models are the most widely used due to their simplicity and low cost. The most adopted static model is described in the standardized INFOGEST protocol (Figure 6), originally proposed by Minekus et al. (2014) and later updated

by Brodkorb et al. (2019). The INFOGEST method simulates the oral, gastric and intestinal phases of digestion under physiologically relevant conditions, including pH, enzymatic activity

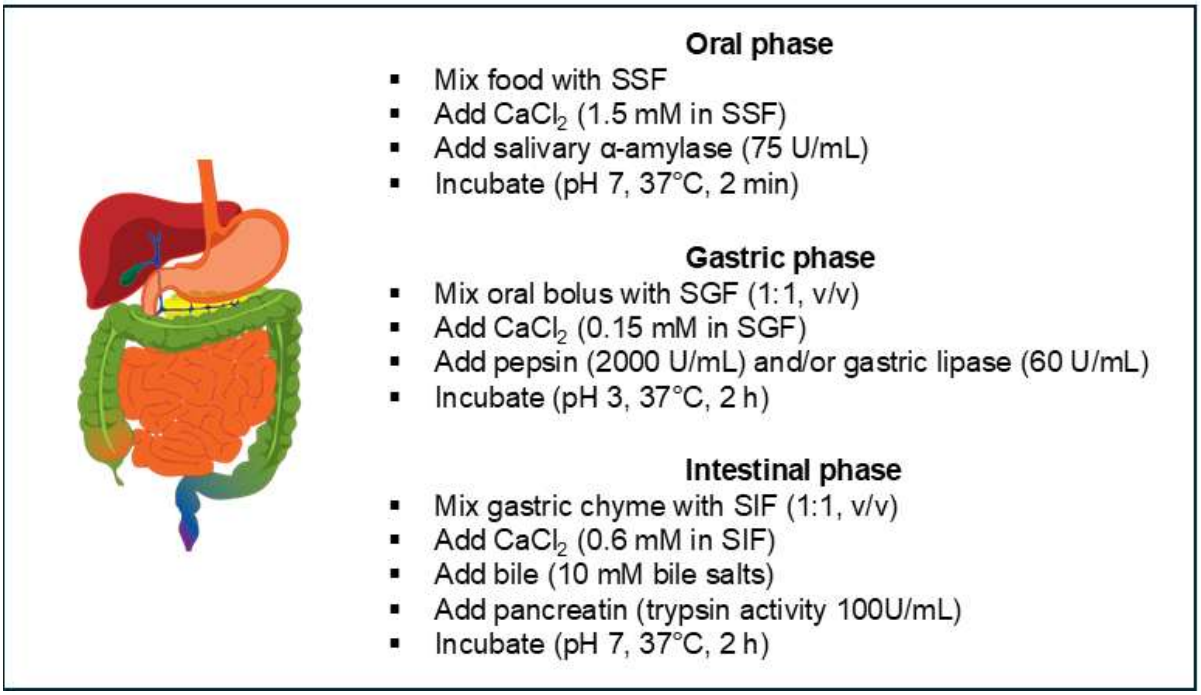

Figure 6. Scheme of the INFOGEST static *in vitro* food digestion protocol. SSF – simulated salivary fluid, SGF – simulated gastric fluid, SIF – simulated intestinal fluid. Based on Brodkorb et al. (2019).

composition of simulated digestive fluids and bile salt concentration. While the protocol is oversimplified for kinetic studies, it can be very useful in rapid, high-throughput screening. Its relevance in predicting the digestion endpoints has been validated through inter-laboratory comparisons and correlations with *in vivo* data (Bohn et al., 2018; Brodkorb et al., 2019; Kondrashina et al., 2024). In addition to the original INFOGEST protocol, which is suitable for the adult population (Brodkorb et al., 2019; Minekus et al., 2014), variants adapted for infants (Ménard et al., 2018) and the elderly (Menard et al., 2023) have been developed. They account for age-related changes in the digestive capability, including enzyme activities, composition of digestive fluids and bile salt concentrations. The semi-dynamic SGID model incorporates critical kinetic aspects of the gastric digestion, i.e., gradual acidification, fluid/enzyme secretion and emptying, offering greater physiological relevance than static systems with moderate complexity and cost (Mulet-Cabero et al., 2020). Fully dynamic SGID models, such as the DiDGI® (Digesteur Dynamique Gastro-Intestinale) and SHIME® (The Simulator of the Human Intestinal Microbial Ecosystem), replicate peristalsis, secretion patterns and compartmental transit using computer-controlled mechanisms. These models provide unmatched simulation fidelity but require specialized infrastructure and are cost-prohibitive for routine use in most laboratories (Reynaud et al., 2021; Zhu, Zhang et al., 2024).

1.4. Modification of bioactive peptides by the Maillard reaction

Peptides may require physical or chemical modifications to mitigate their poor physicochemical stability, high susceptibility to proteolytic degradation and bitter taste, thereby enhancing their applicability in food and pharmaceutical formulations (Pei et al., 2022; Yu et al., 2018). Physical modification methods involve encapsulation of protein hydrolysates/peptides in microcapsules composed of polymers such as starch, polylactic acid and ethyl cellulose or their encapsulation in liposomes formed from phospholipid bilayers, which enhance peptide stability, protect against enzymatic degradation and facilitate controlled release and absorption in the gastrointestinal tract (Pei et al., 2022). Chemical modification methods of peptides involve unnatural amino acid substitution, cyclization, N/C-terminal cleavage, acylation, phosphorylation, sulfitolysis and amino-carbonyl reaction (Maillard reaction). These methods aim to improve peptide solubility and resistance to proteolytic degradation, reduce bitterness and prolong systemic stability (Pei et al., 2022; Wu et al., 2021; Yap & Gan, 2020). According to Wu et al. (2021), the Maillard reaction may be one of the most industrially viable methods of modifying biopeptides, owing to both nutritional and economic concerns.

The Maillard reaction refers to a complex, three-stage cascade (Figure 7) of non-enzymatic browning reactions occurring between the carbonyl groups of reducing saccharides and the amino groups of amino acids, peptides or proteins. It has been associated with both positive (e.g. desirable changes in flavour and aroma) and negative (e.g. nutritional deterioration, formation of toxic compounds) consequences, depending on the processing conditions and the nature of the reactants involved (Staroszczyk, 2023). Growing evidence suggests that under controlled conditions, the Maillard reaction may serve as an effective means of enhancing the functionality of bioactive peptides. When performed at moderate temperatures (typically 80-120 °C) for up to several hours, the Maillard reaction can enhance bioactivity and bioavailability of peptide conjugates, while also improving their sensory characteristics (Arihara et al., 2017; Fu et al., 2020; Wu et al., 2021). For instance, when crab shell protein hydrolysates were glycated with Fru at 100 °C for 1 h, a significant increase in antioxidant activity was observed, along with the emergence of antibacterial properties not exhibited by the unmodified peptides (Jiang et al., 2018). According to Chen et al. (2020), Maillard reaction products (MRPs) obtained by heating snapper scale protein hydrolysates with Xyl at 100 °C for 4 h exhibited enhanced antioxidant activity and significantly greater hepatoprotective effects in ethanol-induced liver injury in mice, compared to non-glycated peptides. Crocodile meat protein hydrolysate glycated with Xyl at 100 °C for 3.2 h also showed markedly improved antioxidant activity and anti-ageing effects in *Drosophila melanogaster*, in comparison with the unmodified hydrolysate (Li et al., 2021). Similarly, when *Lentinula edodes*

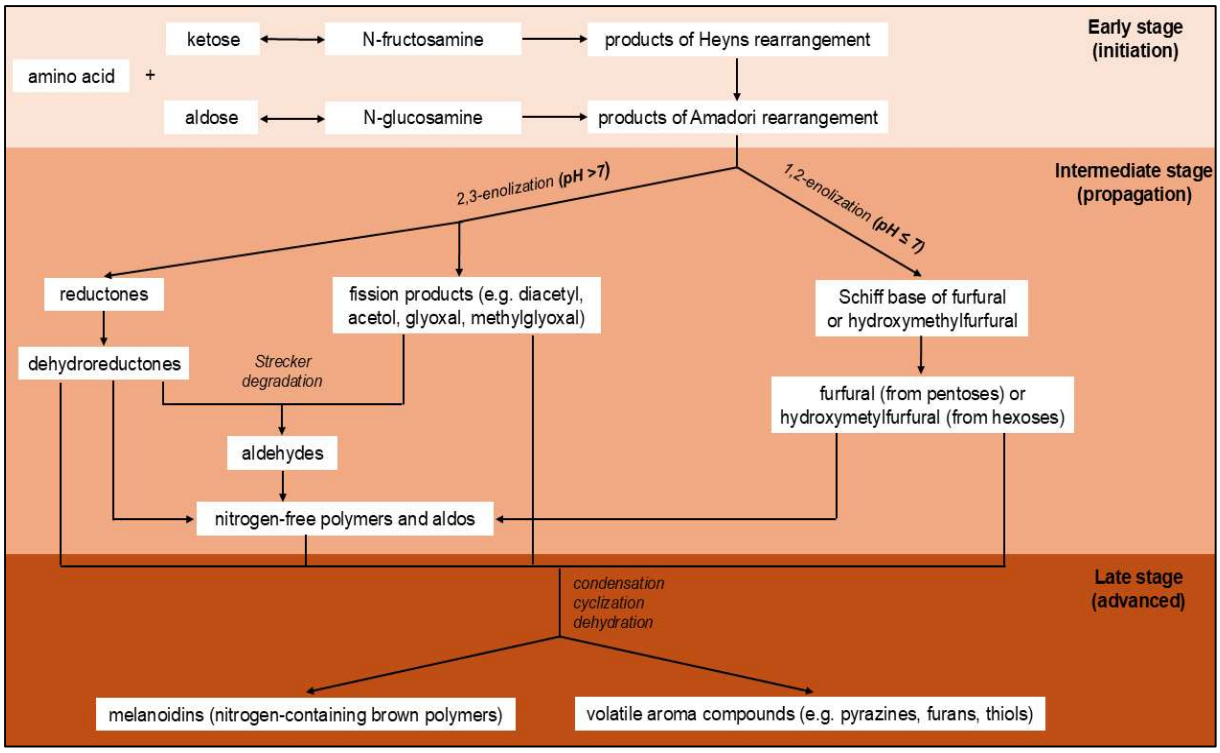

Figure 7. Schematic overview of the Maillard reaction, illustrating major pathways and representative products formed during early, intermediate and late stages. Based on Hodge (1953) and Staroszczyk (2023).

mushroom protein hydrolysates were subjected to the Maillard reaction with Xyl and Cys at 120 °C for 100 min, the resulting MRPs exhibited a marked increase in antioxidant and antimicrobial activities, although their α-amylase and α-glucosidase inhibitory activity decreased. Moreover, the MRPs obtained showed an enhancement of umami, caramel and meaty notes, alongside improved overall taste acceptability, compared to the non-glycated hydrolysate (Qiu et al., 2024). According to Gómez-Estaca et al. (2021), glycation of shrimp protein hydrolysate with Glc at 100 °C for at least 40 min significantly improved its antioxidant properties, but eliminated POP inhibitory activity, a function characteristic of the original hydrolysate. These findings collectively emphasize that while the Maillard reaction can enhance certain bioactive properties of peptides, it may compromise others, underscoring the importance of careful process optimization to balance desired functionalities.

2. Keratins

2.1. Physicochemical properties and classification of native keratins

Keratins (gr. *keras* – horn) are structural proteins of animal origin, primarily localized in the epidermis and its cornified appendages such as feathers, hair, horns and claws. They are among the most abundant proteins in mammals, reptiles and birds, along with collagen and elastin. However, unlike the latter two proteins, keratins are distinguished by a remarkably high Cys content, ranging from 7 to 22% (Kornitłowicz-Kowalska & Bohacz, 2011). Regardless of origin and type, native keratins are characterised by very high physical and chemical durability, mechanical strength, resistance to hydrolysis by conventional proteases (such as trypsin, pepsin and papain), slow biodegradability, and insolubility in most polar and non-polar solvents (Sinkiewicz et al., 2018). These properties result from numerous intra- and intermolecular cross-links formed by disulfide and hydrogen bonds, as well as hydrophobic and ionic interactions (Figure 8) (Shavandi et al., 2017). These molecular features lead to the self-assembly of keratins into an intricately ordered filament-matrix framework (Wang, Yang et al., 2016).

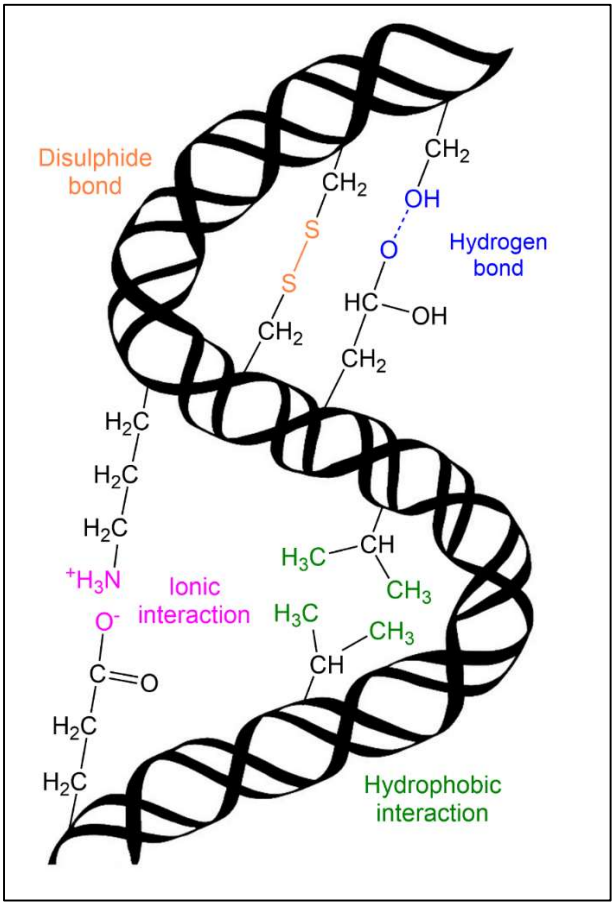

Figure 8. Molecular interactions stabilising keratin structure. Based on Shavandi et al. (2017).

The diverse physicochemical properties of keratins stem from differences in their amino acid composition and structural organization, which render this protein group highly heterogeneous. The first of their classification criteria is based on mechanical and structural characteristics, distinguishing between soft and hard keratins (Sinkiewicz et al., 2018). **Soft keratins** possess a relatively low Cys content (approx. 10-14%) and form networks with weaker cross-linking, which imparts them with flexibility and elasticity. They are typically found in hair, wool and the outermost layers of the skin, where they provide soft and pliable structural support. **Hard keratins** have a higher Cys content (up to 22%), resulting in a denser network of disulfide bonds that imparts increased mechanical strength and stiffness. These proteins occur in hardened body parts such as feathers, horns, claws, beaks and nails, ensuring toughness and defence against chemical and physical challenges (Sinkiewicz et al., 2018).

The second classification criteria consider the secondary structure of keratins, distinguishing α, β and γ-keratins. **α-Keratins** are fibrous proteins, rich in Cys residues, with molecular weight (MW) between 40 and 70 kDa (Sinkiewicz et al., 2018). They are composed of four right-handed α-helices that coil into a larger, left-handed super-helix (protofibril) stabilized by hydrogen bonds running parallel to the helix axis and disulfide bonds connecting adjacent polypeptide chains (Ferraro et al., 2016). The protofibrils assemble into intermediate filaments about 7 nm in diameter (Wang, Yang et al., 2016). These structural proteins are commonly found in wool, quills, hair, horns, fingernails, hooves and stratum corneum (Sinkiewicz et al., 2018). **β-Keratins** are fibrous proteins, rich in short-chain amino acid residues such as Ser, Gly, Pro and Ala, with MW ranging from 10 to 22 kDa. They are composed of polypeptide chains that mainly adopt a β-sheet conformation, with stability provided mostly by dense hydrogen bonds (Sinkiewicz et al., 2018). The β-sheets pack into filaments approx. 3 nm in diameter (Wang, Yang et al., 2016). These protective proteins occur mostly in feathers, beaks, claws and scales, and they are more difficult to extract than α-keratins (Sinkiewicz et al., 2018). In addition to fibrous α- and β-keratins, keratinous tissues contain **γ-keratins**, also known as amorphous or matrix keratins. These are globular proteins of relatively low MW, divided into two groups: those rich in Cys, with MW 11-26 kDa and those rich in Cys, Gly and Tyr, with MW 6-9 kDa (Ferraro et al., 2016). Unlike α- and β-keratins, which form ordered fibrous structures, γ-keratins act as matrix proteins embedding the fibrous keratins. In this role, they stabilize the hierarchical organization of keratinous tissues and contribute to their mechanical resilience (Wang, Yang et al., 2016). γ-Keratins occur in the external layer of the hair cuticle (Sinkiewicz et al., 2018).

2.2. Characteristics of the most abundant keratin raw materials

2.2.1. Introduction

Quantifying the total availability of keratin-rich biomasses according to waste type is challenging due to inconsistent and incomplete data, but the poultry and pork industries are considered the leading producers (Sharma & Kumar, 2019). The total amino acid composition of selected keratin-rich biomasses, i.e., chicken feathers, sheep wool and pig hair, is shown in Table 2.

Table 2. Total amino acid composition of native chicken feather, sheep wool and pig hair keratins. Based on Banasaz & Ferraro (2024).

Amino acid	Amino acid composition [mol%]		
	Chicken feathers	Sheep wool	Pig hair
Ala	4.01	5.20	4.90
Arg	6.16	6.24	7.65
Asx	5.23	5.93	6.05
Cys	7.16	13.10	10.75
Glx	8.76	11.10	12.55
Gly	6.31	8.56	9.25
His*	0.40	0.79	-
Ile*	4.28	2.98	3.15
Leu*	7.38	7.20	6.95
Lys*	1.11	2.66	2.60
Met*	0.25	0.54	0.65
Phe*	4.40	2.48	2.30
Pro	8.84	6.60	7.15
Ser	8.93	10.80	11.30
Thr*	3.77	6.53	6.95
Trp*	0.97	-	-
Tyr	2.44	3.78	3.85
Val*	6.12	5.68	4.85

** – essential amino acid*

2.2.2. Chicken feathers

Chicken feather is the most important keratin source worldwide (Sharma & Kumar, 2019). Each year, more than 40 million tons of waste feathers are incinerated, emitting sulfur dioxide and carbon dioxide into the atmosphere (Aradoaei et al., 2024), and this amount is expected to rise further due to increasing global demand for poultry meat. Feathers represent 5-10% of the total body weight of mature chickens, and are composed of around 90% protein (keratin), along with approx. 7% water and up to 3% lipids (Sharma & Kumar, 2019). Although several types of feather keratins have been identified, they show only minor differences in amino acid composition, with basic and acidic residues primarily located at the terminal ends of the polypeptide chains and hydrophobic residues concentrated in the central region (Sinkiewicz et al., 2018). The most abundant amino acid residues in the chicken feather keratin are Ser (8.93 mol%), Pro (8.84 mol%), Glu (8.76 mol%), Leu (7.38 mol%) and Cys (7.16 mol%) (Banasaz & Ferraro, 2024). The whole feathers are composed of roughly 32% α-helices, 54% β-sheets and random coils and about 14% β-turns (Moktip et al., 2025). The predominant β-keratin, which makes up the majority of the feather's overall keratin composition and accumulates mainly in the barbs and barbules, has a MW of around 10 kDa and is distinguished by extensive β-sheet content that ensures high stiffness and tensile strength critical for avian flight. On the other hand, α-keratin, which contributes less to the overall feather structure and is mainly found in the rachis and calamus, features MW of 40-70 kDa and is defined by a high content of α-helical regions that provide flexibility to these parts of the feather (Wang, Yang et al., 2016).

2.2.3. Sheep wool

The global sheep population is estimated at 1.27 billion, yielding around 1.9 million metric tons of wool annually (Giteru et al., 2023). Wool fibres consist mostly of proteins (95-98%), alongside minor amounts of lipids (0.1%) and mineral compounds (0.5%) (Giteru et al., 2023). The dominant amino acid residues in wool are Cys (13.10 mol%), Glx (11.10 mol%), Ser (10.80 mol%), Gly (8.56 mol%) and Leu (7.20 mol%) (Banasaz & Ferraro, 2024). In terms of secondary structure, the whole wool fibres comprise roughly 58% α-helices, 38% β-sheets and 4% disordered structures (Cardamone, 2010). The outer layer of wool, known as the cortex, constitutes approx. 85% of the fibre's mass and is composed of intermediate filament proteins, characterized by a predominantly α-helical structure (Sharma & Kumar, 2019). These filaments are embedded within a high-sulfur protein matrix formed by keratin-associated proteins, which are rich in sulfur or glycine/tyrosine residues and organized in β-sheet structures that support the filament-matrix architecture (Cardamone, 2010). Wool shares similarities with other mammalian soft keratins, showing moderate stiffness and good flexibility. Two major fractions of wool keratin are distinguished based on sulfur content: a low-sulfur fraction (50-60% of fibre

mass) derived from the microfibrils, and a high-sulfur fraction (20-30%) originating from the matrix (Cardamone, 2010). The predominant α-keratin in wool includes two principal subunits: Type I acidic (40-50 kDa) and Type II basic/neutral (55-60 kDa) (Sharma & Kumar, 2019).

2.2.4. Pig hair

An average pig produces about 0.9 kg of hair, which consists primarily of keratin (approx. 90%) (Sharma & Kumar, 2019). In Europe alone, where around 110 million pigs are raised, the annual production of pig hair is estimated at 99 000 tonnes (Banasaz & Ferraro, 2024). Pig hair keratin shares similarities with other mammalian keratins, such as those in wool and human hair, and is also based on α-keratin (Sharma & Kumar, 2019). Very few studies are available on the detailed physicochemical analysis of pig hair. However, existing data indicate that the most abundant amino acid residues in pig hair keratin are Glx (12.55 mol%), Ser (11.30 mol%), Cys (10.75 mol%), Gly (9.25 mol%) and Arg (7.65 mol%) (Banasaz & Ferraro, 2024). Secondary structure analysis of pig hair keratin revealed the presence of roughly 38% α-helices, 32% β-sheets, 19% β-turns and about 10% disordered regions (Mohan et al., 2022). Two principal subunits of α-keratin have been identified in pig hair keratin: Type I, with MWs between 45-50 kDa, and Type II, with MWs between 55-60 kDa. These structural features, along with the high Cys content, impart mechanical behaviour similar to that of wool (Banasaz & Ferraro, 2024; Wang, Yang et al., 2016).

2.3. Methods used for keratin extraction

2.3.1. Introduction

Keratin is increasingly recognised for its potential in various applications such as bioactive peptides, biomedical materials, cosmetic formulations and biodegradable films (Moktip et al., 2025; Sinkiewicz et al., 2018). However, the strongly cross-linked structure of native keratin renders it insoluble and chemically inert. Effective keratin extraction methods require the application of intensive chemical, biological and/or physical treatments (Figure 9) to disrupt its rigid architecture, enabling solubilization for downstream applications (Ossai et al., 2022; Shavandi et al., 2017). Many methods of keratin extraction have been developed, varying in complexity, economic viability, and their impact on the physicochemical and biological properties of the protein preparations obtained. The choice of extraction method plays a critical role in the efficiency of keratin recovery, preserving its functional integrity, molecular structure and bioactivity (Ferraro et al., 2016; Sinkiewicz et al., 2018). Consequently, optimizing extraction conditions is crucial for tailoring keratin-based products for specific applications.

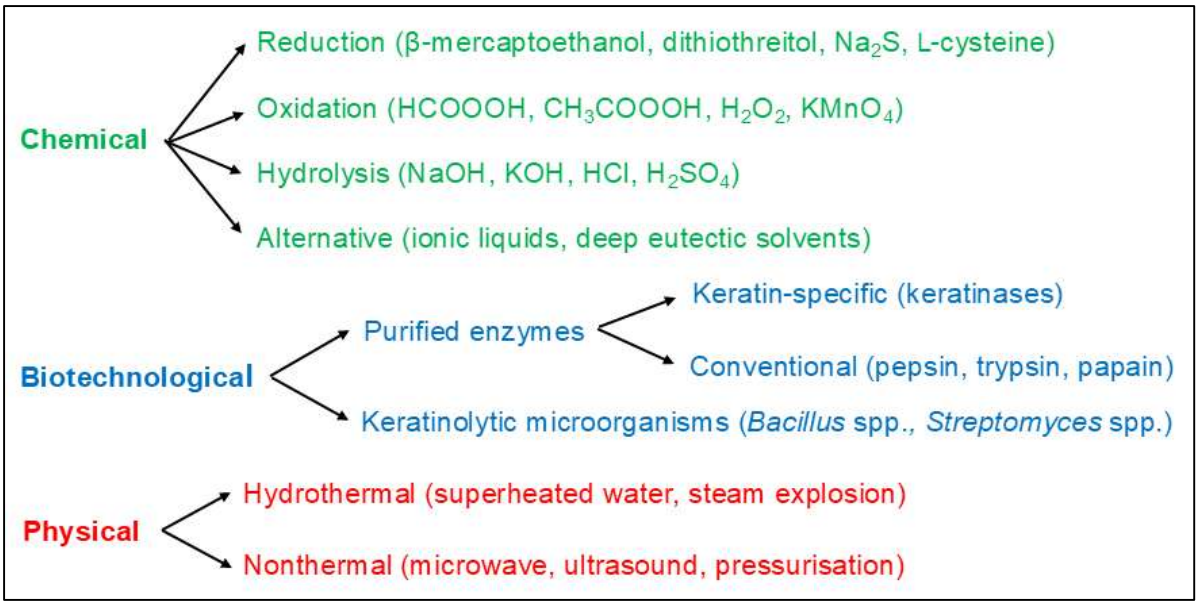

Figure 9. Overview of methods used for keratin extraction. Based on Shavandi et al. (2017), Sinkiewicz et al. (2018) and Moktip et al. (2025).

2.3.2. Chemical methods

Chemical methods are widely used and effective approaches for extracting keratin. These methods rely on chemical agents that disrupt disulfide, hydrogen and peptide bonds, thereby converting insoluble native keratin into soluble protein preparations. Depending on the reagents used and reaction conditions, chemical treatments yield keratin preparations with different MW, functional groups, techno-functional properties and bioactivities. The most commonly applied methods include disulfide bond reduction or oxidation, as well as chemical hydrolysis using acids or alkalis (Moktip et al., 2025; Sinkiewicz et al., 2018).

Reductive extraction is among the oldest keratin isolation techniques and one of the most established approaches for the protected keratin extraction, i.e., preferential cleavage of cross-links (disulfide and hydrogen bonds) while minimizing peptide bond fragmentation (Shavandi et al., 2017). The principle of this method involves reversible cleavage of the disulfide linkages (-S-S-) within the keratin macromolecule, resulting in thiolated derivatives – primarily cysteine thiol residues (-Cys-SH) and, to a lesser extent, cysteine-S-sulfonate residues (-Cys-SSO_3^-), also known as Bunte salts (Moktip et al., 2025). The most commonly used reducing agents include β-mercaptoethanol, dithiothreitol, Na_2S and L-Cys. The keratin derivatives resulting from reductive extraction are referred to as kerateines. Upon removal of the reducing agent, the reduced disulfide bonds can be re-oxidised, resulting in secondary cross-linking, thus allowing for prolonged biological persistence *in vivo*, lasting weeks to months (Sinkiewicz et al., 2018). Reductive extraction provides high keratin yield and the

capacity to produce keratin isolates in which the proteins are solubilized due to partial unfolding, yet maintain the continuity of their peptide backbone. However, access to disulfide bonds is hindered by keratin's compact hydrogen-bonded network, thus usually necessitating the use of chaotropic agents, such as urea, thiourea, transition metal hydroxides or surfactants, to enable effective reduction. These agents increase the chemical complexity of the extraction solution and raise concerns regarding environmental safety and cost, especially when using conventional reductants like β-mercaptoethanol, dithiothreitol and Na_2S (Shavandi et al., 2017; Sinkiewicz et al., 2018). A more sustainable alternative to the traditional reducing agents can be L-Cys (papers **A2-A4**), a naturally occurring amino acid regarded as food-grade, non-toxic and relatively inexpensive. Keratin preparations obtained through L-Cys extraction exhibit high potential for food and pharmaceutical applications, where biocompatibility is critical (Ghaffari-Bohlouli et al., 2023; Pourjavaheri et al., 2019; Taraszkiewicz, Sinkiewicz, et al., 2025; Taraszkiewicz, Sommer, et al., 2025; Wang, Li et al., 2016; Xu & Yang, 2014; Zhang et al., 2022).

The mechanism of **oxidative extraction**, another common chemical method of keratin isolation, involves the irreversible destruction of disulfide bonds using oxidizing agents, which leads to keratin solubilization. During this process, Cys residues undergo stepwise oxidation to more hydrophilic forms – sulfenic acid (-SOH), sulfinic acid ($-SO_2H$) and ultimately cysteic acid (also known as cysteine sulfonic acid, $-SO_3H$) (Banasaz & Ferraro, 2024; Sinkiewicz et al., 2018). Reagents commonly used for this method include organic peracids (e.g. peracetic and performic acid), hydrogen peroxide and potassium permanganate. The oxidised keratin derivatives, known as keratoses, are generally more polar and hygroscopic compared to their reduced counterparts. However, they show lower stability under acidic or alkaline conditions, are incapable of secondary cross-linking and are prone to rapid *in vivo* degradation, typically within a few days to a few weeks (Banasaz & Ferraro, 2024; Sinkiewicz et al., 2018). Moreover, the keratin yield from oxidative extraction is generally lower than in the case of reductive extraction and the aggressive nature of oxidative agents poses risks of unintended side reactions in the extracted protein, resulting in destruction of Cys, His, Met, Phe, Ser, Thr, Trp and Tyr (Banasaz & Ferraro, 2024; Ghaffari-Bohlouli et al., 2023).

Acid/alkaline hydrolysis, previously described in terms of its applications for bioactive peptide production, serves as one of the most straightforward methods of keratin extraction. Keratin is easily solubilized through hydrolysis in strong acids and alkalis, however, the recovered products differ substantially from intact keratin (Sinkiewicz et al., 2018). In contrast to reductive and oxidative extractions, which allow for a more selective cleavage of cross-links, chemical hydrolysis also severely disrupts the peptide backbone in a non-specific manner, resulting in hydrolysates consisting of amino acids, peptides, peptones, proteoses, salts and

sulfides (Ossai et al., 2022; Sinkiewicz et al., 2018). Acidic hydrolysis proceeds rapidly and with high efficiency but leads to the degradation Trp, Ser, Thr, Tyr, Cys, Asn and Gln, with the final composition of the hydrolysate strongly influenced by reaction time and substrate characteristics (Shavandi et al., 2017; Sinkiewicz et al., 2018). Alkaline hydrolysis is slower than acidic but allows for the recovery of nearly all amino acids present in the native keratin when conducted under mild conditions. However, when conducted under more aggressive conditions or prolonged reaction time, it can lead to the degradation of Asn, Arg, Ser, Thr and Gln, as well as induce chemical modifications such as deamination, desulfuration, and the formation of non-proteinogenic residues like lysinoalanine, ornithine and lanthionine. In addition, the cleavage of disulfide bonds during the process may generate an unpleasant sulfide odour (Banasaz & Ferraro, 2024; Shavandi et al., 2017; Sinkiewicz et al., 2018). Although poorly suited for the recovery of structurally intact peptides, chemical hydrolysis continues to find utility in large-scale operations where high solubility and low MW fractions are prioritized over structural integrity. Such hydrolysates have found application in the development of organic fertilizers, media for microbial cultivation and biodegradable films used as packaging materials (Sinkiewicz et al., 2018; Song et al., 2014; Stiborova et al., 2016).

Alternative approaches to keratin solubilization, e.g., using ionic liquids and deep eutectic solvents, have also been explored in recent years. These methods offer improved selectivity and milder reaction conditions, facilitating the partial preservation of labile amino acids and reducing the environmental burden. However, their practical application remains limited due to high cost, high solvent viscosity, and challenges related to product recovery and purification (Banasaz & Ferraro, 2024; Moktip et al., 2025).

2.3.3. Biotechnological methods

Biotechnological approaches to keratin extraction rely on enzymatic reactions that fragment the complex protein matrix. These methods include: fermentation with keratin-degrading microbial strains, enzymatic hydrolysis using purified keratinolytic enzymes (keratin-specific proteases, keratinases) and enzymatic hydrolysis using purified proteolytic enzymes (conventional proteases). Regardless of method, and just like with chemical extraction methods, structural disruption of native keratin via chemical or physical pre-treatment improves hydrolysis efficiency (Moktip et al., 2025; Ramnani & Gupta, 2007; Stiborova et al., 2016).

Microbial hydrolysis, previously described in terms of bioactive peptides formation, is among the most widely utilized methods of keratin solubilization. It employs keratinolytic microorganisms secreting enzymes that degrade native keratin, typically under mesophilic to thermophilic conditions (30-70 °C) and neutral to moderate alkalinity (pH 7-11) (Moktip et al., 2025; Nnolim et al., 2020). The mechanism of microbial keratinolysis is not fully known, but

two routes were proposed. Kunert (1976) described a two-step mechanism for *Microsporum gypseum*, consisting of sulfitolysis and proteolysis. According to this author, the fungi first produce HSO_3^- ions (originating from Cys catabolism) that break the disulfide bonds, leading to keratin denaturation, and then, the peptide bonds are broken by keratinases (endopeptidases) (Figure 10A). The second keratinolysis mechanism, proposed by Yamamura et al. (2002) for *Stenotrophomonas* sp. D-1, involves the reduction of disulfide bonds catalysed by disulfide reductases, followed by peptide bond hydrolysis by serine proteases (Figure 10B). Many keratinolytic organisms have been discovered, among which *Bacillus* spp., particularly *B. licheniformis*, *B. subtilis* and *B. pumilus*, are the most studied and considered promising for industrial application due to the Generally Recognized as Safe (GRAS) status and relatively high enzymatic activity (Nnolim et al., 2020). Other important examples of keratinolytic microbes are *Streptomyces*, *Actinomadura* and *Nocardiopsis*, known for their tolerance to extreme conditions (up to 70 °C, pH 10-11) (Kornikłowicz-Kowalska & Bohacz, 2011; Moktip et al., 2025). Thermophilic strains such as *Fervidobacterium islandicum* and *Thermoactinomyces* spp. demonstrated high keratin-degrading activity at temperatures > 80 °C (Moktip et al., 2025; Saeed et al., 2024).

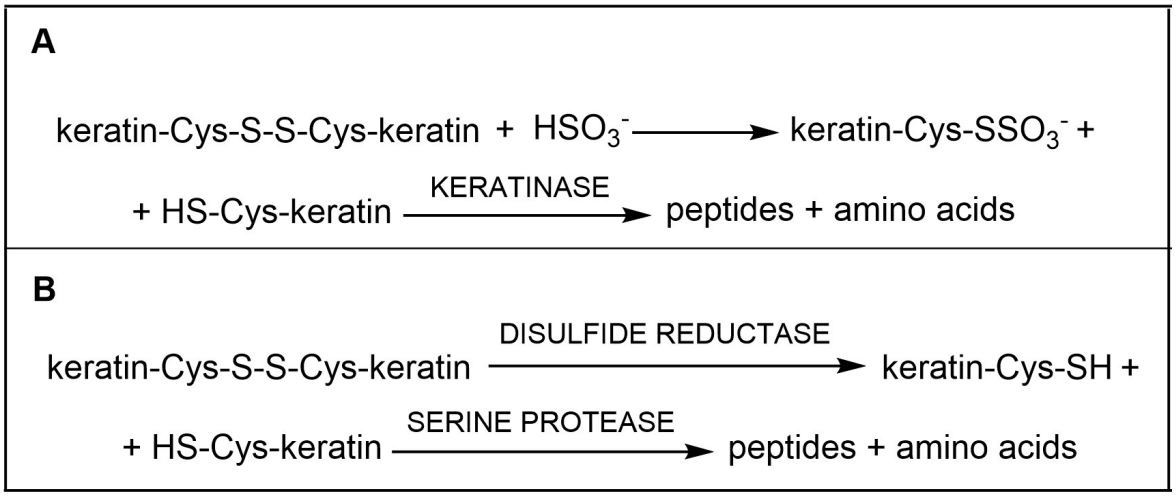

Figure 10. Mechanisms of microbial keratinolysis proposed by **A)** Kunert (1976) and **B)** Yamamura et al. (2002).

Enzymatic hydrolysis using **purified keratinolytic enzymes** offers a targeted tool for keratin degradation. They often exhibit both proteolytic activity (cleaving peptide bonds) and sulfitolytic/reducing activity (disrupting disulfide bonds), which enables more effective breakdown of keratin, compared to non-specific (conventional) proteases (Moktip et al., 2025; Nnolim et al., 2020). Commercial keratinases are usually derived from *B. licheniformis*, *B. subtilis*, *Streptomyces* spp. and *Fervidobacterium* spp. (Moktip et al., 2025; Nnolim et al., 2020; Saeed et al., 2024). However, most are not practical for industrial applications due to the high cost and low activity (Saeed et al., 2024).

Enzymatic hydrolysis using **purified proteolytic enzymes** (conventional proteases), such as pepsin, trypsin and papain, has also been explored for keratin hydrolysis. While conventional proteases are inexpensive and widely available, their application in keratin hydrolysis remains limited by their inability to disrupt disulfide bonds. However, when used on pre-treated substrates or in the presence of reducing agents, they may offer a cost-effective solution for producing broad-spectrum bioactive peptide mixtures (Callegaro et al., 2019; Guo et al., 2020; Ramnani & Gupta, 2007; Sinkiewicz et al., 2018).

2.3.4. Physical methods

Physical methods for keratin solubilization rely on the application of thermal or mechanical energy that opens the tightly cross-linked structure, thereby facilitating its conversion into soluble fragments. These approaches are often used in combination with chemical or biotechnological treatments to improve extraction efficiency and shorten processing time (Perța-Crișan et al., 2021; Shavandi et al., 2017; Sinkiewicz et al., 2018). Key techniques include mechanical comminution (e.g. grinding or milling), which increases surface area and exposes intra-fibrillar disulfide bonds (Giteru et al., 2023; Sharma & Kumar, 2019), hydrothermal treatment using pressurized water or steam at 140-220 °C and 0.1-3 MPa, which disrupts supramolecular structures, as well as microwave, ultrasound and pressure-assisted extractions, which facilitate matrix breakdown and improve solubilization efficiency (Ossai et al., 2022; Sinkiewicz et al., 2018). Although considered eco-friendly due to minimal or no use of chemical reagents, these methods are inherently associated with partial breakdown of the peptide backbone, drastic Cys depletion, deterioration of amino acids susceptible to thermal processing and a decline in MW of extracted keratin (Perța-Crișan et al., 2021; Sinkiewicz et al., 2018).

RESEARCH PART

3. Aim, hypotheses and scope

3.1. Aim

The dissertation aimed to assess the suitability of chicken feather keratin as a precursor of bioactive peptides and MRPs through a hybrid *in silico-in vitro* approach.

3.2. Hypotheses

Based on literature reports and insights gained from pilot experiments, the following research hypotheses were formulated:

H1. *In silico* methods can accurately predict the results of feather keratin hydrolysis with commercial proteases, enabling rational pre-selection of enzymes for *in vitro* hydrolysis.

H2. Mild L-Cys reduction followed by hydrolysis with conventional proteases can efficiently convert waste chicken feathers into water-soluble, low MW peptides with enhanced bioactivity and desirable techno-functional properties.

H3. A two-step peptide release approach involving mild L-Cys reduction followed by enzymatic hydrolysis selectively relaxes keratin's secondary structure and cleaves its primary structure, respectively.

H4. Mild thermal treatment of keratin hydrolysate in the presence of D-Glc or D-Xyl can generate nutritionally safe MRPs with improved antioxidant properties and meat-like aroma.

H5. The sequential application of keratin reduction, proteolysis and Maillard reaction can act synergistically to enhance its antioxidant potential.

H6. Keratin-derived peptides and MRPs can remain bioaccessible, nutritionally safe and retain antioxidant activity after SGID, supporting their potential use as functional ingredients in nutraceutical formulations.

3.3. Research tasks

To achieve the adopted aim and verify the research hypotheses, the following research tasks were planned:

1. *In silico* analysis of chicken feather and pig hair keratin sequences to predict peptide release profiles, assess potential bioactivities and toxicity, and identify optimal conventional proteases for subsequent *in vitro* hydrolysis.
2. Production of a high-purity, water-soluble, digestible keratin isolate (KI) with preserved peptide backbone from chicken feathers using reductive extraction with L-Cys.

3. *In vitro* enzymatic hydrolysis of KI using trypsin, pepsin, chymotrypsin, subtilisin and papain to obtain water-soluble hydrolysates rich in low MW peptides.
4. Production of MRPs by reacting the enzymatic keratin hydrolysate with D-Glc or D-Xyl under controlled thermal conditions.
5. Evaluation of the techno-functional, structural and thermal properties of the obtained preparations at different processing stages.
6. *In vitro* assessment of the antioxidant, cytotoxic and mutagenic potential of the obtained preparations at different processing stages.
7. Evaluation of antioxidant activity, nutritional safety, bioaccessibility and digestibility of selected keratin preparations from different processing stages under physiologically relevant conditions using the INFOGEST SGID protocol.

The overview of research workflow and analytical methods applied in the original papers constituting the dissertation is presented in Figure 11.

3.4. Justification

Developing efficient methods for the valorisation of feather waste aligns with circular economy principles while providing an opportunity to produce bioactive peptides. The amino acid profile of feather keratin combines features strongly associated with health-promoting biological effects, naturally predisposing it to yield potent peptides with antioxidant, antihypertensive, antidiabetic and neuroprotective properties, among others. However, enzymatic hydrolysis, the preferred method of peptide production, has been largely ineffective due to the keratin's highly cross-linked structure, while specific keratinases remain impractical owing to their high cost and insufficient standalone activity. Thus, keratin-derived peptide release has been typically relied on harsh chemical/physical treatments or fermentation methods, which risk compromising product quality and functionality. The dissertation explores a novel approach for controlled peptide release, in which keratin is first reduced with L-Cys and then hydrolysed with conventional proteases. Moreover, keratin's high Cys and low Lys content create favourable conditions for Maillard-driven enhancement of its bio-functional properties, previously unexplored. The former promotes the formation of MRPs with pleasant, meat-like aroma (Lasekan et al., 2013) and suppresses acrylamide generation (Augustine & Bent, 2022), while the latter limits the essential amino acid losses during glycation (van Lieshout et al., 2025). As the nutraceutical potential of peptides depends on their stability during gastrointestinal digestion, this challenge was directly addressed in the dissertation. For the first time, the INFOGEST protocol, the most accurate static model of human-like digestion, was applied to feather keratin-derived peptides. This allowed for systematic evaluation of their activity, bioaccessibility and nutritional safety across all processing stages (isolate, hydrolysate, MRP), providing novel insights into their suitability for nutraceutical applications.

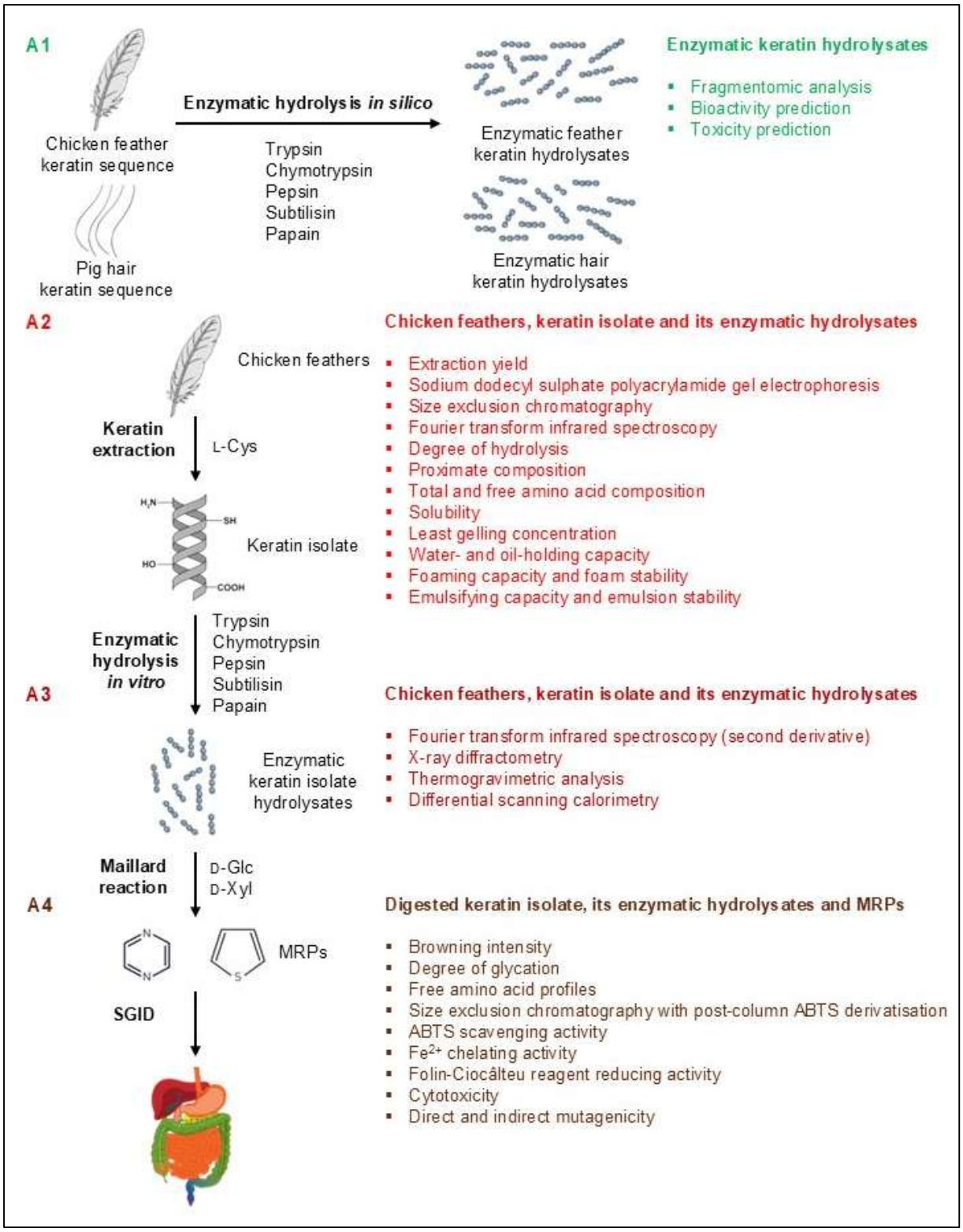

Figure 11. Overview of the research workflow and main analyses described in the original papers (**A1**-**A4**) constituting the doctoral dissertation. MRPs – Maillard reaction products, SGID – simulated gastrointestinal digestion.

4. Overview of papers constituting the doctoral dissertation

4.1. [Paper A1] Prediction of bioactive peptides from chicken feather and pig hair keratins using *in silico* analysis based on fragmentomic approach

An *in silico* analysis of the primary structure of chicken feather and pig hair keratins (paper **A1**) established the computational foundation of the dissertation. Initially, whole keratin sequences were analysed for the presence of known bioactive peptides using the BIOPEP-UWM database. The occurrence of bioactivity types was quantified as the frequency of bioactive fragments (parameter A), i.e., the number of annotated bioactive sequences relative to the total amino acid count in the intact keratin chain. Fifteen and ten different bioactivities were present in the chicken feather and pig hair keratin sequences (Table 1, **A1**), respectively, pointing to considerable latent functionality potentially available for release in both proteins.

In the next step, both keratins were hydrolysed *in silico* with 5 conventional proteases, i.e., trypsin, chymotrypsin, pepsin, subtilisin and papain, using the BIOPEP-UWM “Enzyme(s) action” tool. The predicted hydrolysates were assessed in terms of theoretical DH (DH_t), the occurrence of fragments bearing known bioactivities (fragmentomic analysis), bioactivity probability (assignment of PeptideRanker score) and safety (toxicity prediction with ToxinPred). This workflow was designed to guide the selection of proteases predicted to yield short, non-toxic peptides that are either intrinsically bioactive or enriched in functionally annotated motifs (with respective “bioactive” or “parent” labels in Table 2, **A1**).

Protease choice markedly shaped the theoretical product profile. For both chicken feather and pig hair keratins, the order of protease effectiveness in hydrolysing the proteins, expressed as DH_t, was: pepsin > papain > subtilisin > chymotrypsin > trypsin. However, release of bioactive peptides was not proportional to DH_t – the most extensive pepsin cleavage generated large amounts of free amino acids, reducing the share of short sequences with functional potential. The toxicity screening involving the ToxinPred tool identified no toxic sequences, regardless of the enzyme used; therefore, emphasis was placed on proteases that theoretically yielded short peptides enriched in bioactive motifs rather than on maximising DH_t. This criterion pointed to subtilisin and papain as the preferred proteases for keratin hydrolysis. Detailed summaries of fragmentomic analysis are provided in Tables 3-7 (**A1**) for chicken feather keratin and Tables 8-12 (**A1**) for pig hair keratin.

Bioactivity profiling of theoretical keratin hydrolysates showed that ACE and DPP-IV inhibitory motifs were the most frequent, and many peptides exhibited both of these activities. This finding mirrors their highest representation in the BIOPEP-UWM database. The keratins were also found be a potential source of peptides with POP inhibitory activity, due to the presence of POP inhibitory motifs (Tables 3-12, **A1**) and their high Pro content. The co-

occurrence of multiple bioactivities within the same sequences highlights the potential of keratin as a source of multifunctional peptides. Such peptides are particularly attractive because they can modulate several physiological pathways. Specific examples of such peptides predicted to be released from keratins were the dipeptides Pro-Gly and Gly-Pro annotated with five activities, i.e., DPP-IV, ACE and POP inhibition as well as antithrombotic and gastric mucosal regulatory effects. These dipeptides have been reported previously in collagen hydrolysates and shown to be bioavailable in humans (Sato et al., 2019), which supports their translational relevance. Chicken feather keratin has already been recognised as a source of bioactive peptides, with several studies describing their release and activity, whereas pig hair keratin remains completely unexplored in this regard.

The *in silico* analysis results indicated that both chicken feathers and pig hair have a potential to serve as precursors of bioactive peptides. However, due to the priority of valorising the waste stream of greatest environmental relevance and the superior availability and production scale of the former, the experimental work (papers **A2**-**A4**) focused exclusively on chicken feather keratin, with pig hair keratin not pursued further.

4.2. [Paper A2] Chemical composition and techno-functional properties of high-purity water-soluble keratein and its enzymatic hydrolysates

The first objective of paper **A2** was to establish L-Cys reduction conditions for chicken feathers that maximised keratin extraction yield, while ensuring water solubility, preserving peptide bond integrity and amino acid quality. The target product was a high-purity keratin extract that could be used directly as a food protein and serve as a substrate for the enzymatic release of potentially food-grade bioactive peptides. Both urea-containing and urea-free extraction solutions were tested across a range of L-Cys concentrations, pH values, temperatures and times. Conditions of feathers treatment with both of these solutions were optimized individually, considering the distinct requirements of each approach suggested by prior studies. The presence of 8 M urea facilitates keratin solubilisation by disrupting hydrogen bonds and unfolding the protein, which renders disulfide bonds more accessible to reduction by L-Cys. In this case, the keratin yield was determined under conditions of 1.5 or 2.0% L-Cys, pH 9.0 or 10.5, temperatures between 30 and 70 °C and reaction times from 1 to 6 h, as guided by earlier reports on successful application of urea-containing extraction solutions (Pourjavaheri et al., 2019; Sinkiewicz et al., 2017; Xu & Yang, 2014). On the other hand, the absence of urea necessitated more aggressive reaction conditions to achieve efficient disruption of disulfide bonds. In this case, the keratin yield was quantified under conditions of 1, 1.5 or 2.0% L-Cys, pH values of 10-12 and a prolonged reaction time of 24 h. The temperature was restricted to 30 °C to reduce the risk of peptide degradation under the stronger alkalinity. These conditions were selected based on the prior study on wool keratin (Zhang et al., 2022), which

demonstrated that high pH and long reaction time are essential for efficient urea-free extraction.

In the urea-containing extraction solution, only the pH had a statistically significant effect on keratin yield, while other parameters had no such effect (Table S1 and S2, **A2**). In the urea-free extraction, both L-Cys concentration and solution pH significantly affected the keratin yield (Table S3, **A2**). Although the efficacy of keratin extraction under the most severe urea-free conditions (2% L-Cys, pH 12) was almost as high as with the urea-containing solution, with respective yields of 88 and 90%, all urea-free extracts were severely degraded, likely due to concurrent alkaline hydrolysis (Fig. 1B, **A2**). In contrast, the keratin extract obtained by 1.5% L-Cys in 8 M urea, pH 10.5 at 30 °C for 1 h had intact protein chains (Fig. 1A, KI, **A2**). Preservation of the peptide backbone and high purity of this extract were further confirmed by HP-SEC, FT-IR, free amino groups quantification via o-phthaldialdehyde (DH_{OPA}) assay, proximate composition analysis, as well as total and free amino acid analyses. HP-SEC indicated mainly intact polypeptide chains with an average MW > 9 kDa and only minor traces of lower MW fragments (Fig. 1C,D, **A2**). A comparison of FT-IR spectra between native feather keratin and the extract confirmed the latter's structural integrity and chemical purity (Fig. 2, **A2**). Consistently, the OPA assay showed minimal formation of free amino groups, yielding a DH_{OPA} value < 1% (Table 1, **A2**). Proximate composition analysis indicated a minor increase in ash content compared to feathers, likely resulting from residual NaOH remaining post-dialysis (Table 1, **A2**). Urea was either fully removed or remained only in trace amounts, as the proximate composition analysis showed that 91% of the extract consisted of organic compounds (degrading in the 102-550 °C range), and the total amino acid analysis, the most accurate protein quantification method recommended by the Food and Agriculture Organization of the United Nations (FAO) (Hayes, 2020), confirmed 91% protein content. Total amino acid analysis indicated retention of both essential and non-essential residues relative to feathers, whereas only trace amounts of free amino acids were detected (Table 3, **A2**). As the keratin extract obtained using 1.5% L-Cys in 8 M urea, pH 10.5, at 30 °C for 1 h combined high extraction yield with preservation of the peptide backbone, high protein purity, and minimal degradation, it was selected from among all others for further work and designated as the keratin isolate (KI).

Once optimal extraction conditions had been established, the resulting KI was hydrolysed *in vitro* with the same conventional proteases as those considered in the *in silico* study (paper **A1**), i.e., trypsin, chymotrypsin, pepsin, subtilisin and papain, to verify the predictions. During preliminary experiments, both hydrolysis reaction conditions (substrate concentration, E/S ratio, temperature, time, pH, titrant) and enzyme inactivation conditions (temperature, time) were optimized to maximise the content of low MW peptides while

maintaining water solubility. The final hydrolysate production conditions common for all enzymes were: KI concentration of 10 mg protein/mL, initial reaction volume of 100 mL, E/S ratio of 1:20 (m/m), hydrolysis time of 1 h, followed by thermal inactivation at 85 °C for 15 min. Enzyme-specific hydrolysis conditions, i.e., pH, temperature and titrant, are summarised in Table 3. Under these conditions, all hydrolysates remained water-soluble both immediately after thermal inactivation and after redissolving the freeze-dried powder. Moreover, the DH was unaffected by the form in which KI was used – the same DH values were obtained when KI was hydrolysed directly after dialysis, after a single freeze-thaw cycle, or after redissolving the freeze-dried isolate (data not shown). Hydrolysis with papain was also attempted under different conditions (pH 7-8, 50-70 °C, E/S ratio 1:20-1:100, 1-3 h) but consistently yielded DH < 2% accompanied by extensive protein aggregation (data not shown). Hence, papain was excluded from subsequent experiments.

Table 3. Conditions for *in vitro* hydrolysis of keratin isolate (KI) with different enzymes.

Parameter	Hydrolysate name			
	KI-T	**KI-C**	**KI-P**	**KI-S**
Enzyme name	trypsin	chymotrypsin	pepsin	subtilisin
Hydrolysis titrant	0.1 M NaOH	0.1 M NaOH	1.0 M HCl	1.0 M NaOH
Hydrolysis pH	8	8	2	9
Hydrolysis temperature [°C]	37	37	37	60

The KI and its enzymatic hydrolysates were characterised in terms of chemical composition and techno-functional properties. Two methods for the determination of the DH were used. The first one was spectrophotometric assay (DH_{OPA}), applicable for soluble fractions only, but regardless of hydrolysis pH (Rutherfurd, 2010), i.e., KI, KI-T, KI-C, KI-P and KI-S. The second one was pH-stat titration ($DH_{pH\text{-}stat}$), suitable regardless of protein solubility but only in the case of proteolysis carried out at pH ≥ 7 (Rutherfurd, 2010), i.e., KI-T, KI-C, KI-S and papain hydrolysates. Significant differences in DH were observed among all keratin preparations (Table 1, **A2**). Experimental DH values for trypsin, chymotrypsin and subtilisin were in close accord with DH_t determined *in silico* whereas pepsin substantially underperformed, indicating limited protease access to pepsin-susceptible bonds in the KI. Papain, despite high DH_t *in silico*, was ineffective *in vitro*.

The results of SDS-PAGE (Fig. 1A, **A2**), HP-SEC (Fig. 1D, **A2**) and DH (Table 1, **A2**) measurements consistently indicated that the order of protease effectiveness in hydrolysing the KI was: subtilisin > pepsin > chymotrypsin > trypsin. The average MW of KI hydrolysates based on HP-SEC data was very strongly inversely correlated with their DH, regardless of DH assay. Such a result indicated that MW reduction arose exclusively from peptide bond scission. The intended outcome of the two-step peptide release approach was therefore achieved – the

L-Cys extraction resulted primarily in the breakdown of disulfide and hydrogen bonds, while enzymatic hydrolysis allowed for selective peptide bond scission.

Unlike the keratin extracted by the original L-Cys reduction protocol proposed by Xu & Yang (2014), the KI obtained using 1.5% L-Cys in 8 M urea, pH 10.5 at 30 °C for 1 h, followed by dialysis and lyophilisation was readily water-soluble and therefore more amenable to controlled enzymatic hydrolysis. Techno-functional profiles of the KI hydrolysates were enzyme- and DH-dependent. KI-P retained a low-solubility window comparable to KI, though its isoelectric point shifted towards higher pH, whereas the remaining hydrolysates were clearly more soluble than KI (Fig. 3A, **A2**). Gelation capacity decreased proportionally with average MW – KI exhibited the lowest gelling concentration at 5%, while KI-P and KI-S, composed of the smallest peptides, did not gel even at 20% (Fig. 3B, **A2**). Water-holding capacity was generally low and inversely related to solubility, and oil-holding was high in KI but declined sharply with DH in the hydrolysates (Fig. 3C, **A2**). All preparations were capable of forming foams, but foaming capacity decreased with increasing DH and was strongly pH-dependent. KI consistently showed the highest foaming ability across the tested pH range, exceeding that of all hydrolysates (Fig. 3D, **A2**). Each preparation also formed emulsions upon homogenisation with oil, yet after centrifugation, stable emulsions were observed only in seven pH variants (spanning the tested pH range). This indicates that emulsifying properties were less robust than foaming properties. Emulsifying capacity showed only a moderate correlation with foaming and no straightforward dependence on solubility – for example, despite limited solubility at pH 5, both KI-T and KI-C still produced measurable emulsions at this pH (Fig. 3E, **A2**). Together, these results indicate that enzymatic hydrolysis generally weakened gelling, foaming and oil-binding properties of KI, while only moderately altering emulsifying behaviour.

The total amino acid profile of KI combined high Cys, supporting antioxidant potential, and high Pro content, favourable for generating potent and bioavailable peptides, together with a high share of hydrophobic residues that often feature in bioactive sequences (Table 3, **A2**). However, low content of some essential amino acids, i.e., His, Lys, Met and Trp, indicated that nutritional applications may benefit from their supplementation. Altogether, the findings of paper **A2** highlight that careful optimisation of every processing step is essential for producing high-quality, bioactive keratin preparations from waste feathers. To further elucidate the physicochemical changes induced by extraction and subsequent proteolysis, chemical/crystalline structure and thermal properties of the resulting keratin preparations were studied, and the results were described in paper **A3**.

4.3. [Paper A3] Changes in the structure and thermal properties of feather keratin induced by L-Cys extraction followed by enzymatic hydrolysis

In the first part of paper **A3**, the effect of reductive extraction under the previously optimised conditions (1.5% L-Cys, 8 M urea, pH 10.5, 30 °C, 1 h) on keratin's chemical and crystalline structure and on its thermal properties was examined. Although the chemical structure of the keratin preparations, as inferred from FT-IR spectra, was briefly described in paper **A2**, paper **A3** provided a substantially expanded analysis. Difference spectra and second-derivative procedure were applied to resolve overlapping bands and capture subtle conformational shifts, and the results were integrated with complementary X-ray diffraction (XRD), thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) data.

The detailed analysis of FT-IR spectral changes caused by the L-Cys extraction process confirmed that KI retained a native-like chemical structure and exhibited mainly conformational relaxation without extensive scission of the peptide backbone (Fig. 1, **A3**). KI showed a broadened ν_{OH}/ν_{NH} band indicating weaker hydrogen bonding, reduced ν_{CH2} signals consistent with the minor peptide backbone disruption (Fig. 1A, **A3**), altered disulfide cross-linking pattern, increased random coil content at the expense of α-helix and β-sheet (Fig. 1B, **A3**), redistribution within the content of β-structures (antiparallel and parallel β-strands) and decrease of α-helix content (Fig. 1D, **A3**). Subsequent XRD analysis confirmed the partial deconstruction of α-helix and β-sheet, an increased content of β-sheets, reduced crystallite size and a decrease in interplanar spacing consistent with post-extraction rearrangement of polypeptide packing (Fig. 1E, **A3**). The crystallinity index (CrI) declined by 44% after extraction, yet the presence of discernible peaks in the KI diffractogram confirmed that some crystallinity was retained.

The changes in keratin structure post-extraction were accompanied by altered thermal behaviour. In KI, the minor derivative thermogravimetry (DTG) minimum at 48 °C disappeared, consistent with easier release of bound water and relaxed hydrogen bonding, and the major DTG minimum at 327 °C shifted to a lower temperature, indicating an earlier onset of thermal decomposition (Fig. 2A, B, **A3**). Although the thermal decomposition pattern post-extraction was retained, KI lost less mass up to 460 °C but more above 460 °C, resulting in ~12% higher total mass loss up to 850 °C (Table 2, **A3**). The post-extraction transition of keratin crystal structure into a less ordered one was also confirmed by DSC analysis. In the first scan (30-105 °C), both feathers and KI exhibited a broad endotherm associated with loss of hydrogen-bonded water. In the KI thermogram, its maximum was shifted to a lower temperature, indicating hydrogen bond rearrangement that facilitated water release (Fig. 2C, **A3**). In the second scan (0-350 °C), the sharp α-helix endotherm at 238 °C seen for feather thermogram became broader and shifted to a lower temperature in the KI thermogram, consistent with

partial loss of α-helical crystallites (Fig. 2D, **A3**). A second endotherm at 245-275 °C, assigned to melting or denaturation of crystalline phases, and a third endotherm at 280-320 °C, indicative of either thermal degradation of disulfide bonds, helix denaturation or total decomposition of keratin, occurred in both feathers and KI.

The second part of **A3** examined how enzymatic hydrolysis of KI with trypsin, chymotrypsin, pepsin and subtilisin affected its chemical/crystalline structure and thermal behaviour. Comparative analysis of the FT-IR spectra of KI and hydrolysates indicated almost no changes in KI-T, minor changes in KI-C and KI-P, and significant changes in KI-S (Fig. 3A-M, **A3**), consistent with progressive peptide bond cleavage and the specificity characteristics of the enzymes. The hydrolysis with the first three enzymes had minimal effect on the secondary structure of keratin, while hydrolysis by subtilisin caused a substantial secondary structure loss (the biggest enrichment in β-turns and unordered coils) (Fig. 3M, **A3**), along with formation of sodium carboxylates (Fig. 3K, **A3**). XRD analysis of hydrolysates confirmed that treatment of KI with enzymes deepened the disruption of α-helix and β-sheet structures, further decreased the keratin's crystal sizes and decreased the CrI, with the biggest changes observed in the KI-S (Fig. 3N, **A3**). The CrI of hydrolysates correlated negatively with their DH and positively with their average MW, linking keratin fragmentation to diminished intermolecular ordering and reduced crystallinity.

Enzymatic hydrolysates showed similar thermal behaviour to that of KI, although the onset of thermal decomposition occurred earlier, with the main DTG minimum shifted to lower temperatures for KI-T and KI-C and remaining similar for KI-P (Fig. 4A-B, Table 2, **A3**). The DTG line in the TGA thermogram of KI-S was split, indicating additional thermal events. Although its decomposition initiated earlier, KI-S exhibited higher high-temperature stability than KI, consistent with sodium carboxylate formation (Table 2, **A3**). Across hydrolysates, the weight loss at 30-460 °C was comparable to KI, but at 460-850 °C, it was lower than in KI (Fig. 5A-B, Table 2, **A3**). In DSC analysis, KI-T and KI-S lacked the first-scan water-loss endotherm observed for KI, KI-C and KI-P (Fig. 4C, **A3**). In the second scan, KI-T and KI-C displayed endotherms near 220 °C, corresponding to α-helix disordering, and near ~300 °C, associated with decomposition, but lacked the 245-275 °C melting/denaturation event observed in feathers and KI, suggesting its overlap with decomposition or its absence (Fig. 4D, **A3**). KI-P, in turn, showed the 245-275 °C melting/denaturation peak but no 220 °C α-helix endotherm (Fig. 4D, **A3**). KI-S showed no crystalline-melting feature in the second scan, consistent with extensive loss of ordered secondary structure (Fig. 4C, D, **A3**). Overall, the findings demonstrate that enzymatic hydrolysis induced distinct modifications of keratin stability and thermal transitions, with the extent of these changes depending on the enzyme used.

4.4. [Paper A4] Valorising feather keratin: Antioxidant activity, cytotoxicity and mutagenicity during processing and gastrointestinal digestion

The paper **A4** aimed to investigate the antioxidant activity and nutritional safety of KI, its enzymatic hydrolysates and products of their transformations occurring during the Maillard reaction and SGID. No standardized method for assessing antioxidant capacity exists and it is advised to employ tests based on different mechanisms (Iwaniak et al., 2025). Therefore, three complementary antioxidant activity assays were used for the keratin preparations: 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assay – reflecting both HAT and SET mechanisms, Fe^{2+} chelating activity assay – representing prooxidative metal chelation and Folin-Ciocâlteu reagent (FCR) reducing activity assay – based solely on the SET mechanism (Munteanu & Apetrei, 2021). Total antioxidant activity measurement of the keratin preparations involved all three assays in standard batch spectrophotometric measurements (off-line), and the results were expressed as μmol equivalents of the most common standard in each assay, i.e., Trolox (TE), ethylenediaminetetraacetic acid (EE) and gallic acid (GAE), respectively (Fig. 3, **A4**). Antiradical activity profiling of the keratin preparations involved only the ABTS assay, coupled with novel HP-SEC analysis (on-line), allowing for simultaneous determination of analytes' MW distribution (Fig. 2A, C, E, G, **A4**) and calculation of MW fractions' relative contribution to antioxidant activity (Fig. 2B, D, F, H, **A4**). The safety assessment of the keratin preparations involved the cytotoxicity assay by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test in human enterocytes (Caco-2) and mutagenicity assay by the Ames test involving *Salmonella* Typhimurium TA98 and TA100. The MRPs were additionally characterized in terms of degree of glycation (DG) and browning intensity. Finally, representative keratin preparations from each processing stage were subjected to SGID using the static INFOGEST protocol to evaluate their digestibility and bioaccessibility under physiologically relevant conditions.

Intact KI on its own possessed the ABTS scavenging, Fe^{2+} chelating and FCR reducing activities (Fig. 3, **A4**). As expected, its enzymatic hydrolysis increased the total antioxidant potency and shifted the contribution to antiradical activity from high to low MW fractions (Fig. 2B, D, F, H, **A4**), consistent with the release of smaller, more redox-active peptides. Accordingly, the total antioxidant activity of KI hydrolysates correlated strongly with DH irrespective of the assay, and KI-S, which had the highest DH and lowest average MW, showed the highest activity. Nevertheless, HP-SEC with post-column ABTS derivatisation indicated that peptides across a range of sizes contributed to activity in every hydrolysate: even in KI-S, the 1-5 kDa fraction accounted for a disproportionate share of antiradical activity relative to its mass, showing that the smallest peptides were not solely responsible.

To examine whether antioxidant potency could be further improved, KI-S was reacted with D-Glc or D-Xyl at 90-120 °C for 1-3 h to trigger the Maillard reaction (Table 1, **A4**). All KI-S/saccharide mixtures remained fully water-soluble after heat treatment. The mixtures heated at 90 °C showed negligible DG (Fig. 6, **A4**) and browning (Fig. 7, **A4**) and were therefore excluded from subsequent experiments. At 105-120 °C, DG and browning increased with temperature and time, and were consistently higher for D-Xyl than D-Glc. In addition, the mixtures heated at 105 and 120 °C developed a distinct meat-like aroma that intensified with increasing severity of the Maillard reaction. These chemical changes reflected the modified antioxidant properties. Relative to KI-S, most Xyl-derived MRPs displayed higher or retained ABTS scavenging, whereas all Glc-derived MRPs showed retained or decreased ABTS scavenging (Fig. 3A, **A4**). Fe^{2+} chelation decreased drastically across all MRPs (Fig. 3B, **A4**), while FCR reducing activity rose proportionally with the Maillard reaction intensity (Fig. 3C, **A4**). HP-SEC with post-column ABTS derivatisation indicated that antiradical activity remained dominated by low MW fractions, with no evidence for the formation of high MW melanoidins (Fig. 2C-F, **A4**). In summary, these results indicated the formation of low MW, electron-donating MRPs, such as reductones and α-dicarbonyls, with enhanced radical scavenging and reducing properties. Since no single MRP was dominant across all three assays, the representative preparation KI-S-X4 (KI-S with Xyl, heated at 105 °C for 1 h) was chosen for further analysis post-SGID due to its highest potency in ABTS scavenging and Fe^{2+} chelation.

Three keratin preparations, representative of successive processing stages, i.e., KI, KI-S and KI-S-X4, were subjected to SGID to assess their digestibility, functional stability and nutritional safety under conditions simulating human digestion. All preparations showed high digestibility, regardless of processing and successive processing steps were associated with a progressive shift of antiradical activity toward the low MW fraction (Fig. 2G-H, **A4**). Relative to pre-digestion activity, SGID led to led to changes in antioxidant potency, which could manifest as either increases or decreases depending on the preparation and assay applied (Fig. 3D-F, **A4**). Nevertheless, antioxidant activity was retained across all processing types and assays, and the strongest post-SGID effect was achieved through the combined application of subtilisin hydrolysis and mild Maillard reaction with Xyl (KI-S-X4), resulting in a preparation enriched in highly active low MW compounds. SGID also liberated substantial amounts of free amino acids, from all keratin preparations (Table 2, **A4**). The highest totals were observed in KI-S-SGID, followed by KI-SGID, indicating that subtilisin pre-hydrolysis enhanced digestibility. KI-S-X4-SGID yielded markedly fewer free amino acids, which likely reflects its reduced protein input – while KI and KI-S contained 500 mg of keratin, KI-S-X4 was a 500 mg keratin–xylose mixture. Although glycation consumes free amino groups, the present design of the experiment did not allow conclusions on its effect on overall digestibility, measured as total amount of free amino acids. No free Pro was detected in any

digest, indicating Pro residues remained peptide-bound (Table 2, **A4**).

Across all processing stages and after SGID, none of the keratin preparations exhibited cytotoxic effects in Caco-2 cells, as assessed by the MTT assay (Fig. 4D, **A4**). Likewise, no evidence of direct or indirect mutagenicity was detected in the Ames test with *Salmonella* Typhimurium strains TA98 and TA100 (Fig. 5, **A4**). These results confirm that keratin preparations were devoid of acute cytotoxicity and mutagenicity under the tested conditions. Together with their antioxidant activity, the nutritional safety data strengthen the potential of feather keratin as a source of bioactive peptides for nutraceutical applications.

4.5. [Paper A5] The biological role of prolyl oligopeptidase and the procognitive potential of its peptidic inhibitors from food proteins

Paper **A5** described POP, a conserved Pro-specific peptidase involved in various physiological and pathological processes in the human body, as well as its peptidic inhibitors originating from milk, meat, fish and plant protein-derived peptides. POP has both enzymatic and non-enzymatic activity, both of which are implicated in the development of neurodegenerative and neuropsychiatric disorders, and its inhibitors are considered to have procognitive and neuroprotective potential. It was demonstrated that the most promising POP inhibitory peptides have a short sequence, an abundance of hydrophobic amino acids and usually contain a Pro residue.

Although no prior studies addressed the experimental measurement of POP inhibitory activity of either chicken feather or pig hair keratin-derived peptides, the amino acid composition of these proteins, dominated by hydrophobic residues and rich in Pro (Table 2), indicates that both keratins are potentially sources of POP inhibitors. Additionally, in the *in silico* study (paper **A1**), the keratins sequences were shown to contain POP inhibitory fragments (Table 1, **A1**) and to potentially release peptides with POP inhibitory motifs upon enzymatic hydrolysis (Tables 3-12, **A1**). The keratin preparations investigated experimentally (papers **A2**-**A4**) also showed strong POP inhibitory potential, supported by the high Pro content of the KI (second most abundant residue, Table 3, **A2**), and the release of Pro-containing peptides during hydrolysis of KI with trypsin, chymotrypsin, pepsin and subtilisin (Table 3, **A2**), and during SGID of KI, KI-S and KI-S-X4 (Table 2, **A4**).

5. Conclusions and future perspectives

The dissertation provided novel insights concerning the valorisation of chicken feather keratin as a sustainable source of functional peptides and MRPs with potential applications in foods and nutraceuticals. The major conclusions from the studies are as follows:

- Enzymatic hydrolysis of feather KI with trypsin, chymotrypsin and subtilisin *in vitro* can achieve the DH value of keratin close to *in silico* predictions, while pepsin and papain release fewer peptides than predicted.
- L-Cys keratin reduction, followed by enzymatic hydrolysis, converts feather waste into water-soluble hydrolysates with improved antioxidant activity, and the choice of enzyme and pH value determine their techno-functional properties.
- Urea-assisted L-Cys extraction relaxes keratin's secondary structure, following hydrolysis with trypsin, chymotrypsin and pepsin affects only the primary structure, while subtilisin modifies both the primary and secondary structures, leading to enrichment of the secondary structure with β-turns and disordered coils.
- Treatment of KI-S and Glc/Xyl mixtures at 90 °C for up to 3 h does not trigger the Maillard reaction, while treatment at 105 and 120 °C for up to 3 h enables production of MRPs with meaty-aroma and improved antioxidant properties.
- Sequential keratin reduction, proteolysis and Maillard reaction can act synergistically to enhance its antioxidant potential while maintaining low cytotoxicity and mutagenicity.
- Keratin-derived peptides and MRPs are bioaccessible and retain antioxidant activity after SGID, supporting their suitability as functional ingredients in food and nutraceutical applications.

Future studies should focus on identifying keratin-derived peptides and MRPs using mass spectrometry, experimental validation of predicted bioactivities beyond antioxidant activity, especially ACE, DPP IV and POP inhibition, as well as the assessment of their sensory properties and bioavailability. Scale-up studies addressing reagent recovery, products purification, cost reduction and the sustainable integration of L-Cys extraction, enzymatic hydrolysis and the Maillard reaction for industrial applications would also be valuable.

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Papers constituting the doctoral dissertation

Paper A1

Taraszkiewicz, A., Sinkiewicz, I., Sommer, A., Dąbrowska, M., & Staroszczyk, H. (2022). Prediction of bioactive peptides from chicken feather and pig hair keratins using *in silico* analysis based on fragmentomic approach. *Current Pharmaceutical Design*, 28(10), 841–851. https://doi.org/10.2174/1381612828999220114150201

Percentage of the doctoral student's contribution: 50%

Description of the doctoral student's contribution: I conceptualized the study, developed the methods, performed all computations and data interpretation, prepared figures and tables, wrote the original draft, and participated in preparation of responses to reviewers.

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RESEARCH ARTICLE

Prediction of Bioactive Peptides from Chicken Feather and Pig Hair Keratins using *In Silico* Analysis Based on Fragmentomic Approach

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Abstract: ***Background*:** Keratin is among the most abundant structural proteins of animal origin, however it remains broadly underutilized.

***Objective*:** Bioinformatic investigation was performed to evaluate selected keratins originating from mass-produced waste products, *i.e*., chicken feathers and pig hair, as potential sources of bioactive peptides.

***Methods*:** Pepsin, trypsin, chymotrypsin, papain, and subtilisin were used for *in silico* keratinolysis with the use of "Enzyme(s) action" and fragmentomic analysis of theoretical products was performed using "Profiles of potential biological activity" in BIOPEP-UWM database of bioactive peptides. Bioactivity probability calculation and toxicity prediction of the peptides obtained were estimated using PeptideRanker and ToxinPred tools, respectively.

***Results*:** Our results showed that the keratins are a potential source of a variety of biopeptides, including dipeptidyl peptidase IV, angiotensin converting enzyme, prolyl endopeptidase inhibitory and antioxidative. Papain and subtilisin were found to be the most appropriate enzymes for keratin hydrolysis. This study presents possible structures of keratin-derived bioactive peptides that have not been previously described.

***Conclusion*:** Our data suggest additional *in vitro* and *in vivo* studies to verify theoretical predictions and further investigate the possibility of using keratin-rich waste as a source of peptide nutraceuticals.

Keywords: Bioactive peptides, bioinformatics, chicken feather, *in silico* analysis, keratin, pig hair.

1. INTRODUCTION

Keratin is among the most common fibrous proteins of animal origin. It is the major structural constituent of skin, hair, nails, claws, hooves, horns, beaks, and feathers. A characteristic feature of keratin is high cystine content. Keratins found in hair, skin, or sheep wool contain 10 to 14% of cystine. They are soft and elastic. On the other hand, keratins obtained from horns, claws, bird feathers, and beaks are hard and stiff, because of higher cystine content up to 22% [1]. The keratin polypeptide chains can form α-helices or β-sheets, therefore these proteins are divided into α-keratins, β-keratins, and amorphous keratins [2].

Management of hardly degradable keratinous wastes, which global production exceeds 40 million tonnes every year, poses significant difficulties. Native keratin is very durable, insoluble in most polar and nonpolar solvents, and highly resistant to hydrolysis by the majority of commercially available proteolytic enzymes. Its stability is the result of numerous intramolecular and intermolecular disulfide crosslinks and hydrogen bonds, as well as a high content of hydrophobic amino acids [3].

According to the available literature, the use of non-specific proteases for keratinolysis should be combined with an appropriate pretreatment and/or redox procedure aimed at destroying the disulfide bonds. For example, the hydrolysis of chicken feather keratin by cheap, commercially available proteolytic enzymes, that is pepsin, trypsin, chymotrypsin, papain and subtilisin, was possible when preceded by chemical pretreatment (with or without additional ultrasound treatment) [4], high-density steam flash-explosion [5] or when the process was accompanied by microorganisms or chemical agents used as a source of redox [6]. Methods of keratin solubilization, enabling its subsequent enzymatic digestion, differ in the process yield, degree of keratin modification, as well as cost-effectiveness, and include alkaline, acid or microbial hydrolysis, reduction or oxidation of disulfide bonds, hydrothermal treatment and combination thereof [7].

Animal by-products with high protein content are used as feed and food components, dietary supplements, drug carriers, cosmetics, biodegradable and functional packaging materials, or so-called bioplastics [3]. Various proteins can also serve as precursors of bioactive peptides. Many physiological roles of dietary proteins are carried out by peptide sequences encrypted inside the native protein. The peptides only exert their action after releasing by hydrolysis *in vivo* or *in vitro*. This process occurs during gastrointestinal digestion or food processing, *e.g*. fermentation, ripening [8].

Bioactive peptides, most often 2-30 amino acid residues in length, are protein-derived fragments which not only serve as nutrients, but can also exert hormone- or drug-like activity [9]. These peptides can regulate the body's important physiological functions through their numerous activities, including antidiabetic, antihypertensive, antioxidative, antimicrobial, antithrombotic or immunomodulatory. Some of the biopeptides, whose efficiency and safety were confirmed in human studies, are used as active ingredients in

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functional foods. Biopeptides can also be obtained by chemical synthesis or through the expression of corresponding genes [10].

Among food-derived bioactive peptides, the angiotensin converting enzyme (ACE; EC 3.4.15.1), dipeptidyl peptidase IV (DPP IV; EC 3.4.14.5) inhibitory and antioxidative peptides have been the most widely studied. These peptides have attracted so much attention due to their antihypertensive [11, 12], antidiabetic [13] and anticancer [14] potential. Reports on the bioactive properties of hydrolysates or peptides from various proteins are abundant, but only several from keratins [15]. Nevertheless, since known bioactive peptides are often predominated by hydrophobic amino acid residues [16], the keratins might be better precursors of biopeptides than has been expected so far.

Biological activity of peptides is the result of their specific amino acid composition. Additionally, a short peptide motif exhibiting bioactive properties, which is included in a longer peptide sequence (*i.e*., the parent peptide), without confirmed bioactivity, often decides on the activity of the entire sequence. For instance, a parent peptide that is composed of a motif with confirmed ACE inhibitory activity may determine the inhibitory potential of the whole sequence. This approach for establishing the unexplored function of a fragment with a known sequence is in accordance with the assumptions of fragmentomics [17] and was recently applied for milk and soybean protein hydrolysates [18, 19].

One of the favorable approaches when studying food protein-derived peptides is related to the use of *in silico* (computer-aided) analyses. Although bioinformatic methods do not allow actual production of peptides, they can be very useful in the quick and cost-effective evaluation of precursor proteins not previously studied as a source of bioactive peptides and the discovery of proper enzymes for their liberation [20]. The final verification and confirmation of *in silico* studies are experimental determinations of the biological activity of hydrolysates and peptides with the use of analytical methods such as electrophoresis, spectrophotometry, chromatography and mass spectrometry [21].

The objective of this *in silico* study was to perform an evaluation of keratins originating from mass-produced waste products, *i.e*., chicken feathers and pig hair as precursors of bioactive peptides, provide a preliminary overview of properties of the possible peptides to be obtained and compare the potential of the selected proteolytic enzymes to release these biopeptides. Although several authors reported enzymatic keratin hydrolysis, the structures of keratin-derived biopeptides are largely unknown. The prediction of products of keratin hydrolysis by selected enzymes facilitates subsequent experimental studies, as it simplifies the identification of their structures and guides in the choice of the most appropriate protease to be applied for the *in vitro* keratinolysis [22]. Additionally, the results described in this article can be used as a reference, to which results of experimental studies can be compared, particularly in order to assay the suitability of applied methods of keratin pretreatment/solubilization used prior to the proteolysis.

2. METHODS

2.1. Sequences of Keratins

The following sequences of keratins were downloaded from the UniProt database of protein sequences (https://www.uniprot.org/uniprot/) [23]: chicken (*Gallus gallus*) feather keratin (97 amino acid residues, excluding initiator methionine, UniProt accession number: P04458), and pig (*Sus scrofa*) hair keratin (84 amino acid residues, fragments, UniProt accession number: O62660). The selected proteins were chosen as representatives of hard and soft keratins, respectively [1]. There are additional sequences of chicken feather keratin available in UniProt, but as they share very high sequence homology, only one of them was chosen for the analyses.

2.2. Frequency of Bioactive Fragments Occurrence in Keratins

The sequences of keratins were examined for the presence of known bioactive peptides using BIOPEP-UWM database (http://www.uwm.edu.pl/biochemia/index.php/en/biopep) [24]. The frequency of occurrence of biopeptides with a given activity in the protein sequences was calculated as A = a/N, where "a" is the number of fragments with a given activity in a protein sequence and "N" is the number of all amino acid residues of a protein sequence.

2.3. *In Silico* Proteolysis

The keratins were theoretically hydrolysed with the BIOPEP-UWM tool called "Enzyme(s) action" [24]. The proteases chosen were pepsin (EC 3.4.23.1) (pH >2), trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1), papain (3.4.22.2) or subtilisin (EC 3.4.21.62). The selection of these enzymes was based on the availability of their cleavage patterns in BIOPEP-UWM and their documented use in keratin hydrolysis [4-6]. Theoretical degree of hydrolysis was calculated as DH_t = d/D, where "d" is the number of hydrolysed peptide bonds and "D" is the total number of peptide bonds in a protein chain.

2.4. Peptide Fragmentomic Analysis

The products of potential keratinolysis, excluding free amino acids, were examined for the presence of bioactive motifs using "Profiles of potential biological activity" in BIOPEP-UWM, defined as the type of bioactivity and location of the fragment in the amino acid sequence.

2.5. Peptide Bioactivity Probability Calculation

The peptides resulting from simulated keratinolysis were investigated to determine the probability of biological activity using PeptiderRanker webserver (http://distilldeep.ucd.ie/Peptide Ranker), based on established structure-function relationships [25]. Score values were assigned from 0 to 1, with a threshold of 0.5, *i.e*., each peptide with score value above 0.5 was described as a promising bioactive peptide.

2.6. Peptide Toxicity Prediction

The potential toxicity of the peptides generated was tested using ToxinPred tool (https://webs.iiitd.edu.in/raghava/toxinpred/index.html) [26]. The support vector machine (SVM, Swiss-Prot) and motif-based toxicity prediction method and the SVM threshold value of 0.0 were chosen.

All bioinformatic tools are freely accessible and were accessed in April 2021.

3. RESULTS AND DISCUSSION

The presence of peptides exhibiting 16 different biological activities was confirmed in the keratin sequences analysed in this study. Ten of them, *i.e*. ACE inhibition, ubiquitin-mediated proteolysis activation, antioxidative, antithrombotic, dipeptidyl peptidase III (DPP III; EC 3.4.14.4) inhibition, DPP IV inhibition, prolyl endopeptidase (PEP; EC 3.4.21.26) inhibition, stomach mucosal membrane activity regulation, renin inhibition and glucose uptake stimulation, were detected in both examined keratins. The values of frequency of bioactive fragment occurrence (parameter A) in intact keratins are summarized in Table **1**.

Table 1. Frequency of bioactive fragment occurrence (A) in intact keratins.

Activity	Chicken Feather Keratin	Pig Bristle Keratin
ACE inhibitor	0.412	0.298
Activating ubiquitin-mediated proteolysis	0.010	0.012
Antibacterial	0.010	n.d.
Anticancer	0.010	n.d.
Antioxidative	0.010	0.024
Antithrombotic	0.041	0.012
Anxiolytic	n.d.	0.012
Chemotactic	0.010	n.d.
DPP III inhibitor	0.052	0.036
DPP IV inhibitor	0.536	0.655
Insulin secretion inhibitor	0.010	n.d.
PEP inhibitor	0.041	0.012
Regulating the stomach mucosal membrane activity	0.041	0.012
Renin inhibitor	0.010	0.012
Stimulating glucose uptake	0.021	0.012
Stimulating vasoactive substance release	0.021	n.d.

n.d. - no data indicating the absence of bioactive fragments in the intact keratins based on BIOPEP-UWM.

Chicken feather keratin amino acid sequence

SCYDLCRPCGPTPLANSCNEPCVRQCQDSRVVIQPSPVVVTLPGPILSSFPQNTAVGSSTSAAVGSILSEE GVPISCGGFGISGLGSRFSGRRCLPC

↓ *in silico* hydrolysis by pepsin (pH > 2)

SCY - D - L - C**RP**CG - **PT** - **PL** - A - N - SCN - E - PC - **VRQ** - CQ - D - SR - V - V - **IQ** - **PSP** - V - V - **VT** - L - **PG** - P - **IL** - S**SF** - **PQ** - N - T - A - **VG** - S**ST** - SA - A - **VG** - S - **IL** - **SE** - E - G - **VP** - ISCG - G - F - G - I**SG** - L - G - S**RF** - SG - **RR**CL – PC

Fig. (1). *In silico* hydrolysis of chicken feather keratin with pepsin (pH > 2); parent peptides are underlined, bioactive motifs are **bolded**.

The occurrence of bioactive motifs within the protein sequence signifies its potential for bioactive peptide production. However, the peptides must be liberated from the protein *via* hydrolysis to exert their biological functions. The complex structure of keratins makes this process particularly difficult. For that reason, the majority of keratin hydrolyses have been performed using either chemical or microbial methods [15]. Because the theoretical proteolysis is based on the specificity of selected enzymes, it cannot be performed when applying acid and alkaline hydrolysis, or microbial fermentation involving multiple enzymes, cutting and recognition sequences are unknown [27]. In this study, keratins from chicken feather and pig hair were subjected to simulated hydrolysis using cheap, commonly used proteases. For illustrative purposes, the products of *in silico* hydrolysis of chicken feather keratin with pepsin are shown in Fig. (**1**).

As a result of theoretical keratinolysis, numerous peptides were generated that were not assigned as bioactive themselves, *e.g.*, CRPCG detected in pepsin-hydrolysed chicken feather keratin. However, a variety of these unexamined peptides comprised of shorter motifs with defined bioactivity (*i.e.*, provided in the BIOPEP-UWM database), as in the case of above-mentioned pentapeptide containing RP dipeptide sequences exhibiting DPP IV and ACE inhibitory properties (Fig. **1**). The occurrence of such peptides is compatible with the fragmentomic conception [17]. Among the known bioactive motifs detected theoretically in the parent peptides, most were dipeptides. Indeed, short motifs match the sequences of longer parent peptides more easily [18, 19].

Table **2** presents the calculated values of DH_t and summarises the numbers of products in the theoretical keratin hydrolysates, including the peptides which sequences were identical with the sequences of previously known peptides collected in BIOPEP-UWM database (named "bioactive" in the table), the parent peptides - which were not bioactive themselves but they contained motifs matching the known peptides previously uploaded to the database, the peptides which were not recognized as bioactive and they did not contain bioactive motifs, and free amino acids. The values of DH_t can be used to estimate the effectiveness of the enzyme to be used in keratin hydrolysis. The highest values were observed in pepsin-catalysed reactions, amounting to 54.2 and 63.9%, and the lowest were noted in trypsin hydrolysates - reaching only 6.3 and 9.6%, for chicken feather and pig hair keratin, respectively. It is important to highlight that the applied computer simulation assumes that all proteins' peptide bonds are hydrolysable, whereas during *in vitro* or *in vivo* proteolysis some of the bonds may actually be resistant to proteases [18]. On the contrary, even in the case of incomplete hydrolysis, the predicted peptides can still be detected during experimental studies [28]. Moreover, the knowledge of the theoretical products of a protein's hydrolysis facilitates their identification after *in vitro* hydrolysis, as the calculation of theoretical retention times of particular peptides during chromatographic separation becomes possible, and interpretation of mass spectra is easier [22]. Lastly, the release of (bioactive) peptides is not always proportional to the degree of hydrolysis of a protein, as extensive hydrolysis leads to the production of large amounts of free amino acids, which do not exhibit as equally potent bioactive properties as peptides they form [29, 30].

Table 2. Comparison of peptides and amino acids released *in silico* from chicken feather and pig hair keratin using different enzymes.

Source	Enzyme	Number of Peptides				Number of Free Amino Acids	DH_t (%)
		Total	Bioactive	Parent	No Activity		
Chicken feather	Pepsin	29	13	8	8	24	54.2
	Trypsin	6	-	6	-	1	6.3
	Chymotrypsin	15	-	10	5	-	14.6
	Papain	26	5	16	5	12	37.5
	Subtilisin	24	6	14	4	12	36.4
Pig hair	Pepsin	18	7	5	6	36	63.9
	Trypsin	8	1	7	-	1	9.6
	Chymotrypsin	14	2	11	1	6	22.9
	Papain	25	10	12	3	8	38.6
	Subtilisin	16	3	13	-	6	25.2

Table 3. Peptides predicted to be released from chicken feather keratin based on *in silico* hydrolysis with pepsin.

Activity	Parent Peptides	Bioactive Motifs
ACE inhibitor	**CRPCG(0.915), SRF(0.906), PG(0.877), PL(0.811), RRCL(0.733)**, **SSF(0.733)**, SG(0.407), PQ(0.393), IL(0.393), ISG(0.285), PT(0.249), VP(0.237), VG(0.168), VRQ(0.092), SST(0.082)	IL, PG, PL, PQ, PT, RF, RP, RR, SF, SG, ST, VG, VP, VR
Antithrombotic	**PG(0.877)**	PG
DPP III inhibitor	**RRCL(0.733)**	RR
DPP IV inhibitor	**CRPCG(0.915), PG(0.877), PL(0.811), RRCL(0.733), SSF(0.733)**, **PSP(0.664)**, PQ(0.393), IL(0.393), PT(0.249), VP(0.237), VG(0.168), IQ(0.124), VRQ(0.092), VT(0.027)	IL, IQ, PG, PL, PQ, PS, PT, RP, RR, SF, SP, VG, VP, VR, VT
PEP inhibitor	**PG(0.877)**	PG
Regulating[†]	**PG(0.877)**	PG
Renin inhibitor	**SSF(0.733)**	SF
Stimulating[‡]	IL(0.393)	IL
Stimulating[§]	SE(0.045)	SE

Bold font is used to indicate peptides which probability of bioactivity is high (Score value > 0.5).
[†]Peptide regulating the stomach mucosal membrane activity.
[‡]Peptide stimulating glucose uptake.
[§]Peptide stimulating vasoactive substance release.

The composition of the theoretical keratin hydrolysis products varied significantly, depending on the protease used. The hydrolysates produced by trypsin and chymotrypsin contain mostly long peptides (> 5 amino acid residues), which is due to their narrow specificity. The hydrolysates produced by subtilisin and papain contained moderate amounts of short peptides (≤ 4 amino acid residues), while pepsin produced the biggest number of dipeptides, but it also released the greatest amount of free amino acids. Generally, protein hydrolysates containing low molecular weight peptides are preferable, as the majority of the described bioactive peptides have short amino acid sequences. Such peptides are also more likely to resist degradation during gastrointestinal digestion, get absorbed in the body, and exhibit their activity *in vivo* [31]. Therefore, it might be suggested that subtilisin and papain are the most appropriate enzymes for the production of bioactive peptides from keratins. The detailed results of fragmentomic analysis of bioactive peptides theoretically released by the applied enzymes are presented in Tables **3-7** and **8-12**, for chicken feather and pig hair keratin, respectively.

The dominant part of keratin-encrypted peptides, represented by the highest values of the parameter A, exhibited DPP IV and ACE inhibitory properties (Table **1**). These peptides are considered useful in the prevention and treatment of metabolic syndrome - a combination of biochemical disorders that increase the risk of developing cardiovascular diseases and type-2 diabetes [10]. Numerous fragments exerting these both activities were found, *e.g.*, GL detected in subtilisin-hydrolysed chicken feather keratin (Table **7**). Among the biopeptides deposited in the BIOPEP-UWM database, the DPP IV and ACE inhibitory peptides constitute the dominant part, as these have been the most widely studied [24].

Inhibition of ACE is one of the established pharmacological strategies of hypertension treatment, as this protease converts angiotensin I to angiotensin II - a potent vasoconstrictor and plays a role in degrading bradykinin - a vasodilator. The commonly used synthetic ACE inhibitors are effective, but some side effects resulting from their long-term usage were reported, such as oppressive dry cough, skin rashes, or taste disturbances. The bioactive peptides exhibiting ACE inhibitory properties can be used as adverse effect free alternatives. Their antihypertensive effectiveness has also been confirmed in a few clinical trials [32]. A significant number of peptides exerting ACE inhibitory activity detected *in silico* in the examined keratins contained glycine, valine, proline, leucine, and isoleucine residues. These amino acids are typical for ACE inhibitors [33].

DPP IV is one of the key enzymes responsible for blood sugar level regulation due to its involvement in the inactivation of incretin hormones, which decrease plasma glucose levels and promote the growth of pancreatic beta cells. Numerous synthetic inhibitors of this enzyme have been developed, useful in the prevention and treatment of type 2 diabetes, but their use is associated with side effects such as musculoskeletal, gastrointestinal or skin-related disorders, as well as allergic reactions. It has been suggested that natural peptides could be a safer alternative for glycemic management [13], just like in the case of ACE inhibitory peptides. Interestingly, in addition to their antidiabetic properties,

Table 4. Peptides predicted to be released from chicken feather keratin based on *in silico* hydrolysis with trypsin.

Activity	Parent Peptides	Bioactive Motifs
ACE inhibitor	**FSGR(0.844)**, **PCGPTPLANSCNEPCVR(0.815)**, V-R(0.029)	AA, AV, EG, FG, FP, GF, GG, GI, GL, GP, GR, GS, GV, IL, IQP, LA, LG, LPG, PG, PL, PQ, PT, QP, SF, SG, SG, ST, TP, VG, VP, VR
Activating[¶]	**PCGPTPLANSCNEPCVR(0.815)**	LA
Anticancer	V-R (0.029)	VVV
Antioxidative	V-R (0.029)	LPGPILSSFPQ
Antithrombotic	**PCGPTPLANSCNEPCVR(0.815)**, V-R(0.029)	GP, PG, PGP
Chemotactic	V-R (0.029)	PGP
DPP III inhibitor	**PCGPTPLANSCNEPCVR(0.815),** V-R(0.029)	GF, LA
DPP IV inhibitor	**CLPC (0.910), SCYDLCR (0.849), PCGPTPLANSC-NEPCVR(0.815)**, QCQDSR(0.266), V-R(0.029)	AA, AV, EG, EP, FP, GF, GG, GI, GL, GP, GV, IL, IQ, IQP, LA, LP, NE, NT, PG, PI, PL, PQ, PS, PT, PV, QD, QN, QP, SF, SI, SP, TA, TL, TP, TS, VG, VI, VP, VR, VT, VV, YD
Insulin secretion inhibitor	V-R(0.012)	PGP
PEP inhibitor	**PCGPTPLANSCNEPCVR(0.815),** V-R(0.029)	GP, PG, PGP
Regulating[†]	**PCGPTPLANSCNEPCVR(0.815),** V-R(0.029)	GP, PG, PGP
Renin inhibitor	V-R(0.029)	SF
Stimulating[‡]	V-R(0.029)	IL
Stimulating[§]	V-R(0.029)	EE, SE

Bold font is used to indicate peptides which probability of bioactivity is high (Score value > 0.5).
V-R- VVIQPSPVVVTLPGPILSSFPQNTAVGSSTSAAVGSILSEEGVPISCGGFGISGLGSR.
[¶]Peptide activating ubiquitin-mediated proteolysis.
[†]Peptide regulating the stomach mucosal membrane activity.
[‡]Peptide stimulating glucose uptake.
[§]Peptide stimulating vasoactive substance release.

Table 5. Peptides predicted to be released from chicken feather keratin based on *in silico* hydrolysis with chymotrypsin.

Activity	Parent Peptides	Bioactive Motifs
ACE inhibitor	**GSRF(0.909), PGPIL(0.837), CRPCGPTPL(0.836), SEEGVPISCG-GF(0.746), SSF(0.733), SGRRCL(0.723),** GISGL(0.481), PQN(0.285), TAVGSSTSAAVGSIL(0.156), EPCVRQCQDSRVVIQPSPVVVTL(0.123)	AA, AV, EG, GF, GG, GI, GL, GP, GR, GS, GV, IL, IQP, PG, PL, PQ, PT, QP, RF, RP, RR, SF, SG, ST, TP, VG, VP, VR
Anticancer	EPCVRQCQDSRVVIQPSPVVVTL(0.123)	VVV
Antithrombotic	**PGPIL(0.837), CRPCGPTPL(0.836)**	GP, PG, PGP
Chemotactic	**PGPIL(0.837)**	PGP
DPP III inhibitor	**GSRF(0.909), SEEGVPISCGGF(0.746), SGRRCL(0.723),** EPCVRQCQDSRVVIQPSPVVVTL(0.123)	GF, RF, RR, RV
DPP IV inhibitor	**PGPIL(0.837), CRPCGPTPL(0.836), SEEGVPISCGGF(0.746), SSF(0.733), SGRRCL(0.723),** GISGL(0.481), PQN(0.285), TAVGSSTSAAVGSIL(0.156), EPCVRQCQDS-RVVIQPSPVVVTL (0.123)	AA, AV, EG, EP, GF, GG, GI, GL, GP, GV, IL, IQ, IQP, PG, PI, PL, PQ, PS, PT, PV, QD, QN, QP, RP, RR, SF, SI, SP, TA, TL, TP, TS, VG, VI, VP, VR, VT, VV
Insulin secretion inhibitor	**PGPIL(0.837)**	PGP
PEP inhibitor	**PGPIL(0.837), CRPCGPTPL(0.836)**	GP, PG, PGP
Regulating[†]	**PGPIL(0.837), CRPCGPTPL(0.836)**	GP, PG, PGP
Renin inhibitor	**SSF(0.733)**	SF
Stimulating[‡]	**PGPIL(0.837)**, TAVGSSTSAAVGSIL(0.156)	IL
Stimulating[§]	**SEEGVPISCGGF(0.746)**	EE, SE

Bold font is used to indicate peptides which probability of bioactivity is high (Score value > 0.5).
[†]Peptide regulating the stomach mucosal membrane activity.
[‡]Peptide stimulating glucose uptake.
[§]Peptide stimulating vasoactive substance release.

Table 6. Peptides predicted to be released from chicken feather keratin based on *in silico* hydrolysis with papain.

Activity	Parent Peptides	Bioactive Motifs
ACE inhibitor	**PG(0.877), PL(0.811), SSF(0.733), PIL(0.642), ANSCNEPCVR(0.553),** VPISCG(0.419), SG(0.407), SIL(0.331), ISG(0.285), PT(0.249), QPSPVVVT(0.208), AVG(0.162), SST(0.082), SEEG(0.059)	AV, EG, IL, PG, PL, PT, QP, SF, SG, ST, VG, VP, VR
Anticancer	QPSPVVVT(0.208),	VVV
Antithrombotic	**PG(0.877)**	PG
DPP IV inhibitor	**PG(0.877), PL(0.811), SSF(0.733), SCYDL(0.706), PIL(0.642), ANSCNEPCVR(0.553),** VPIS-CG(0.419), SIL(0.331), PT(0.249), QPSPVVVT(0.208), AVG(0.162), QDSR(0.149), QNT(0.069), SEEG(0.059), VVI(0.041)	AV, EG, EP, IL, NE, NT, PG, PI, PL, PS, PT, PV, QD, QN, QP, SF, SI, SP, VG, VI, VP, VR, VT, VV, YD

(Table 6) Contd….

Activity	Parent Peptides	Bioactive Motifs
PEP inhibitor	**PG(0.877)**	PG
Regulating[†]	**PG(0.877)**	PG
Renin inhibitor	**SSF(0.733)**	SF
Stimulating[§]	SEEG(0.059)	EE, SE
Stimulating[‡]	**PIL(0.642)**, SIL(0.331)	IL

Bold font is used to indicate peptides which probability of bioactivity is high (Score value > 0.5).
[†]Peptide regulating the stomach mucosal membrane activity.
[‡]Peptide stimulating glucose uptake.
[§]Peptide stimulating vasoactive substance release.

Table 7. Peptides predicted to be released from chicken feather keratin based on *in silico* hydrolysis with subtilisin.

Activity	Parent Peptides	Bioactive Motifs
ACE inhibitor	**RF(0.987), CGGF(0.985), PGPIL(0.837), CRPCGPTPL(0.836), GL(0.809), GRRCL(0.804),** IL(0.393), GS(0.341), GIS(0.269), AA(0.191), VPIS(0.162), PQNTA(0.132), VIQPS(0.115), VGS(0.106), VRQCQDS(0.085), EEG(0.045)	AA, EG, GF, GG, GI, GL, GP, GR, GS, IL, IQP, PG, PL, PQ, PT, QP, RF, RP, RR, TP, VG, VP, VR
Antithrombotic	**PGPIL(0.837), CRPCGPTPL(0.836)**	GP, PG, PGP
Chemotactic	**PGPIL(0.837)**	PGP
DPP III inhibitor	**RF(0.987), CGGF(0.985), GRRCL(0.804)**	GF, RF, RR
DPP IV inhibitor	**CGGF(0.985), PGPIL(0.837), CRPCGPTPL(0.836), GL(0.809), GRRCL(0.804), CNEPC(0.575)**, IL(0.393), GIS(0.269), AA (0.191), VPIS(0.162), PQNTA(0.132), VIQPS(0.115), VGS(0.106), VRQCQDS(0.085), VTL(0.062), TS (0.047), EEG (0.045)	AA, EG, EP, GF, GG, GI, GL, GP, IL, IQ, IQP, NE, NT, PG, PI, PL, PQ, PS, PT, QD, QN, QP, RP, RR, TA, TL, TP, TS, VG, VI, VP, VR, VT
Insulin secretion inhibitor	**PGPIL(0.837)**	PGP
PEP inhibitor	**PGPIL(0.837), CRPCGPTPL(0.836)**	GP, PG, PGP
Regulating[†]	**PGPIL(0.837), CRPCGPTPL(0.836)**	GP, PG, PGP
Stimulating[‡]	**PGPIL(0.837)**, IL(0.393)	IL
Stimulating[§]	EEG(0.045)	EE

Bold font is used to indicate peptides which probability of bioactivity is high (Score value > 0.5).
[†]Peptide regulating the stomach mucosal membrane activity.
[‡]Peptide stimulating glucose uptake.
[§]Peptide stimulating vasoactive substance release.

DPP IV inhibitors have been recently suggested as novel, potential antihypertensive agents [12].

The preventive potential of keratin-derived peptides against metabolic and cardiovascular disorders could be further enhanced due to the presence of antithrombotic [34], glucose uptake stimulating [35], and vasoactive substance release stimulating [36], as well as renin inhibitory peptides [37] in the analysed protein sequences. However, the low value of occurrence frequency calculated for above-mentioned bioactivities represent a small number of these peptides within the examined keratins.

The analysed keratins were found to be a potential source of peptides exhibiting DPP III inhibitory properties. This enzyme catalyzes the hydrolysis of various biomolecules such as adrenocorticotropin, angiotensins, and enkephalins and its inhibitors are believed to be promising in pain management. Some of its inhibitors showed an antinociceptive potential, thus they may serve as natural pain modulators [38].

PEP is an intracellular serine protease involved, *e.g.*, in learning and memory, cell division and differentiation, which has been linked to some neurological disorders including schizophrenia and depression. Its activity is also higher in the brains of Alzheimer's patients. Therefore, PEP inhibitors are expected to be used as therapeutic agents for memory deficits and cognitive dysfunctions related to aging and neurodegenerative diseases. Most of its inhibitors are synthetic, substrate-like molecules based on the N-acyl-L-prolyl-pyrrolidine structure, however, a few protein-derived peptidic inhibitors have also been described - most of which have contained at least one proline residue [39]. The examined keratins were found out to be a potential source of such peptides due to the presence of some PEP inhibitory motifs in their amino acid sequences (Tables **3-12**) as well as the fact that keratins are proline-rich proteins [3]. Special attention should be paid to multifunctional peptides, as they are involved in regulating a variety of physiological functions. Among the peptides detected theoretically in analysed keratins, PG and GP were the most promising, exhibiting five different activities including DPP IV, ACE, and PEP inhibitory, regulating stomach mucosal membrane action and antithrombotic activity. These dipeptides were previously identified in collagen hydrolysates and their bioavailability was confirmed in human studies [40].

Following the BIOPEP-UWM based analyses, the Peptide Ranker was used to evaluate the products of potential keratinolysis for their probability of being bioactive. The results presented in this paper were rounded to three decimal places. The score values ranging from 0.012 to 0.999 are provided in brackets next to sequences of potentially released parent peptides (Tables **3-12**). It was found that previously known bioactive sequences, such as EK, VS, or AS (DPP IV inhibitors deposited in the BIOPEP-UWM database) (Table **12**) have had low Score values - suggesting little probability of bioactivity. The curator of Peptide Ranker advised that the predictive approach based on molecular docking may only be suitable when studying the competitive inhibitors [41]. Despite the fact that Peptide Ranker cannot describe the type of peptide bioactivity, it can be useful in screening for the most promising (parent) peptides, as well as the structure-activity relationship studies [28, 42, 43]. The following theoretically released peptides: PC, PCG, CL, CR, CY, SCY, QC, ISCG, CQ - derived from chicken feather keratin and CN - from pig hair keratin have had high Score values of 0.934, 0.933, 0.879, 0.865, 0.831, 0.656, 0.599, 0.569, 0.540 and 0.634, respectively, while their bioactivity is unknown. These short, cysteine-containing and keratin-specific peptides are particularly worth further investigation.

Table 8. Peptides predicted to be released from pig hair keratin based on *in silico* hydrolysis with pepsin.

Activity	Parent Peptides	Bioactive Motifs
ACE inhibitor	**RPC(0.901), PG(0.877), PL(0.811), RL(0.626)**, IPA(0.432), SY(0.262), VPSSC-Q(0.228), VSSG(0.106), VRQ(0.092)	IP, IPA, PG, PL, RL, RP, SG, SY, VP, VR
Antithrombotic	**PG (0.877)**	PG
DPP IV inhibitor	**WF(0.999), RPC(0.901), PG(0.877), PL(0.811), RL(0.626)**, VCPN(0.448), IPA(0.432), SY(0.262), VPSSCQ(0.228), IQ(0.124), VSSG(0.106), VRQ(0.092)	IP, IPA, IQ, PA, PG, PL, PN, PS, RL, RP, SY, VP, VR, VS, WF
PEP inhibitor	**PG (0.877)**	PG
Regulating[†]	**PG (0.877)**	PG

Bold font is used to indicate peptides which probability of bioactivity is high (Score value > 0.5).
[†]Peptide regulating the stomach mucosal membrane activity.

Table 9. Peptides predicted to be released from pig hair keratin based on *in silico* hydrolysis with trypsin.

Activity	Parent Peptides	Bioactive Motifs
ACE inhibitor	**P-K(0.601)**, SQQQEPLVCPN(0.428), ETMQFLNDR(0.222), LASYLEK(0.186), DNAELER(0.125), VR(0.115)	AEL, AF, EG, EK, EK, GA, GI, IP, IPA, LA, LEK, LN, LPG, NG, PG, PL, QG, SG, SY, VP, VR
Activating[¶]	LASYLEK(0.186)	LA
Antioxidative	**P-K(0.601)**, DNAELER(0.125)	EL, FC
Antithrombotic	**P-K(0.601)**	PG
Anxiolytic	LASYLEK(0.186)	YL
DPP III inhibitor	ETMQFLNDR(0.222), LASYLEK(0.186)	FL, LA, YL
DPP IV inhibitor	**P-K(0.601)**, SQQQEPLVCPN(0.428), ETMQFLNDR(0.222), LASYLEK(0.186), DNAELER(0.125), VR(0.115), QLER(0.113), QIQER(0.092)	AE, AF, AS, AT, DN, DR, EG, EK, EP, ET, FL, FN, GA, GI, IP, IPA, IQ, LA, LN, LP, LV, MQ, NA, ND, NE, NG, NW, PA, PG, PL, PN, PS, QE, QF, QG, QI, QL, QQ, SY, TL, TM, TV, VP, VR, VS, WF, YL
PEP inhibitor	**P-K(0.601)**	PG
Regulating[†]	**P-K(0.601)**	PG
Renin inhibitor	ETMQFLNDR(0.222)	QF
Stimulating[‡]	SQQQEPLVCPN(0.428)	LV

Bold font is used to indicate peptides which probability of bioactivity is high (Score value > 0.5).
P-K- PCVPSSCQGITLPGACNIPATVSSGNWFCEGAFNGNEK.
[¶]Peptide activating ubiquitin-mediated proteolysis.
[†]Peptide regulating the stomach mucosal membrane activity.
[‡]Peptide stimulating glucose uptake.

Table 10. Peptides predicted to be released from pig hair keratin based on *in silico* hydrolysis with chymotrypsin.

Activity	Parent Peptides	Bioactive Motifs
ACE inhibitor	**CEGAF(0.715), RPCVPSSCQGITL(0.649), PGACN(0.624)**, DRL(0.476), ASY(0.222), IPATVSSGN(0.121), AEL(0.098), ERQIQERSQQQEPL(0.068), EKVRQL(0.064), EKETM(0.055)	AEL, AF, EG, EK, GA, GI, IP, IPA, KE, PG, PL, QG, RL, RP, SG, SY, VP, VR
Antioxidative	AEL(0.098)	EL
Antithrombotic	**PGACN(0.624)**	PG
DPP IV inhibitor	**QF(0.946), CEGAF(0.715), RPCVPSSCQGITL(0.649)**, **PGACN(0.624)**, DRL(0.476), VCPN(0.448), ASY(0.222), IPATVSSGN(0.121), AEL(0.098), ERDN (0.069), ERQIQERSQQQEPL(0.068), EKVRQL(0.064), EKETM(0.055)	AE, AF, AS, AT, DN, DR, EG, EK, EP, ET, GA, GI, IP, IPA, IQ, KE, KV, PA, PG, PL, PN, PS, QE, QF, QG, QI, QL, QQ, RL, RP, SY, TL, TM, TV, VP, VR, VS
PEP inhibitor	**PGACN(0.624)**	PG
Regulating[†]	**PGACN(0.624)**	PG
Renin inhibitor	**QF(0.946)**	QF

Bold font is used to indicate peptides which probability of bioactivity is high (Score value > 0.5).
[†]Peptide regulating the stomach mucosal membrane activity.

A comprehensive analysis of keratin-derived bioactive peptides is not available in the literature. A similar, yet less detailed *in silico* study of chicken feather keratin has been performed by other authors [44]. Their results only partially go along with the results presented in this article. The considerably greater amount of data on keratin-derived biopeptides in this study, as compared with that reported in the cited work, is due to the number of new peptides that were submitted to the BIOPEP-UWM since the submission of the cited article. The most significant difference is that in this study the predominating biopeptide activities were DPP IV and ACE inhibitory, while Choińska *et al.* reported mostly ACE inhibitory peptides but only few DPP IV inhibitors.

The literature reports from experimental studies confirm the existence of ACE and DPP IV inhibiting peptides in chicken feather hydrolysates. Enzymatic hydrolysate of chicken feather meal exhibited stronger ACE inhibitory activity than keratin hydrolysate obtained from a mixture of horns and hooves of cows and buffaloes [45]. The feather keratin <6.5 kDa peptides obtained *via* acid hydrolysis had the ability to inhibit ACE activity by approx. 50%

Table 11. Peptides predicted to be released from pig hair keratin based on *in silico* hydrolysis with papain.

Activity	Parent Peptides	Bioactive Motifs
ACE inhibitor	**AF(0.973), PG(0.877), ACNIP(0.608)**, PCVPSSC(0.481), ASYL(0.423), NG(0.388), QG(0.388), CEG(0.335), QEPL(0.279), VSSG(0.106), AEL(0.098), EKVR(0.038), NEKET(0.027)	AEL, AF, EG, IP, KE, NG, PG, PL, QG, SG, SY, VP, VR
Antioxidative	AEL(0.098)	EL
Antithrombotic	**PG(0.877)**	PG
Anxiolytic	ASYL(0.423)	YL
DPP III inhibitor	ASYL(0.423)	YL
DPP IV inhibitor	**NWF(0.989), AF(0.973), QF(0.946), PG(0.877), ACNIP(0.608)**, PCVPSSC(0.481), VCPN(0.448), ASYL(0.423), NG(0.388), QG(0.388), CEG(0.335), QL(0.292), QEPL(0.279), NDR(0.182), QI(0.131), VSSG(0.106), DN(0.103), AEL(0.098), QER(0.082), AT(0.071), EKVR(0.038), NEKET(0.027)	AE, AF, AS, AT, DN, DR, EG, EK, EP, ET, IP, KE, KV, ND, NE, NG, NW, PG, PL, PN, PS, QE, QF, QG, QI, QL, SY, VP, VR, VS, WF, YL
PEP inhibitor	**PG(0.877)**	PG
Regulating†	**PG(0.877)**	PG
Renin inhibitor	**QF(0.946)**	QF

Bold font is used to indicate peptides which probability of bioactivity is high (Score value > 0.5).
†Peptide regulating the stomach mucosal membrane activity.

Table 12. Peptides predicted to be released from pig hair keratin based on *in silico* hydrolysis with subtilisin.

Activity	Parent Peptides	Bioactive Motifs
ACE inhibitor	**RPC(0.901), CEGAF(0.715), PGACNIPAT(0.626),** CQGITL (0.489), NDRL(0.407), QQQEPL(0.237), NGNEKETMQF(0.206), VRQL(0.161), VPS(0.153), ERDNAEL(0.096), EK(0.025)	AEL, AF, EG, EK, GA, GI, IP, IPA, KE, NG, PG, PL, QG, RL, RP, VP, VR
Antioxidative	ERDNAEL(0.096)	EL
Antithrombotic	**PGACNIPAT(0.626)**	PG
DPP IV inhibitor	**GNW(0.944), RPC(0.901), CEGAF(0.715), PGACNIPAT(0.626)**, CQGITL (0.489), VCPN(0.448), NDRL(0.407), QQQEPL(0.237), NGNEKETMQF(0.206), VRQL(0.161), VPS(0.153), AS(0.125), ERDNAEL(0.096), ERQIQERS(0.068), VS(0.044), EK(0.025)	AE, AF, AS, AT, DN, DR, EG, EK, EP, ET, GA, GI, IP, IPA, IQ, KE, MQ, NA, ND, NE, NG, TM, NW, PA, PG, PL, PN, PS, QE, QF, QG, QI, QL, QQ, RL, RP, TL, VP, VR, VS
PEP inhibitor	**PGACNIPAT(0.626)**	PG
Regulating†	**PGACNIPAT(0.626)**	PG
Renin inhibitor	NGNEKETMQF(0.206)	QF

Bold font is used to indicate peptides which probability of bioactivity is high (Score value > 0.5).
†Peptide regulating the stomach mucosal membrane activity.

[46]. Feather hydrolysate produced by *Chryseobacterium* sp. kr6 exerted the ability to inhibit ACE and DPP IV activity by 65 and 44%, respectively, as well as radical scavenging properties [47]. Similarly, feather keratin hydrolysate fermented by *Bacillus* spp. exhibited ACE and DPP IV inhibitory and antioxidative activities [48]. Feather meal produced by *Bacillus* strains also exhibited DPP IV inhibitory as well as antioxidant properties [49]. The extracted keratin from chicken feathers by high-density steam flash explosion was hydrolyzed with various proteases to yield peptides, some of which matched the sequences of previously identified bioactive peptides deposited in BIOPEP-UWM [5].

Majority of studies on keratin hydrolysates have been focused on their antioxidant properties. Keratin hydrolysate from chicken feather meal had strong 2,2'-diphenylo-1-picryl-hydrazyl (DPPH) radical scavenging properties [45]. Keratin hydrolysate obtained after fermentation of chicken feathers with *Bacillus pumilus* A1 also revealed DPPH scavenging activity, as well as reducing power [50]. Likely, isolated octapeptide SNLCRPCG from keratin hydrolysate obtained through submerged cultivation of chicken feathers with *Bacillus subtilis* S1-4 had antioxidant properties against DPPH and 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radicals, as well as its ability to reduce Fe(III) and chelate Fe(II) ions [51]. According to Alahyaribeik & Ullah [4], DPPH scavenging activity of the feather keratin hydrolysate produced by Alcalase after 30 minutes was higher than the activity of hydrolysates obtained after 15 or 60 minutes. The keratin hydrolysate obtained during cultivation of *Chryseobacterium* sp. kr6 on chicken feather containing medium showed the ability to reduce ABTS radicals and Fe(III) ions [52]. It was also confirmed the antioxidant activity of peptide LPGPILSSFPQ from the hydrolysate. This peptide has been the only one antioxidative peptide detected in chicken feather keratin in this *in silico* study (Tables **1** and **4**), meaning that the structures of keratin-derived antioxidative peptides remain largely unknown.

There are also reports of antimicrobial properties of keratin hydrolysates. The amphiphilic peptide derived from feather hydrolysate obtained using a mixture of *Thermoactinomyces* strains effectively inhibited the growth of *Fusarium solani*, *Fusarium oxysporum*, *Mucor* sp. and *Aspergillus niger* - common plant pathogens [53]. In turn, feather hydrolysates and nanoparticles produced on its basis exhibited inhibitory properties against *Staphylococcus aureus* and *Escherichia coli* [54]. Chicken feather keratin hydrolysate obtained *via* fermentation with *Paenibacillus woosongensis* TKB2 contained a potent bactericidal peptide with a molecular weight of 4.6 kDa. Its minimum inhibitory concentration and minimum bactericidal concentration values against multiple antibiotic-resistant *Staphylococcus aureus* were 22.5 and 90 μg/mL, respectively [55]. Its sequence was established to be CNEPCVRQCQDSRVVIQPSPVVVTLPGPILSSFPQNTAVGSSTSA and it was the only antimicrobial peptide which was found *in silico* in the keratins analysed in this study, as the structures of others are unknown.

Pig hair keratin and its hydrolysates have been studied less extensively. The amino acid sequence of this protein that we accessed from UniProt database is not complete and represents only a fragment of this keratin. However, it can be assumed that we have analysed the vast majority of it, as the mentioned fragment's molecular weight in the database is 9.45 kDa. The molecular weight of this keratin is reported at the level of ca. 10.86 kDa [56]. To the best of our knowledge, only four studies describing the enzymatic hydrolysis of pig hair have been published so far, although the bioactive properties or chemical structures of the obtained products have not been investigated [57-60].

Data from toxicological studies regarding the safety of keratin hydrolysates are scarce. Markers of oxidative stress such as the rate of thiobarbutiric acid reagent substances and activity of antioxidant enzymes were decreased in rats fed with feather hydrolysate produced by *Bacillus pumilus* A1 compared to controls, and no adverse effects of such diet were observed [61]. Likely, it was established that administration of acid-hydrolysed feather keratin to mice improved liver, kidney, and heart antioxidant capability, as well as inhibited lipid peroxidation in a dose-dependent manner [62]. The anti-staphylococcal peptide derived from feather keratin at 90 μg/mL concentration did not show any adverse effects against neither red blood cell membrane nor human colon adenocarcinoma (HT-29) cells [55]. Similarly, the enzymatic hydrolysates of feather keratin displayed no cytotoxicity against human neuroblastoma cells (SK-N-MC) at a concentration of up to 0.312 mg/mL [4]. Likewise, feather hydrolysate produced by *Bacillus licheniformis* displayed no cytotoxicity to *gingival* fibroblast blood cell lines at concentration up to 5 mg/mL [63]. Pig hair keratin isolate was established to be non-toxic to human skin cells [56]. The results of ToxinPred analyses demonstrated that none of the analysed *in silico* keratin-derived peptides were predicted to be toxic and therefore, these peptides could potentially be appropriate for feed, food or pharmaceutical applications.

CONCLUSION

Keratins extracted from waste biomass could be a valuable potential source of various bioactive peptides. The perspectives are promising considering the great abundance of these proteins. The keratin-derived bioactive peptides predominantly exhibited DPP IV and ACE inhibitory properties. Among the proteases analysed, subtilisin and papain seem to be the most appropriate enzymes for the generation of keratin-derived biopeptides. The literature reports from experimental investigations confirm the existence of bioactive peptides in keratin hydrolysates. The results of this study encouraged additional studies to confirm the theoretical predictions and further inspect the use of keratin-rich wastes as a source of peptidic nutraceuticals. Hence, second part of our study, involving the selection of optimal keratin processing conditions, determination of structures and bioactivities of peptides obtained, followed by *in vivo* studies, including bioactivity, bioavailability, and safety assessment, is being prepared.

AUTHORS` CONTRIBUTIONS

Antoni Taraszkiewicz, Conceptualization, data curation, formal analysis, investigation, methodology, software, writing-original draft, Izabela Sinkiewicz, formal analysis, methodology, supervision, writing-review and editing, Agata Sommer, writing-review and editing, Małgorzata Dąbrowska, investigation, Hanna Staroszczyk, project administration, supervision, writing-review and editing.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No animals/humans were used for studies that are basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data supporting the findings of the article is available within the article.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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Paper A2

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Chemical composition and techno-functional properties of high-purity water-soluble keratein and its enzymatic hydrolysates

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ABSTRACT

This study compared the effectiveness of urea-containing and urea-free L-cysteine solutions in extracting high-quality feather keratin and evaluated commercial proteases for producing keratin-derived bioactive peptides. The urea-assisted extraction was crucial for achieving high structural integrity and yield of soluble keratin. The keratin isolate exhibited oil-holding capacity of 9.37 g/g, foaming capacity of up to 127 %, and emulsifying capacity of up to 49 %. Its proteolysis with trypsin, chymotrypsin, pepsin and subtilisin resulted in peptides with average molecular weight between 2.10 and 5.96 kDa and degree of hydrolysis from 6 to 36 %. The subtilisin hydrolysate had the highest degree of hydrolysis, 63 % of peptides <1 kDa, and excellent solubility across a wide pH range, but negligible water and oil-binding, foaming, and emulsifying properties. This study highlights the need to optimize each step in keratin extraction and hydrolysis processes to produce high-quality bioactive keratin preparations for diverse applications, including food and pharmaceutical.

1. Introduction

Keratins are structural proteins of the animal epidermis and its products. Their characteristic feature, distinguishing them from similar proteins such as collagen and elastin, is a high cysteine content (Ferraro et al., 2016). Keratins are extensively cross-linked with disulphide and hydrogen bonds and contain a high amount of hydrophobic amino acid residues. These properties result in a high stability of native keratins, rendering them insoluble in most solvents, indigestible and slowly biodegradable (Sharma & Kumar, 2019). α-Keratins, rich in cysteine structural proteins ranging in molecular weight (MW) between 40 and 70 kDa, consist of four α-helices coiled into a super-helix. These proteins are commonly found in wool, quills, hair, horns, fingernails, hooves and stratum corneum. β-Keratins, rich in short-chain amino acid residues protective proteins, are comprised of polypeptide chains within the 10–20 kDa MW range. These proteins, maintaining structural stability mainly through dense hydrogen bonds, are commonly found in feathers, beaks, claws and scales (Sharma & Kumar, 2019; Sinkiewicz et al., 2018; Wang, Yang, et al., 2016).

An enormous amount of keratin-rich waste biomass is generated globally, especially within the poultry industry which produces more than 8.5 billion tons of feathers annually (Jagadeesan et al., 2023). Chicken feathers contain 90 % raw keratin and constitute up to 10 % of the body mass of mature birds. Despite their abundance, the feather by-products remain substantially underutilized, presenting a considerable challenge in effective waste management and resource optimization (Sharma & Kumar, 2019). The majority of feather waste is disposed through incineration or composting, contributing to environmental pollution, while feather management methods are primarily for low-value applications, such as fertilizers and animal feed of inferior nutritional quality (feather meal). Such underutilization not only signifies a lost opportunity for the development of novel keratin-based bioproducts but also heightens environmental concerns linked to inefficient waste disposal (Ossai et al., 2022).

Abbreviations: ATR FT-IR, attenuated total reflectance Fourier transform infrared; DTT, dithiothreitol; DH, degree of hydrolysis; HP-SEC, high performance size exclusion chromatography; LGC, least gelling concentration; MW, molecular weight; OPA, o-phthaldialdehyde; pI, isoelectric point; SDS, sodium dodecyl sulphate; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; TCA, trichloroacetic acid.

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Many techniques for keratin extraction have been developed, including chemical, physical or biological treatments and their combinations. These processes differ in the keratin yield, the physicochemical properties of protein preparations obtained and cost-effectiveness (Callegaro et al., 2019; Sinkiewicz et al., 2018). Keratin preparations containing digestible protein show great promise for use in value-added products within the food and pharmaceutical industry, such as novel dietary protein (Dias et al., 2022; Giteru et al., 2023; Houltham et al., 2014; Santos et al., 2024), source of bioactive peptides with antihypertensive and antidiabetic potential (Callegaro et al., 2019; Sinkiewicz et al., 2018) or functional additive due to its antioxidant, antimicrobial, foaming, emulsifying and fat-binding properties (Bouhamed et al., 2020; Sinkiewicz et al., 2018). However, the efficient production of keratin preparations suitable for such applications poses a challenge as native keratins are insoluble in solutions that do not trigger their degradation (Sinkiewicz et al., 2018). Thus, most feather solubilization methods yield low-quality keratin, often contaminated with unnatural amino acids or sulphur-containing odorous compounds, limiting its potential applications (Crum et al., 2018; Shavandi et al., 2017).

Reductive extraction stands out among the keratin isolation techniques. Not only for a high keratin yield but also for preserving the protein backbone through preferential cleavage of disulphide and hydrogen cross-links while minimizing peptide bond fragmentation. As a result of reductive extraction, soluble derivatives called kerateins are formed (Shavandi et al., 2017; Sinkiewicz et al., 2018). Among the most promising reducing agents that allow for keratein production is L-cysteine. Unlike most traditional reducing agents like β-mercaptoethanol, dithiothreitol (DTT) or sodium sulphide, L-cysteine is non-toxic, cost-effective, eco-friendly and widely available through sustainable industrial fermentation processes. L-Cysteine-extracted keratin could, therefore, be suitable for high-value applications, including the food and pharmaceutical industry (Ghaffari-Bohlouli et al., 2023; Xu & Yang, 2014).

Hydrolysis is a crucial tool in protein processing, releasing bioactive peptides and enhancing the protein's functional and nutritional value. Peptides produced through partial proteolysis have smaller MW and altered functional (e.g. solubility, emulsification, gelation) and biological (e.g. bioactivity, digestibility, allergenicity) attributes compared to intact proteins (Barac et al., 2012; Lorenzo et al., 2018; Tavano, 2013). While chemical hydrolysis is cost-effective and efficient, it is time-consuming, difficult to control and risks forming non-specific or toxic by-products (Momen et al., 2021; Stiborova et al., 2016). Microbial hydrolysis, though milder and more specific, is slow, and protein metabolism by microorganisms can reduce the total protein yield (Lasekan et al., 2013; Stiborova et al., 2016). Enzymatic hydrolysis provides superior control, specificity and repeatability, combined with rapid reaction speed. It produces highly nutritious hydrolysates free from toxic by-products and can be particularly cost-effective in the case of waste materials from mainstream food processing, considering processors are already charged for their disposal at landfills (Hayes, 2021; Lorenzo et al., 2018). However, commercial keratinases, while uniquely capable of cleaving native keratin, are not yet practical for industrial applications, due to the high cost and low activity (Saeed et al., 2024).

The objective of this study was to obtain a high-purity, water-soluble preparation of feather keratin and then generate bioactive peptides showing potential for food-grade applications. To address challenges in enzymatic keratin hydrolysis, this study proposes a novel two-step approach. First, soluble keratin was extracted through reduction with L-cysteine, with or without 8 M urea, and then hydrolysed using conventional proteases: trypsin, chymotrypsin, pepsin, subtilisin or papain. This approach contrasts with chemical and microbial methods typically used for keratin hydrolysis, potentially offering improved feasibility and cost-effectiveness. This investigation integrates our previous theoretical (in silico) study (Taraszkiewicz et al., 2022), which indicated that chicken feathers are a potential source of peptides with 15 bioactivities (mainly dipeptidyl peptidase IV, angiotensin-converting enzyme and prolyl oligopeptidase inhibitory and antioxidant), with experimental (in vitro) results of the keratin enzymatic hydrolysis, contributing to a comprehensive understanding of the process. Selected structural and techno-functional properties of the resulting hydrolysates, relevant to potential applications in the food or pharmaceutical industries, were also assessed.

2. Materials and methods

2.1. Materials

Commercial detergent Ludwik® was purchased from Grupa Inco S. A., Poland. L-Cysteine, urea, trypsin from porcine pancreas (1500 U/mg), α-chymotrypsin from bovine pancreas (≥40 U/mg protein), pepsin from porcine gastric mucosa (≥2500 U/mg protein), subtilisin A from *Bacillus licheniformis* (≥2.4 U/g, Alcalase® 2.4 L), papain from papaya latex (≥10 U/mg protein), acrylamide, ammonium persulphate, bromophenol blue, HCl, L-leucine, L-glycine, o-phthaldialdehyde (OPA), sodium dodecyl sulphate (SDS), sodium tetraborate, tetramethylethylenediamine, trichloroacetic acid (TCA), Tris, HPLC-grade acetonitrile, trifluoroacetic acid and protein standards were purchased from Sigma-Aldrich, USA. Acetic acid, glycerol, methanol and NaOH were acquired from POCH, Poland. Prestained Protein Marker II (6.5–200 kDa) was purchased from AppliChem, Germany. DTT and Coomassie Brilliant Blue R-250 were acquired from Fluka, USA. Food-grade refined sunflower oil was supplied by Rustica, Poland.

2.2. Obtaining keratin preparations

2.2.1. Pretreatment of feathers

White chicken feathers supplied by a local company (Drobful, Poland) were pretreated based on previous methods (Dąbrowska et al., 2022; Sinkiewicz et al., 2017). The feathers were first washed in warm tap water with detergent and rinsed with distilled water, then dried at 50 °C overnight, cut into 2–3 cm filaments and finally milled into 0.75 mm pieces using an ultra-centrifugal mill (Retsch, Type ZM 200, Germany).

2.2.2. Keratin extraction

The extraction of keratin was performed based on reduction methods reported previously (Sinkiewicz et al., 2017; Xu & Yang, 2014; N. Zhang et al., 2022). Either urea-containing or urea-free solution of L-cysteine was used for this purpose. In both cases, the pretreated feathers were mixed at a ratio of 1:20 (*w/v*) with extraction solution and shaken at 200 rpm (Thermo Forma 420 Orbital Shaker, Thermo Scientific, USA). In the case of urea-containing solution, the extraction was carried out with 1.5 or 2.0 % (w/v) L-cysteine and 8 M urea, adjusted to pH 9.0 or 10.5 with NaOH, at 30, 40, 50, 60 or 70 °C for 1, 3 or 6 h. In the case of urea-free solution, the extraction was carried out with 1.0, 1.5 or 2.0 % (w/v) L-cysteine, adjusted to pH 10.0, 11.0 or 12.0 with NaOH, at 30 °C for 24 h. After the reaction was completed, in both cases, the mixture was centrifuged (5000 rpm, 10 min) and filtered to separate the solution of soluble keratin from the feather residue. The filtrate was purified against distilled water using Spectra/Por dialysis membranes of regenerated cellulose (MWCO 3.5 kDa) at 4 °C for 3 days, freeze-dried and stored at 4 °C.

2.2.3. Keratin extraction yield

The keratin extraction yield [%] was determined as the weight loss of the raw material (feather solubilization) based on the measurement of the dry mass of undissolved feathers post keratin reduction processes (Sinkiewicz et al., 2017).

2.2.4. Enzymatic hydrolysis of keratin isolate

For the enzymatic hydrolysis only one keratin extract was chosen from the range of extracts produced, based on high yield, good structural

integrity and cost-effectiveness. This extract was obtained in the process with 1.5 % L-cysteine, 8 M urea, pH 10.5 at 30 °C for 1 h, labelled keratin isolate. This isolate was dissolved in distilled water to reach 10 mg protein/mL, and heated to the optimal temperature of each enzyme, followed by pH adjustment using either 1 M HCl or NaOH. Hydrolysis reactions with a different single enzyme: trypsin (KI-T), chymotrypsin (KI-C), pepsin (KI-P) and subtilisin (KI-S), were performed separately. For all the enzymatic reactions, the enzyme to substrate ratio was 1:20 (*w*/w) and the reaction time was 1 h. The hydrolysis was carried out in triplicate at pH 8 and 37 °C (KI-T and KI-C), at pH 2 and 37 °C (KI-P), or at pH 9 and 60 °C (KI-S). The pH was kept constant during the whole reaction using a pH-stat titrator (T70, Mettler Toledo, USA) with 0.1 M NaOH (KI-T and KI-C), 1 M HCl (KI-P), or 1 M NaOH (KI-S). After the reaction, keratin hydrolysates obtained were heated at 85 °C for 15 min to inactivate the enzymes, replicates pooled, freeze-dried and stored at 4 °C. Preliminary experiments with papain revealed that keratin hydrolysates produced by this enzyme did not reach the degree of hydrolysis (DH) > 2 % and regardless of hydrolysis conditions excessive protein aggregation occurred (data not shown).

2.3. Chemical composition and structural properties

2.3.1. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE analysis was performed according to Laemmli (1970). The keratin extracts obtained with urea-free solution, the keratin isolate, and its enzymatic hydrolysates were dissolved in distilled water at 20, 10, and 30 mg/mL, respectively. Variability in the concentration was necessary to visualize the particular protein bands, due to differences in protein integrity between the samples. The stacking and separating gels were 5 and 15 %. The gels were stained with 0.25 % Coomassie Brilliant Blue R-250 dissolved in a mixture of methanol, distilled water and acetic acid (50:40:10; *v*/v) and then destained using a mixture of methanol, distilled water and acetic acid (40:53:7; v/v). Pre-stained mixture of 10–240 kDa proteins was used as an MW marker.

2.3.2. High performance size exclusion chromatography (HP-SEC)

The samples or protein standards were dissolved in a mobile phase composed of acetonitrile, Milli-Q water and trifluoroacetic acid (30:60.9:0.1; v/v) to a final concentration of 0.5 mg/mL and centrifuged (12,000 rpm, 10 min). 30 μL of supernatant was injected into ReproSil 50 SEC column (300 × 8 mm, 5 μm), with a fractionation range of 0.5–10 kDa and eluted at a flow rate of 1 mL/min. Agilent liquid chromatograph (model 1200) with a diode array detector at 215 nm was used. The column was calibrated with: aprotinin (6.5144 kDa), bovine insulin (5.7335 kDa), bovine insulin oxidized, chain B (3.4959 kDa), bacitracin A (1.4227 kDa) and bradykinin (1.0602 kDa).

2.3.3. Attenuated total reflectance Fourier transform infrared (ATR FT-IR) spectroscopy

The FT-IR spectra of the pretreated feathers, the keratin isolate and its enzymatic hydrolysates were recorded in the range of 400–4000 cm^{-1} on FT-IR spectrophotometer (Invenio-R, Bruker, USA). Sixty four scans were recorded for each spectrum, with a resolution of 4 cm^{-1}. Prior to the analysis, the samples were conditioned in a desiccator over P_2O_5 for 7 days.

2.3.4. Degree of hydrolysis

Two methods for the determination of the DH were used. DH_{OPA} was measured based on quantification of free -NH_2 groups, according to Bavaro et al. (2021) with a slight modification. 10 μL of the sample was mixed with 200 μL of reagent consisting of 0.8 mg/mL OPA, 38.1 mg/mL sodium tetraborate, 1 mg/mL SDS and 0.88 mg/mL DTT. After 15 min incubation, A_{340} was measured using a microplate reader (Synergy HT, BioTek Instruments, Inc., USA). L-Leucine was used to generate a calibration curve. DH_{OPA} was calculated as:

$$DH_{OPA}\ [\%] = (h_1/h_2) \times 100\%$$

where: h_1 and h_2 – concentration of -NH_2 groups in the sample and in the keratin isolate subjected to oxidation with performic acid followed by complete hydrolysis with HCl according to ISO 13903:2005, respectively [mmol -NH_2/g protein].

$DH_{pH\text{-}stat}$ was measured based on the volume of NaOH solution added during the hydrolysis to maintain a constant pH (Teshnizi et al., 2020) and calculated according to Adler-Nissen (1986):

$$DH_{pH-stat}\ [\%] = (V_b \times N_b)/(m_p \times h \times \alpha) \times 100\%$$

where: V_b – NaOH volume [mL], N_b – NaOH concentration [mol/L], m_p – protein mass [g], h – total number of peptide bonds [7.7 mEq/g protein], α – degree of dissociation of α-NH_2 groups.

2.3.5. Proximate composition

Moisture and ash content of the feathers, the keratin isolate and its enzymatic hydrolysates were determined using Leco TGA701 gravimetric oven (Leco Instruments UK Ltd., UK) (Pérez-Vila et al., 2024). 0.8 g of each powder was heated to 102 °C at a rate of 1 °C/min and held at 102 °C for 4 h to determine the moisture content, and then heated to 550 °C at a rate of 15 °C/min and held at 550 °C for 4 h to determine the ash content. Protein content was determined by total amino acid analysis (2.3.6.) in the feathers and the keratin isolate, and by subtraction of moisture and ash content in the enzymatic hydrolysates.

2.3.6. Amino acid analysis

Analysis of the total amino acid profile in the feathers and the keratin isolate was performed by Sciantec Analytical Services Ltd. (Cawood, UK) according to ISO 13903:2005. The analysis of the free amino acid profile (except Trp) in the keratin isolate and enzymatic hydrolysates followed the method of Mounier et al. (2007). An equal volume of the redissolved sample and 24 % (*w/v*) TCA were mixed, left for 10 min and then centrifuged (15,000 rpm, 10 min). The supernatant was removed, and the sample was diluted 1:2 with the internal standard, norleucine, to give a final concentration of 125 nmol/mL norleucine. Free amino acids were quantified using a Jeol JLC-500/V amino acid analyser (Jeol (UK) Ltd., Garden City, Herts, UK) fitted with a Jeol Na^+ high performance cation exchange column.

2.4. Techno-functional properties.

2.4.1. Solubility

The solubility of the keratin isolate and its enzymatic hydrolysates was measured according to Bouhamed et al. (2020), with a slight modification. The sample was dissolved in distilled water to a final concentration of 10 mg protein/mL, pH was adjusted to 2–9 using 0.1–2 M HCl or NaOH and then the mixture was centrifuged (5000 rpm, 30 min). The protein content of the supernatant was determined using the Biuret method. Solubility was expressed as a percentage of soluble protein content relative to the total protein content in the sample.

2.4.2. Gelling properties

The least gelling concentration (LGC) of the keratin isolate and its enzymatic hydrolysates was determined according to Taragjini et al. (2022). The samples were dissolved in distilled water to reach 1, 5, 10, 15 or 20 % (m/m), heated at 90 °C for 1 h in a heat block (Red-Hot 35, DNA Gdańsk, Poland) and then cooled down at 4 °C for 2 h. The LGC was defined as the minimal concentration at which the sample did not slip from the inverted tube.

2.4.3. Water- and oil-holding capacity

The water- and oil-holding capacity of the keratin isolate and its enzymatic hydrolysates were measured according to Ma et al. (2022), with a slight modification. The powdered samples (100 and 50 mg) were

mixed with 1 mL distilled water and 1.5 mL sunflower oil, respectively, vortexed for 1 min, left for 30 min and then centrifuged (5000 rpm, 30 min). The supernatant was eliminated and the sample was weighted.

2.4.4. Foaming properties

The foaming properties of the keratin isolate and its enzymatic hydrolysates were measured according to Bouhamed et al. (2020) with a modification. 100 mg of powdered sample was dissolved in distilled water to a final volume of 10 mL, pH was adjusted to 5, 7 or 9 using 0.1–1 M HCl or NaOH and the mixture was homogenized (12,000 rpm, 1 min).

The foaming capacity and foam stability were calculated as follows:

$$\text{Foaming capacity } [\%] = (V_B - V_A)/V_A \times 100\%$$

$$\text{Foam stability } [\%] = (V_C - V_A)/(V_B - V_A) \times 100\%$$

where: V_A, V_B and V_C – the total volume of the mixture before, directly after and 30 min after homogenization, respectively [mL].

2.4.5. Emulsifying properties

The emulsifying properties of the keratin isolate and its enzymatic hydrolysates were measured according to Taragjini et al. (2022) with a modification. 100 mg of powdered sample was dissolved in distilled water to a final volume of 10 mL, pH was adjusted to 5, 7 or 9 using 0.1–1 M HCl or NaOH and then 15 mL of sunflower oil was added. The mixtures were homogenized (12,000 rpm, 1 min), centrifuged (7000 rpm, 5 min), heated at 85 °C for 15 min, and re-centrifuged. The emulsifying capacity and emulsion stability were calculated as follows:

$$\text{Emulsifying capacity } [\%] = (V_1 - V_0)/V_0 \times 100\%$$

$$\text{Emulsion stability } [\%] = (V_2/V_1) \times 100\%$$

where: V_0 – the total volume of the mixture before homogenization [mL], V_1 and V_2 – the volume of the emulsion layer after centrifugation, heating and re-centrifugation, respectively [mL].

2.5. Statistical analysis

The experimental results were processed using SigmaPlot 11.0 (SYSTAT Software, Germany) through one-way analysis of variance (ANOVA) with a significance level of $p < 0.05$.

3. Results and discussion

3.1. Obtaining keratin preparations

3.1.1. Keratin yield obtained with urea-containing solution of L-cysteine

In the first part of this study, urea-containing solution of L-cysteine was used to extract the keratin. Urea is commonly used in reductive keratin extraction to unfold the protein and facilitate the exposure of disulphide bonds to reducing agents (Shavandi et al., 2017; Sinkiewicz et al., 2018). Initially, the effects of reaction temperature and time on keratin yield were investigated while maintaining a constant composition of the extraction solution previously proposed (Pourjavaheri et al., 2019; Xu & Yang, 2014). This solution, comprising 2.0 % (*w/v*) L-cysteine and 8 M urea at pH 10.5, consistently yielded approx. 90 % keratin, even at the mildest reaction conditions tested (Table S1). Increasing the temperature up to 70 °C and time up to 6 h did not enhance the keratin yield. Therefore, in pursuit of greater cost-effectiveness, the effects of decreasing the L-cysteine concentration from 2.0 to 1.5 % and lowering the pH from 10.5 to 9.0, on the keratin yield were examined while the temperature was 30 °C and the reaction time was 1 h. The decreased concentration of L-cysteine had no significant effect on the keratin yield ($p > 0.05$), while lowered pH led to the decrease of the keratin yield to approx. 86 % ($p < 0.05$, Table S2). Such a result likely reflects the finite number of disulfide bonds in keratin, which, once reduced, render further increases in L-cysteine concentration ineffective. The lower keratin yield at pH 9 likely results from reduced deprotonation of the -SH group, decreasing nucleophilicity and reactivity, thereby impairing disulfide bond reduction (Xu & Yang, 2014). Thus, in terms of keratin yield with urea-containing L-cysteine solutions, the optimal composition of the extraction solution was 1.5 % L-cysteine, 8 M urea, pH 10.5, and the optimal reaction conditions were 30 °C for 1 h, at which the keratin yield reached about 90 %. Xu and Yang (2014) reported a maximal feather keratin yield of 75 % under similar extraction conditions, i.e. 10 % L-cysteine based on the weight of feathers (equivalent to approx. 0.6 % in solution), 8 M urea, pH 10.5 at 70 °C for 12 h. Pourjavaheri et al. (2019) obtained a maximal yield of 66 % when treating the feathers with 2.0 % L-cysteine, 8 M urea, pH 10.5 at 40 °C for 6 h. Qin et al. (2023) achieved a maximal keratin yield of 63 % using 15 % L-cysteine, 8 M urea, pH 10.5, with ultrasonic treatment at 200 W for 4 h. In the reductive feather keratin extraction with urea and different reducing agents, Sinkiewicz et al. (2017) obtained the yield of 84, 82, 78 and 63 % with β-mercaptoethanol, sodium bisulphite, DTT and sodium m-bisulphite, respectively. The differences in keratin yield reported by these studies can be attributed to variations in the reducing agents used and their concentration, reaction conditions (e.g. temperature, pH, solid-liquid ratio), and additional treatment methods such as ultrasound, which can enhance solubilization but also lead to over-degradation under prolonged exposure. Furthermore, differences in feather pretreatment, such as grinding to increase surface area, and variations in the calculation methods for keratin yield may have also contributed to these discrepancies.

3.1.2. Keratin yield obtained with urea-free solution of L-cysteine

In the second part of the study, the keratin extraction with urea-free solution of L-cysteine was performed. According to Zhang et al. (2022), wool keratin can be effectively extracted at room temperature using only 2 % L-cysteine at pH > 10, which lowers the price by eliminating 8 M urea, but with extraction time prolonged to 24 h. According to these authors, the wool keratin yield obtained at such conditions was between 5 and 95 %, increasing with pH. In our study, the keratin yield obtained at 30 °C after 24 h reaction ranged from 14 to 88 %, increasing with L-cysteine concentration and pH of the extraction solution (Table S3). According to Dąbrowska et al. (2022) alkaline-hydrolysed feather keratin yield ranged from 11 to 41 % at room temperature and, according to Sinkiewicz et al. (2017), from 29 to 94 % at 70 °C. In both of these studies and similar to Table S3, NaOH concentration was the major factor determining the extraction yield.

3.2. Chemical composition and structural properties

3.2.1. SDS-PAGE

3.2.1.1. Electrophoretic profiles of keratin obtained with urea-containing solution of L-cysteine. SDS-PAGE was used to evaluate the keratin integrity and to estimate the MW of the peptides obtained. Only the keratin isolate produced in the optimized process with 1.5 % L-cysteine, 8 M urea, pH 10.5 at 30 °C for 1 h was chosen for analysis as it had a high keratin yield with lower temperature, less time (Table S1), and lower L-cysteine content (Table S2) than other extracts. The profile of the keratin isolate showed that it had a well-preserved protein backbone and was relatively pure (Fig. 1A, KI), as intact bands at expected MW were clearly seen. The most intense band at 10 kDa corresponded to β-keratin (the dominant component of feathers) and those at 50–70 kDa were most likely α-keratins (Sharma & Kumar, 2019).

The profiles of KI-T and KI-C indicated that hydrolysis of the keratin isolate with trypsin and chymotrypsin led to peptides of 8–13 kDa. In the profiles of KI-P and KI-S only weak bands were visible, indicating the presence of low MW peptides that are not detectable by the staining

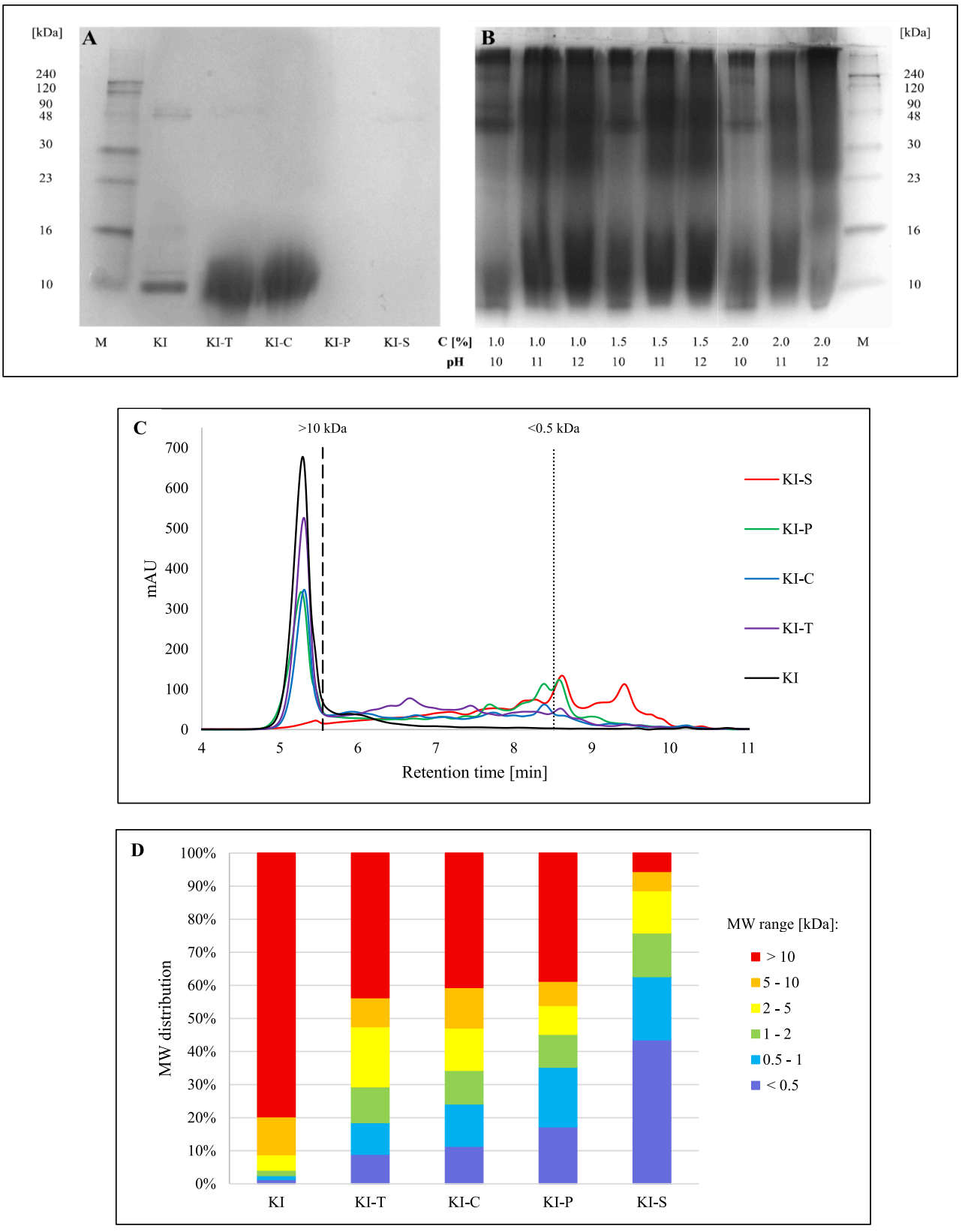

Fig. 1. MW distribution of keratin preparations: (A) SDS-PAGE electropherogram of the keratin isolate and its enzymatic hydrolysates, (B) SDS-PAGE electropherogram of keratin extracts obtained with urea-free solutions, (C) HP-SEC chromatogram of the keratin isolate and its enzymatic hydrolysates, (D) MW distribution profiles of the keratin isolate and its enzymatic hydrolysates. KI – keratin isolate.

employed. Based on the specificity of the proteases applied, such a result was expected. Trypsin has the narrowest substrate specificity, followed by chymotrypsin, having a slightly wider substrate preference and ending up with pepsin and subtilisin, both having wide substrate specificity (Minkiewicz et al., 2019).

Xu and Yang (2014) isolated feather keratin with urea-containing L-cysteine solution, visualising β-keratin only or both α- and β-keratin bands, depending on extraction conditions. Nonetheless, smears indicating protein degradation due to non-specific alkaline hydrolysis were present in all samples obtained by these authors. Pourjavaheri et al. (2019) also used urea-containing L-cysteine solution and extracted feather keratin with higher purity, as indicated by the absence of smears. However, only band corresponding to β-keratin was observed. The differences in electrophoretic keratin patterns obtained in the aforementioned studies, in comparison with the keratin isolate, may again be a result of different feather pretreatment but also keratin reduction conditions and isoelectric precipitation rather than dialysis.

3.2.1.2. Electrophoretic profiles of keratin obtained with urea-free L-cysteine solutions. As the keratin yield obtained in the urea-free extraction varied greatly (Table S3), all 9 extracts were analysed by SDS-PAGE to evaluate the protein integrity (Fig. 1B). In contrast to the electrophoretic pattern of the keratin isolate, obtained with urea-containing solution, all electrophoretic profiles of extracts produced with urea-free solutions were visible as smeared bands. This indicates that protein degradation has occurred, likely due to alkaline hydrolysis caused by high pH and long reaction time, regardless of reaction conditions tested (Fig. 1B). The degree of keratin degradation, represented by the band smearing intensity, was moderate at pH 10 and severe at pH 11 and 12. These degradation products, while likely of limited usefulness in high-value sectors, could potentially still be suitable for applications where protein integrity is less critical, such as fertilizers (Sinkiewicz et al., 2018). Zhang et al. (2022) obtained L-cysteine-extracted keratin from wool at pH ranging from 7 to 13.5 without urea. According to the authors, the keratin yield increased with rising pH levels, but the integrity of the polypeptide chain was maintained up to pH of 12, leading to the extraction yield of approx. 70 %. As Fig. 1B clearly shows, undegraded feather keratin could not be obtained without urea. The differences in the protein profiles of L-cysteine-extracted feather and wool keratins, obtained under similar reduction conditions, probably result from varying proportions of keratin types in these sources. In feathers, β-keratin, the stiffer and less extractable one, is predominant, while in wool, α-keratin, easier to solubilize, is more abundant (Sharma & Kumar, 2019; Sinkiewicz et al., 2018).

From the range of keratin extracts obtained with urea-containing and urea-free solutions of L-cysteine, only the keratin isolate was chosen for the assessment of structural and functional properties, based on the high yield, purity, structural integrity and cost-effectiveness.

3.2.2. HP-SEC

The sharpness of the keratin isolate peak (Fig. 1C, black line) and its retention time observed in the chromatogram at close proximity to the upper fractionation range of the column (10 kDa) confirm the preservation of the polypeptide backbone during extraction. A slight tail on the right side of the peak indicates the presence of only a small fraction of keratin hydrolysis products. The retention time of the peak mostly overlaps with the void volume of the column, indicating that most analytes were close to or higher than 10 kDa in size. The β-keratin of chicken feathers, the dominant component of feathers, comprises 97 amino acid residues and has the MW of 9.9 kDa (Bateman et al., 2023). The sizes of peptides were the highest in the keratin isolate and lower in the enzymatic hydrolysates, with the average MW of 9.04, 5.96, 5.79, 5.23 and 2.10 kDa for the keratin isolate, KI-T, KI-C, KI-P and KI-S, respectively (Fig. 1D). Qin et al. (2023) reported that keratin isolated by ultrasound-assisted extraction with 15 % L-cysteine, 8 M urea, pH 10.5 exhibited a broad MW distribution. Depending on ultrasonic power and time, the majority of peptides were within the 0.5–3 kDa range, reflecting significant protein fragmentation. The MW of protein hydrolysates significantly influences their bioactive properties. Low MW peptides generally exhibit reduced immunogenicity, as well as improved solubility, bioavailability, and bioactivity, except for antimicrobial activity, which is typically attributed to peptides containing 10–50 amino acid residues (Apostolopoulos et al., 2021; Malinowska-Pańczyk, 2023).

3.2.3. Degree of hydrolysis

Protein DH in the keratin preparations was measured using two methods, and the results obtained, DH_{OPA} and $DH_{pH\text{-}stat}$, were compared to theoretical DH_t values calculated in our in silico study (Taraszkiewicz et al., 2022, Table 1). The OPA assay confirmed that the DH_{OPA} value of the keratin isolate was below 1 %. The actual keratin DH may have been even lower, considering the reactivity of the OPA reagent towards residual L-cysteine remaining after extraction and ε-NH_2 groups of lysine, even in intact protein (Rutherfurd, 2010).

The effectiveness of keratin hydrolysis by the proteases applied, indicated by their DH_{OPA} values, aligned with the results from HP-SEC (Fig. 1). A strongly linear relationship ($R^2 > 0.98$) between the hydrolysates' average MW determined by HP-SEC and their DH was noted, regardless of the DH assay. This suggests that the reduction in the keratin isolate MW post-enzymatic hydrolysis resulted solely from the breakdown of peptide bonds. In contrast, according to Zhang et al. (2015), the MW decrease in duck feather keratin caused by alkaline hydrolysis resulted not only from the disruption of peptide bonds but also from the disulfide and non-covalent bonds.

$DH_{pH\text{-}stat}$ of KI-P could not be measured due to the hydrolysis at pH 2. The $DH_{pH\text{-}stat}$ values of KI-T, KI-C and KI-S showed a very strong correlation ($R^2 = 0.99$) with their respective DH_{OPA} values. However, the values of $DH_{pH\text{-}stat}$ were higher than those of DH_{OPA}, likely due to the poor reactivity of the OPA reagent with proline and cysteine in comparison with other amino acids. The values of DH_{OPA} obtained for hydrolysates rich in free cysteine and free proline, and peptides containing these amino acids, may, therefore, be underestimated (Rutherfurd, 2010).

At least one of the experimental DH values of KI-T, KI-C and KI-S aligned closely with the DH_t values (Table 1). In contrast, the value of DH_{OPA} in KI-P was much lower than its DH_t, suggesting that the peptide bonds susceptible to pepsinolysis, although present in the keratin chain, were largely inaccessible. A discrepancy between the theoretical and experimental results of protein hydrolysis is common (Iwaniak et al., 2024). The in silico proteolysis is based only on the specificity of enzymes, whereas the actual complete enzyme characteristics are more complex. The computer simulation assumes that all peptide bonds are

Table 1
DH and proximate composition of feathers, the keratin isolate and its enzymatic hydrolysates.

Parameter [%]	Feathers	KI	KI-T	KI-C	KI-P	KI-S
Moisture	9.7 ± 0.05^{A}	7.7 ± 0.03^{C}	5.6 ± 0.03^{E}	5.6 ± 0.04^{E}	8.5 ± 0.02^{B}	5.9 ± 0.01^{D}
Ash	0.6 ± 0.1^{E}	1.6 ± 0.03^{D}	4.0 ± 0.04^{B}	3.9 ± 0.06^{B}	2.0 ± 0.05^{C}	24.3 ± 0.36^{A}
Protein	83.8 ± 0.95^{C}	91.0 ± 0.08 ^{A}	90.5 ± 0.06 ^{A}	90.5 ± 0.09 ^{A}	89.5 ± 0.03^{B}	69.8 ± 0.03^{D}
DH_{OPA}	–	0.8 ± 0.05^{E}	5.9 ± 0.34^{D}	6.9 ± 0.35^{C}	7.6 ± 0.62^{B}	20.5 ± 0.55^{A}
$DH_{pH\text{-}stat}$	–	–	8.4 ± 0.19^{C}	12.8 ± 0.15^{B}	–	36.2 ± 0.11^{A}
DH_t*	–	–	6.3	14.6	54.2	36.4

The values in the columns marked with various superscript letters (A-E) differ significantly ($P < 0.05$).
KI – keratin isolate.
* Adapted from Taraszkiewicz et al. (2022).

cleavable, while under experimental conditions some of them may be resistant to hydrolysis and the protein-enzyme interactions can be impeded by the complexity of the protein 3D structure (Iwaniak et al., 2020). Additionally, in the case of feathers, the experimental DH referred to all feather proteins present in the keratin isolate, while in our theoretical study, only β-keratin was analysed (Taraszkiewicz et al., 2022).

3.2.4. FT-IR

In all the FT-IR spectra of keratin preparations obtained, bands typical for proteins were observed (Table 2, Fig. 2). The FT-IR spectrum of the keratin isolate (Fig. 2, KI) is like the spectrum of the pretreated feathers (Fig. 2, Feather), with only slightly lower absorbance at approx. 2935 and 2850 cm^{-1}, and Amide *I*-III regions. No new bands appeared in the spectrum of the keratin isolate compared to the spectrum of native keratin present in the pretreated feathers. This indicated that the former has similar functional groups to the latter (N. Zhang et al., 2022), confirming the high purity of the keratin isolate and lack of significant peptide bond scission during the extraction (Wang, Li, et al., 2016; Y. Zhang et al., 2015). However, a partial transition from α-helix to β-sheet configuration could have taken place in the keratin isolate, as evidenced by a slight shift in the Amide *I* region towards higher wavenumbers (Alahyaribeik & Ullah, 2020a; Martínez-Hernández et al., 2005; Wang, Li, et al., 2016).

In the enzymatic hydrolysates, changes in the Amide *I* and Amide II band intensity, and the right shoulder in the Amide A band indicated an increase in the amount of free -NH_2 groups, confirming the peptide bond hydrolysis. This is consistent with the results of HP-SEC and DH. A strong negative correlation ($R^2 = 0.84$) between DH_{OPA} and the band absorbance corresponding to the Amide I region was observed, while a moderate positive correlation ($R^2 = 0.69$) existed between DH_{OPA} values and the band absorbance corresponding to —COO^- group. The FT-IR spectrum of KI-P does not show a peak around 1390 cm^{-1}, unlike the remaining hydrolysates. This difference is likely due to the pH of KI-P being below the pI, causing protonation of the —COOH group, whereas in the other hydrolysates, the pH was above the pI, resulting in its deprotonation. The prominent bands at 530–540 cm^{-1} found in all keratin preparations suggest that even with extensive disruption of peptide bonds, the keratin continues to be strongly cross-linked by disulfide bonds.

3.2.5. Proximate composition

The feathers showed a moisture content of approx. 10 %, similar to previous findings (Sharma & Kumar, 2019). The keratin isolate and its enzymatic hydrolysates exhibited lower moisture level, from 5.6 to 8.5 % (Table 1), typical of powdered proteins (Rao et al., 2016) and ensuring microbiological stability.

The ash content of the feathers, initially 0.6 %, gradually increased with subsequent processing steps. In the keratin isolate, it rose by 1 %, likely due to residual NaOH remaining post-dialysis, and in the enzymatic hydrolysates, it increased proportionally with the DH ($R^2 > 0.88$), up to nearly 25 % in the case of KI-S (Table 1). The high ash content is a typical characteristic of extensively hydrolysed proteins from both plant and animal sources, as reaching high DH requires the significant addition of acids or bases for pH adjustment (Chalamaiah et al., 2012; Tawalbeh et al., 2023).

The protein content of the feathers and the keratin isolate reached approx. 84 and 91 %, respectively. High purity of the keratin isolate was confirmed, as its moisture, ash and protein content reached a total of 100 %. This result made it possible to calculate the protein content of the enzymatic hydrolysates by subtracting their moisture and ash contents from the total sample mass. The protein content in the hydrolysates decreased with the increasing DH, due to the increasing content of inorganic salts (ash) (Table 1).

3.2.6. Total amino acid composition

Table 3 details the total amino acid profile of the feathers and the keratin isolate. Published data on whey protein isolate, a complete, easily digested protein of high nutritional value is included for comparison (Norton et al., 2012). The presented profiles of the feathers and the keratin isolate were similar to the profiles of feathers and keratin preparations reported previously (Sharma & Kumar, 2019). The content of most amino acids was higher in the keratin isolate than in the feathers, except for Glx, His, Lys, Met and Tyr, indicating that these amino acid residues are likely concentrated in the parts of feathers that are more difficult to solubilize. Both the feathers and the keratin isolate contained all essential and non-essential amino acids (EAA and NEAA, respectively), however the content of His, Lys, Met and Trp was too low to consider the keratin isolate a complete protein. The total EAAs content of whey protein isolate was about 1.5 times higher than that of the keratin isolate. The keratin isolate's level of branched-chain amino acids: Ile, Leu and Val was only about 13 % lower than in whey protein isolate, indicating its potential in reducing post-exercise muscle damage and soreness (Salem et al., 2024). The keratin isolate's high Cys content no doubt contributes to it's antioxidant capacity, beneficial for cellular defence and immune functions (Callegaro et al., 2019; Iwaniak et al., 2024; Sinkiewicz et al., 2018). The high content of Arg and Cys in the keratin isolate suggests its potential use in supplements for low birth weight infants, who require elevated levels of these amino acids in their diet (Kaushik et al., 2016; M. Wang et al., 1999). A much higher Arg:Lys ratio (1:0.06 in the keratin isolate vs. 1:2.3 in whey protein isolate) indicates potential cardiovascular benefits, as a higher ratio is commonly linked to reduced cholesterolemic and atherogenic effects of food proteins (Friedman, 2018; Kaushik et al., 2016). Additionally, the high Pro content indicates the keratin isolate's potential as a precursor of bioactive peptides. Studies focusing on the structure-activity

Table 2
Band assignments in the FT-IR spectra of pretreated feathers, keratin isolate and its enzymatic hydrolysates.

Region	Position [cm^{-1}]						Band assignment	Reference
	Feather	KI	KI-T	KI-C	KI-P	KI-S		
Amide A	3281	3281	3281	3281	3279	3279	ν_{NH}, ν_{OH}, α-helix	(Martínez-Hernández et al., 2005; Pourjavaheri et al., 2015, 2019)
Amide *B*	3073	3073	3073	3061	3061	3061	asymmetric ν_{NH}	(Martínez-Hernández et al., 2005)
	2930	2961	2961	2961	2961	2959	asymmetric ν_{CH3}	(Martínez-Hernández et al., 2005; Pourjavaheri et al., 2015, 2019)
	2936	2936	2936	2936	2936	2936	symmetric ν_{CH3}	(Martínez-Hernández et al., 2005; Pourjavaheri et al., 2015, 2019)
	2853	2878	2878	2878	2878	2876	ν_{CH2}	(Martínez-Hernández et al., 2005)
Amide *I*	1630	1634	1636	1636	1638	1632	$\nu_{C=O}$, α-helix, β-sheet	(Pourjavaheri et al., 2019)
Amide II	1514	1518	1528	1526	1518	1518	δ_{NH}, ν_{CH}, β-sheet	(B. Ma et al., 2016; Martínez-Hernández et al., 2005; Pourjavaheri et al., 2019)
	1447	1449	1450	1450	1450	1450	δ_{CH2} or δ_{CH3}	(Martínez-Hernández et al., 2005)
	1389	1389	1392	1391	–	1391	δ_{CH3}, -COO^-	(Kristoffersen et al., 2019, 2020; Martínez-Hernández et al., 2005)
Amide III	1232	1234	1236	1236	1232	1238	ν_{CN}, δ_{NH}, ν_{CC}, $\delta_{C=O}$	(B. Ma et al., 2016; Martínez-Hernández et al., 2005)
	1159	1159	1157	1159	1169	1159	$\nu_{S=O}$	(Alahyaribeik & Ullah, 2020b)
	1070	1061	1072	1047	1070	1047	ν_{CC}	(Martínez-Hernández et al., 2005)
	548	548	538	536	534	542	ν_{SS}	(Alahyaribeik & Ullah, 2020b)

KI – keratin isolate.

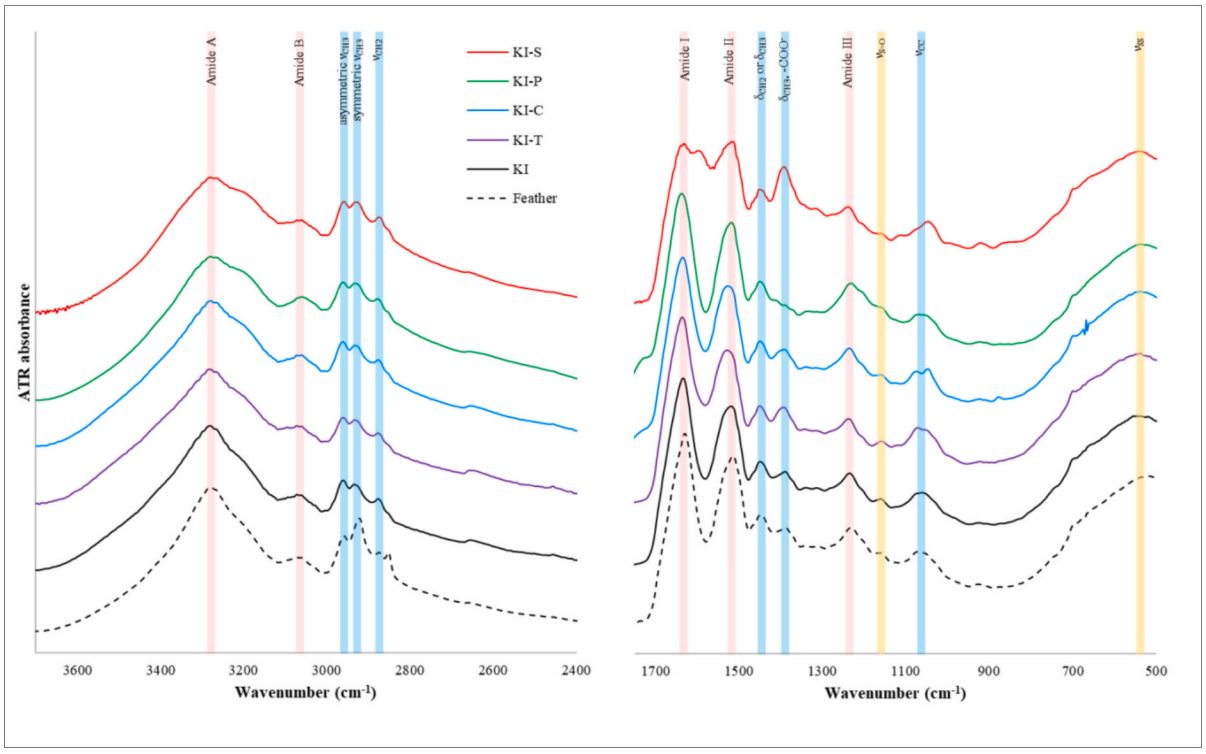

Fig. 2. FT-IR spectra of the pretreated feathers, the keratin isolate and its enzymatic hydrolysates. KI – keratin isolate.

Table 3
Total and free amino acid profiles of the feathers, the keratin isolate and enzymatic hydrolysates.

Amino acid	Total amino acid content [g/100 g]			Free amino acid content [mg/g]				
	Feather	KI	WPI	KI	KI-T	KI-C	KI-P	KI-S
Ala	3.66	4.07	4.41	0.03	0.07	0.08	0.05	1.49
Arg	5.70	6.40	2.16	0.02	3.04	0.13	0.10	0.54
Asx	5.28	5.41	9.53	0.00	0.08	0.00	0.39	0.00
Cys[a]	5.93	7.82	2.25	6.24	2.99	3.16	5.99	4.33
Cya[a]	–	–	–	0.97	4.98	8.76	11.69	11.23
Glx	8.69	8.40	15.19	0.01	0.05	0.00	0.12	2.87
Gly	6.15	7.00	1.62	0.03	0.05	0.07	0.09	0.28
His[b]	0.50	0.20	1.80	0.18	1.80	1.45	1.19	6.06
Ile[b]	4.09	4.40	5.57	0.01	0.08	0.75	1.23	6.93
Leu[b]	6.82	7.20	9.80	0.02	0.05	0.05	0.48	0.86
Lys[b]	1.50	0.40	8.18	0.00	1.06	1.72	0.16	0.85
Met[b]	0.48	0.20	1.80	0.03	0.08	0.18	0.32	1.71
Phe[b]	4.08	4.70	2.97	0.10	1.92	3.06	1.54	4.22
Pro	8.78	10.10	5.03	0.00	0.00	0.00	0.00	0.00
Ser	9.77	11.90	4.23	0.03	0.07	0.11	0.11	1.28
Thr[b]	3.96	4.20	5.75	0.01	0.05	0.12	0.09	0.66
Trp[c]	0.41	0.40	1.53	–	–	–	–	–
Tyr	1.84	1.60	2.70	0.01	0.15	0.06	0.03	0.58
Val[b]	6.16	6.50	5.39	0.00	0.00	0.55	0.00	1.74
ΣEAA	28.00	28.20	42.79	0.28	3.18	4.94	3.58	20.09
ΣNEAA	55.80	62.70	47.11	7.33	11.48	12.37	18.57	22.59
ΣBCAA	17.07	18.10	20.77	0.03	0.12	1.34	1.71	9.54
TOTAL	83.80	90.90	89.90	7.68	16.52	20.25	23.58	45.62

ΣEAA, ΣNEAA, ΣBCAA – sum of essential, non-essential and branched chain amino acids, respectively.
KI – keratin isolate.
WPI – whey protein isolate accessed from Norton et al. (2012).
[a] For the total amino acid analysis Cys content was determined after performic acid oxidation (as Cya).
[b] Essential amino acid.
[c] For the free amino acid analysis Trp could not be quantified accurately by Joel JLC-500/V.

relationship of peptides highlight Pro residue as an important contributor to their inhibition of angiotensin-converting enzyme, dipeptidyl peptidase IV (Iwaniak et al., 2018) and prolyl oligopeptidase (Taraszkiewicz et al., 2024), and antioxidant activity (Sinkiewicz et al., 2018). Furthermore, the presence of Pro residue in peptides increases their resistance to proteolytic degradation. These peptides may have longer half lives in the gut lumen increasing their opportunity to cross the gut barrier and enter the bloodstream (become bioavailable)

(Udenigwe et al., 2021).

3.2.7. Free amino acid composition

The most abundant free amino acids in the keratin isolate were Cys and Cya – its oxidized derivative (Table 3). These were likely residuals from the keratin extraction and dialysis processes. The total content of other free amino acids was below 0.5 mg/g, confirming that the keratin isolate was not significantly hydrolysed during extraction. The total content of free amino acids in the enzymatic hydrolysates was directly proportional to DH_{OPA} values ($R^2 = 0.99$). KI-T exhibited the highest content of free Arg, consistent with our prediction that Arg would be the only free amino acid released from β-keratin by trypsin (Taraszkiewicz et al., 2022). In KI-C, the total free amino acid content was about 20 mg/g, despite our predictions that chymotrypsin could only release peptides from β-keratin (Taraszkiewicz et al., 2022), suggesting these free amino acids were α-keratin-derived. The highest total content of free amino

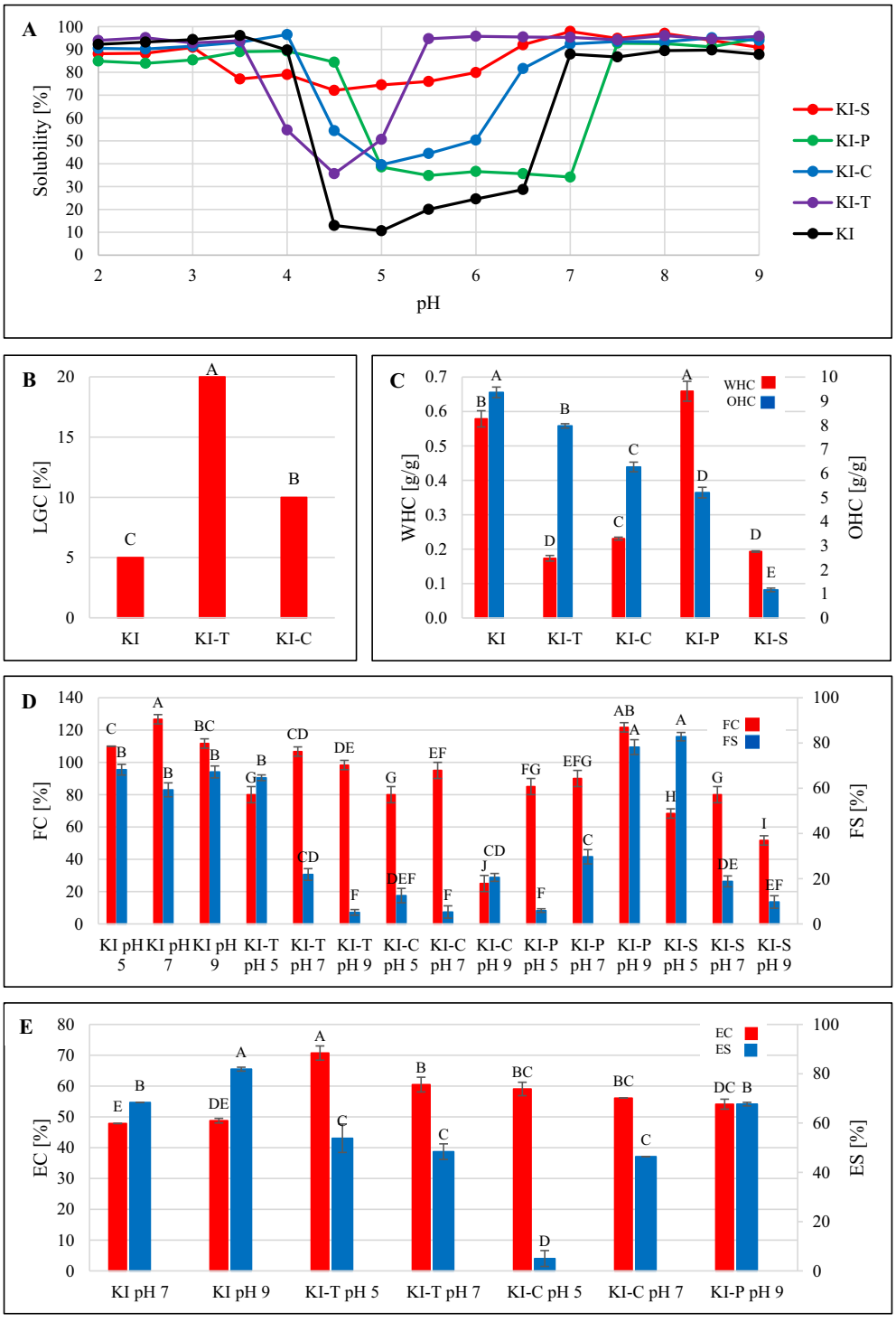

Fig. 3. Techno-functional properties of keratin preparations: (A) protein solubility [%] as function of pH; (B) gelling properties, LGC – least gelling concentration; (C) water and oil holding properties, WHC – water holding capacity, OHC – oil holding capacity; (D) foaming properties, FC – foaming capacity, FS – foam stability; (E) emulsifying properties, EC - emulsifying capacity, ES – emulsion stability. KI – keratin isolate. Values marked with different superscript letters (A-J) differ significantly ($p < 0.05$).

acids was detected in KI-S, as expected from the hydrolysate with the highest DH. However, with its MW fraction <0.5 kDa making up over 40 % (Fig. 1D), it is likely that this fraction predominantly comprised low MW peptides rather than free amino acids. None of the hydrolysates contained free Pro, even though it was the second most abundant amino acid in the keratin isolate (Table 3). This means that all Pro residues remained incorporated in peptides, indicating the keratin isolate's potential as a promising precursor of bioavailable peptides with high bioactivities (Udenigwe et al., 2021).

3.3. Techno-functional properties

3.3.1. Solubility

The keratin isolate exhibited a typical U-shaped solubility profile, with the highest amount of precipitated protein close to the isoelectric point (pI), at pH of 4.5–5.0 (Fig. 3A). The enzymatic hydrolysis of the keratin isolate caused a reaction condition-dependent shift in the pI. KI-P had a similar low-solubility pH range to the keratin isolate, although the pI shifted towards higher pH. The solubility of the remaining enzymatic hydrolysates was clearly improved in comparison with the keratin isolate, as the low-solubility pH range was narrower, and a lower amount of precipitate was noted at the pI (Fig. 3A). KI-S exhibited high solubility over the wide pH range, due to the presence of peptides with the lowest MW that are highly soluble (Fig. 1). Esparza et al. (2018) and Bouhamed et al. (2020) determined the pI of keratin at pH 4 in the case of protein resulting from DTT-assisted reduction and acid hydrolysis, respectively. Dąbrowska et al. (2022) reported the pI range at pH 3.4–4 for keratin obtained through alkaline hydrolysis. According to Xu and Yang (2014), the feather keratin extracted with the urea-containing solution of 0.6 % L-cysteine, pH 10.5 at 70 °C for 12 h, purified by isoelectric precipitation, was water-insoluble, unlike the keratin isolate extracted under very similar conditions. According to Shavandi et al. (2017), the conditions of water-soluble keratin production must be strictly controlled, as even small changes in the ratio of reducing and denaturing agents can cause protein precipitation.

3.3.2. Gelling properties

Gelling properties are crucial for proteins in food applications, where forming self-supporting gels is highly valued. Proteins with a low LGC typically exhibit strong gel-forming capability (Kamani et al., 2024; Taragjini et al., 2022). The keratin isolate, containing peptides of the highest MW (Fig. 1), had the lowest LGC at 5 %, indicating superior gel formation. Conversely, KI-P and KI-S, composed of the smallest peptides, failed to form gels even at 20 % (Fig. 3B), highlighting the necessity of large peptide size for structural integrity in gel networks (Damodaran et al., 2007). However, KI-T had the LGC of 20 %, twice that of KI-C, despite having a higher MW (Fig. 1). This suggests that peptide composition and their mutual interactions also affected gelling capacity. Moreover, gelling properties are influenced by pH, ionic strength and the presence of other solutes, which affect electrostatic and hydrophobic interactions within proteins (Kamani et al., 2024; Taragjini et al., 2022). While protein hydrolysates rarely form heat-induced gels, except for gelatine with an average MW > 20 kDa (Damodaran et al., 2007), various keratin-based gels have been reported at concentrations of 5–20 % (Shavandi et al., 2017). For comparison, the LGCs of bovine gelatine, chickpea, egg white, soybean, lentil and whey protein preparations were 3, 6, 10, 10, 13 and 14 %, respectively (Aydemir & Yemenicioğlu, 2013).

3.3.3. Water- and oil-holding capacity

Water- and oil-holding capacity are useful in determining protein powder's efficacy in preventing fluid leakage during processing and storage (Bouhamed et al., 2020; Kamani et al., 2024). The water-holding capacity of the keratin preparations obtained ranged between 0.17 and 0.66 g/g, with KI-P exhibiting the highest water retention (Fig. 3C). A clear inverse correlation was observed between water-holding capacity and solubility: the keratin isolate and KI-P, with the lowest solubility, had the highest water-holding capacity values. Conversely, the highly soluble hydrolysates – KI-T, KI-C and KI-S showed worse water retention. Such an inverse relationship is common. Highly soluble proteins tend to have more hydrophilic regions, making them more dispersible in water but less capable of forming networks that trap water (Damodaran et al., 2007). Bouhamed et al. (2020) reported similar water-holding capacity values for acid-hydrolysed feather keratin, ranging from 0.6 to 1.1 g/g. Aydemir and Yemenicioğlu (2013) reported the water-holding capacity of whey, egg white, lentil, chickpea, soybean and bovine gelatine at 0.00, 0.14, 1.04, 6.46, 7.94 and 8.84 g/g, respectively.

The keratin isolate demonstrated a remarkably high oil-holding capacity of 9.37 g/g, while the enzymatic hydrolysates showed a DH-dependent oil-holding capacity decrease (Fig. 3C). A robust inverse correlation between keratin DH and oil-holding capacity was evident ($R^2 > 0.94$), suggesting that lower peptide sizes resulted in poorer oil-binding capacities of the hydrolysates. Bouhamed et al. (2020) reported the oil-holding capacity of acid-hydrolysed feather keratin at 2–2.5 g/g, notably lower than that of the keratin isolate and most of its enzymatic hydrolysates. Wattie et al. (2018) found that feather keratin-based cryogels achieved a maximum oil-holding capacity of 10.76 g/g. According to Aydemir and Yemenicioğlu (2013), the oil-holding capacity of bovine gelatine, soybean, whey, egg white, lentil and chickpea protein preparations was 1.12, 1.16, 1.59, 6.37, 8.62 and 13.37 g/g respectively.

3.3.4. Foaming properties

All keratin preparations analysed formed foams, but their foaming properties strongly depended on pH and DH (Fig. 3D). Higher DH generally corresponded to lower foaming capacity, although not uniformly at all pH levels. At pH 5 and 7, a relatively strong linearity ($R^2 > 0.68$) was observed, but this correlation diminished at pH 9 ($R^2 < 0.24$), irrespective of the DH assay. At pH 7, the keratin isolate and all hydrolysates, except KI-P, exhibited the highest foaming capacity values. KI-P showed an increase in foaming capacity as the pH rose, peaking at pH 9 with foaming capacity of 122 %, coupled with stability of 88 %. In contrast, KI-S at pH 9 demonstrated the poorest performance, with the foaming capacity of only 52 % and stability of 67 %. At all pH levels tested, the keratin isolate outperformed the hydrolysates, maintaining some of the highest foaming capacity and achieving foam stability exceeding 60 %. For instance, the keratin isolate at pH 5 showed foaming capacity of 110 % and stability of 83 %, underscoring its superior foaming capabilities. Among the enzymatic hydrolysates, relatively high foam stability was observed only at certain pH values: at pH 5 in KI-T and KI-S, and at pH 9 in KI-P. In KI-C, the foam stability was low regardless of pH.

Bouhamed et al. (2020) reported the foaming capacity values of acid-hydrolysed feather keratin ranging from 55 % to 130 %, with stability from 0 % to 50 %, depending on hydrolysis conditions and protein concentration. Aydemir and Yemenicioğlu (2013) reported that the foaming capacity of egg white, lentil, soy, chickpea, whey and bovine gelatine protein preparations were 24, 34, 36, 44, 44 and 60 %, respectively. Their foam stability ranged between 90 and 100 %. In comparison, the keratin isolate's foaming capacity and foam stability values at pH 7 (127 % and 77 %, respectively) were remarkably high, indicating superior foaming properties. This comparison highlights the potential of keratin preparations obtained, especially the isolate, in applications requiring robust foam formation. On the other hand, the enzymatic hydrolysates displayed foaming properties ranging from moderate to poor, heavily influenced by pH.

3.3.5. Emulsifying properties

Emulsifying properties are crucial for numerous applications, where proteins stabilize oil-water mixtures. All keratin preparations obtained formed emulsions after homogenization with sunflower oil. However, only 7 out of the 15 tested pH variants formed emulsions that could be

measured after centrifugation (Fig. 3E), while in the remaining preparations, complete separation of the protein and oil layer occurred post-centrifugation. None of the preparations formed a measurable emulsion layer at all three tested pH variants, and KI-S did not form a measurable emulsion layer at any of the pH variants tested, indicating the pH sensitivity of the keratin-stabilized emulsions. The emulsifying properties of the keratin preparations were influenced by the DH and pH. The keratin isolate demonstrated a relatively high emulsifying capacity of 49 % at pH 7 and pH 9, with corresponding emulsion stability of 68 % and 82 %, but did not form a measurable emulsion layer at pH 5. In contrast, KI-T and KI-C formed measurable emulsions at pH 5 and 7, but not at pH 9, while KI-P – only at pH 9. A moderate correlation between emulsifying and foaming capacity ($R^2 = 0.61$) was noted, suggesting that proteins with good foaming abilities also stabilize emulsions effectively.

According to Damodaran et al. (2007), the emulsifying and foaming properties of proteins generally improve after partial hydrolysis, due to solubility improvement, up to DH of approx. 10 %. In this study, the keratin hydrolysates did not show a clear relationship between solubility at the given pH and the emulsifying capabilities. Despite all keratin preparations obtained, except KI-S, being largely insoluble at pH 5, KI-T and KI-C were still capable of forming measurable emulsions at this pH. In contrast, the keratin isolate and KI-P could only form emulsions in the pH range where they were highly soluble. According to Lin et al. (2022), peptides with MW above 5 kDa exhibit better emulsifying properties than smaller peptides. Our results align with these observations, as the hydrolysates with the lowest MW, exhibited significantly worse emulsifying and foaming properties.

4. Conclusions

This study significantly improved the L-cysteine-assisted feather keratin reduction method, enhancing keratin yield, quality and solubility. Urea-containing solution of L-cysteine was critical in achieving the high structural integrity of the keratin isolate, significantly reducing extraction time under alkaline conditions compared to urea-free extraction. The optimized method produced the keratin isolate with substantial potential for food and pharmaceutical applications due to its high purity, enzymatic digestibility, unique amino acid composition and desirable functional attributes, particularly gelling, oil-binding and foaming properties. The amino acid profile of the keratin isolate indicated its suitability for bioactive peptide production, with a high content of hydrophobic amino acids and Cys for high antioxidant potential, and a high Pro content enhancing peptide bioavailability. Nonetheless, supplementation with His, Lys, Met and Trp was found to be recommended for a better balanced profile of EAAs.

Enzymatic hydrolysis increased the DH of the keratin isolate up to 36 %, leading to improved solubility but limited gelling capacity and worsened oil-holding capacity in a DH-dependent manner. The foaming and emulsifying properties of KI-T, KI-C and KI-P differed to those of the keratin isolate, and these differences were a function of DH and pH. KI-S had the highest DH, the highest content of low MW peptides with potentially high bioactivity and bioavailability, and excellent solubility, but its foaming, emulsifying, water and oil-binding properties were negligible. A comparison of experimental DH and DH_t values demonstrated good agreement for trypsin, chymotrypsin and subtilisin, confirming the reliability of in silico predictions for these proteases. Conversely, pepsin and papain exhibited significantly lower experimental DH, highlighting their limited efficiency under the tested conditions. These findings validate the utility of bioinformatic approaches for enzyme performance prediction while emphasizing the need to optimize specific enzymes like pepsin and papain.

The presented study emphasizes the importance of optimizing each step in the keratin extraction and hydrolysis processes to produce high-quality, bioactive protein preparations from feather waste. Ongoing investigations evaluate the performance of the obtained keratin preparations during thermal processing, simulated gastrointestinal digestion and intestinal absorption.

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CRediT authorship contribution statement

Antoni Taraszkiewicz: Writing – original draft, Visualization, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Izabela Sinkiewicz:** Writing – review & editing, Supervision, Resources, Methodology, Formal analysis. **Agata Sommer:** Writing – review & editing, Visualization, Software, Resources, Methodology, Investigation, Formal analysis, Data curation. **Barbara Kusznierewicz:** Methodology. **Linda Giblin:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Hanna Staroszczyk:** Writing – review & editing, Supervision, Project administration, Methodology.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that support the findings of this study are openly available in Bridge of Knowledge at https://doi.org/10.34808/7548-pd20.

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Appendix A. Supplementary data

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Table S1

Effect of reaction temperature and time on the % keratin yield extracted with urea-containing solution of 2% (w/v) L-cysteine at pH 10.5.

Time [h]	Temperature [°C]				
	30	40	50	60	70
1	91.9 ± 0.57^{Aa}	90.5 ± 0.30^{Aa}	91.1 ± 1.58^{Aa}	90.3 ± 0.50^{Aa}	90.2 ± 1.07^{Ab}
3	91.4 ± 0.12^{Aa}	90.0 ± 5.20^{Aa}	91.1 ± 0.24^{Aa}	91.2 ± 1.27^{Aa}	89.9 ± 0.77^{Aa}
6	91.2 ± 0.81^{Aa}	90.8 ± 0.25^{Aa}	91.0 ± 1.73^{Aa}	90.6 ± 0.96^{Aa}	90.3 ± 1.10^{Aa}

Different uppercase letters (A) within the same row indicate significant differences due to reaction temperature, while different lowercase letters (a-b) within the same column indicate significant differences due to the reaction time ($P < 0.05$).

Table S2

Effect of L-cysteine concentration and pH on the % keratin yield extracted with urea-containing solution of L-cysteine at 30°C for 1 h.

pH	L-Cysteine concentration [%]	
	1.5	2.0
9.0	85.4 ± 1.82^{Ab}	87.1 ± 0.74^{Ab}
10.5	90.8 ± 1.01^{Aa}	91.9 ± 0.57^{Aa}

Different uppercase letters (A) within the same row indicate significant differences due to L-cysteine concentration, while different lowercase letters (a-b) within the same column indicate significant differences due to pH of extraction solution ($P < 0.05$).

Table S3

Effect of L-cysteine concentration and pH on the % keratin yield extracted with urea-free solution of L-cysteine at 30°C for 24 h.

pH	L-Cysteine concentration [%]		
	1.0	1.5	2.0
10.0	13.8 ± 0.86^{Bc}	16.3 ± 1.10^{Bc}	25.4 ± 1.78^{Ac}

11.0	24.7 ± 1.00^{Cb}	43.0 ± 3.90^{Bb}	65.7 ± 2.66^{Ab}
12.0	30.4 ± 2.42^{Ca}	56.2 ± 0.33^{Ba}	87.8 ± 2.31^{Aa}

Different uppercase letters (A-C) within the same row indicate significant differences due to L-cysteine concentration, while different lowercase letters (a-c) within the same column indicate significant differences due to pH of extraction solution ($P < 0.05$).

Paper A3

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Description of the doctoral student's contribution: I curated the dataset, secured funding, assisted with sample preparation, FT-IR, TGA and DSC measurements, project administration, literature search and manuscript revision.

Changes in the structure and thermal properties of feather keratin induced by L-Cys extraction followed by enzymatic hydrolysis

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Keywords: Keratin isolate, Keratin enzymatic hydrolysate, Protein secondary structure, Crystallinity, Thermal properties

ABSTRACT

High-purity water-soluble keratein was extracted from chicken feathers with urea-containing L-Cys solution followed by enzymatic hydrolysis with commercial proteases. The L-Cys extraction resulted in a decrease of the α-helix share in favor of the β-sheet and both crystal structures tended to transform into less ordered ones as the ATR FT-IR spectra and the XRD diffractograms revealed. Changes in the keratin structure led to a decrease in the protein thermal stability, which decomposed more slowly but gradually, as TGA and DSC thermograms confirmed. The hydrolysis of extracted keratin with trypsin, chymotrypsin,

pepsin and subtilisin resulted in peptides with modified secondary structure, with subtilisin making the most profound changes. Although the secondary structure of the protein transformed into smaller peptide fragments by hydrolysis with subtilisin included only β-turns, this hydrolysate showed the highest thermal stability and the highest resistance to degradation above 300°C due to the formation of thermally stable sodium carboxylate.

1. Introduction

Keratin is a structural protein found abundantly in various industrial by-products, including feathers, hair, hooves, horns, wool, and nails (Vineis et al., 2019). These keratin-rich biomass wastes are now increasingly recognized for their potential to be transformed into high-value bioproducts. Successfully extracted and appropriately processed from biomass keratin can serve not only as a biomaterial for medical applications and sustainable food packaging but also as a source of bioactive peptides with nutraceutical potential (Ossai et al., 2022; Sinkiewicz et al., 2018). Chicken feathers are the most abundant keratin waste worldwide, generated in quantities exceeding several million tons annually (Jagadeesan et al., 2023). The low cost, high protein content, and widespread availability make them an attractive raw material for keratin extraction and utilization.

The chemical and supramolecular structure of feather keratin is highly complex. There are two principal structural forms of feather keratin: β-keratin and α-keratin. Predominant β-keratin with a molecular weight of approx. 10 kDa, concentrated mainly in the barbs and barbules, is rich in β-sheet regions that provide rigidity and tensile strength essential for avian flight. α-Keratin with a molecular weight 40-70 kDa, typical for the rachis and calamus, is rich in α-helix regions that confer feathers flexibility (Wang et al., 2016). Keratin is stabilized by an extensive network of intra- and intermolecular disulfide, hydrogen, and ionic bonds, as well as hydrophobic interactions (Shavandi et al., 2017). These cross-links create an insoluble

and chemically inert biopolymer, necessitating the application of severe chemical, physical and biological treatments to disrupt the tightly bound structure and solubilize the keratin for downstream processing (Moktip et al., 2025).

In recent years, the interest in using keratin as a promising source of bioactive peptides with antioxidant, antimicrobial, antihypertensive and antidiabetic properties, among others has steadily increased (Moktip et al., 2025). However, the approach to obtaining keratin-derived bioactive peptides is quite different from that of conventional food proteins. For the latter (e.g., proteins of milk, fish, plant), bioactive peptides are most commonly obtained via enzymatic hydrolysis of protein isolates/concentrates, due to the many advantages offered by this approach, i.e., high specificity of the reaction, mild processing conditions, preservation of nutritional value and the ability to tailor peptide profiles with precision (Kadam et al., 2024; Mora & Toldrá, 2023). This strategy has rarely been applied to keratin, mainly because extracting keratin isolates that retain native protein structures without extensive degradation is challenging (Sinkiewicz et al., 2018). As a result, most studies attempting to generate keratin-derived bioactive peptides from chicken feathers have relied on chemical or microbial hydrolysis, which, while effective in breaking down the protein, suffer from low specificity, risk of amino acid degradation, by-product formation, and limited process control (Lasekan et al., 2013; Lorenzo et al., 2018; Stiborova et al., 2016). Reports on the enzymatic hydrolysis where the substrate is a high-purity keratin isolate remain scarce.

It is well established that the biological activity of peptides is attributed first of all to their molecular weight, amino acid composition and sequence. However, some studies indicate that secondary structure also affects the peptide activity. According to Yang et al. (2017), Jiang et al. (2018) and Ma et al. (2020), a higher content of β-structures (β-sheet and β-turn) and a lower content of α-helix and random coil are associated with enhanced antioxidant properties. Likely, the α-helix into β-sheet transformation affects the exposure

and alignment of amino acid residues that can neutralize free radicals and increase molecular flexibility. On the contrary, the majority of potent antimicrobial peptides have an α-helical structure, as their amphipathic nature facilitates membrane insertion and disruption of microbial cells. At the same time, peptides rich in the β-sheet, although less flexible, provide enhanced stability and resistance to proteolytic degradation, making them advantageous under certain conditions requiring prolonged antimicrobial action (Li et al., 2021; Nayab et al., 2022; Zhang et al., 2021). These findings suggest that retaining or selectively modifying these structural forms could play a significant role in modulating the functionality of keratin-derived peptides.

As we have already reported, *in silico* hydrolysis of chicken feather keratin with trypsin, chymotrypsin, pepsin and subtilisin could release peptides with up to 15 distinct biological activities, in particular those with antihypertensive, antidiabetic and antioxidant potential (Taraszkiewicz et al., 2022). In the follow-up study, we optimized an efficient method to produce a high-purity keratin isolate, then we obtained its hydrolysates using the same proteases, and finally, we analysed their chemical composition and techno-functional properties (Taraszkiewicz et al., 2025). The present study aims to extend this work by providing deeper physicochemical characteristics of the keratin preparations produced. Although studies on the structure of keratin, including feather keratin, have been reported previously by many authors, to the best of our knowledge, this is the first attempt to describe the structure and thermal properties of enzymatic hydrolysates obtained from L-Cys extracted feather keratin, with trypsin, chymotrypsin, pepsin and subtilisin. The results obtained offer insights into the structure–function relationships of keratin-derived peptides, which are relevant for their development as functional food components or pharmaceutical ingredients.

2. Materials and methods

104

105 *2.1. Materials*

106 L-Cysteine (L-Cys), urea, trypsin from porcine pancreas (specific activity 1500 U/mg), α-
107 chymotrypsin from bovine pancreas (specific activity ≥ 40 U/mg protein), pepsin from
108 porcine gastric mucosa (specific activity ≥ 2500 U/mg protein), subtilisin A from *Bacillus*
109 *licheniformis* (specific activity ≥ 2.4 U/g, Alcalase® 2.4L), and HCl were purchased from
110 Sigma-Aldrich, USA. NaOH was obtained from POCH, Poland.

111

112 *2.2. Development of keratin preparations*

113 *2.2.1. Pretreatment of feathers*

114 Waste white chicken feathers, kindly supplied by a local company (Drobful, Kczewo,
115 Poland), were cleaned and shredded based on methods described previously (Sinkiewicz et
116 al., 2017; Taraszkiewicz et al., 2025). The feathers were washed in tap water at 50°C with
117 detergent, rinsed with distilled water, dried at 55°C overnight, cut into 20-30 mm long
118 filaments, and finally ground using an ultra-centrifugal mill (Retsch, Type ZM 200) with a
119 mesh diameter of 0.75 mm. The pretreated feathers were stored at 4°C.

120

121 *2.2.2. Keratin isolate formation*

122 The keratin extraction from pretreated feathers was performed by the reduction method
123 previously reported (Sinkiewicz et al., 2017; Taraszkiewicz et al., 2025; Xu & Yang, 2014).
124 Briefly, extraction with a solution of 1.5 (w/v) L-Cys as a reducing agent in 8 M urea medium
125 was carried out at pH 10.5, 30°C for 1 hr. These conditions were selected in preliminary
126 studies, based on the high reaction yield (90.8%), the good structural integrity of the keratin
127 obtained, and the cost-effectiveness of the process (Taraszkiewicz et al., 2025).

The pretreated feathers were mixed at a ratio of 1:20 (w/v) with the extraction solution and shaken at 200 rpm (Thermo Forma 420 Orbital Shaker, Thermo Scientific, USA). After the reaction was completed, the mixture was centrifuged (5000 rpm, 25°C, 10 min) and filtered to separate the solution of soluble keratin from the insoluble feather residue. The resulting filtrate was purified against distilled water using Spectra/Por dialysis membranes of regenerated cellulose (MWCO 3.5 kDa) at 4°C for 3 days, and then freeze-dried. The keratin isolate obtained (KI) was stored at 4°C for further study.

2.2.3. Keratin hydrolysates formation

Keratin hydrolysates were prepared following the previously described method (Taraszkiewicz et al. 2025). The KI was diluted with distilled water to reach 10 mg protein/mL determined by biuret assay. The hydrolysis reaction of KI with enzyme, i.e., trypsin (KI-T), chymotrypsin (KI-C), pepsin (KI-P) and subtilisin (KI-S), used in the 20:1 (w/w) KI to enzyme ratio, lasted 1 h. The pH and temperature of the reaction were adjusted to the maximum activity of the enzymes, i.e., in the case of KI-T and KI-C to pH 8 and 37 °C, KI-P to pH 2 and 37 °C, and KI-S to pH 9 and 60°C. The pH was kept constant during the hydrolysis using a pH-stat titrator (T70, Mettler Toledo, USA) with 0.1 M NaOH (KI-T and KI-C), 1 M HCl (KI-P), or 1 M NaOH (KI-S). Each hydrolysis reaction was carried out in triplicate. After hydrolysis, keratin hydrolysates were heated to 85°C for 15 min to inactivate the enzymes, the triplicates pooled, freeze-dried, and stored at 4°C for further analysis.

2.3. Methods

2.3.1. Attenuated Total Reflectance Fourier Transformation Infrared spectroscopy (ATR FT-IR)

The ATR FT-IR spectra of pretreated feathers and keratin preparations (KI and keratin hydrolysates) were recorded on an Invenio® spectrophotometer (Bruker, USA). For each spectrum 64 scans with a resolution of 4 cm^{-1} were collected. All samples were thoroughly homogenized in an agate mortar and conditioned before their analysis for 7 days in a desiccator over P_2O_5. For each sample aliquot, three to five replicate spectra were recorded to assess precision and ensure the reproducibility of each sample.

The second derivatives of the spectra were calculated using the Savitzky-Golay algorithm (27 data points, ca. 25 cm^{-1}, and a third-order polynomial with nine points) to resolve the overlapping bands of individual vibrations in the region 1800–1200 cm^{-1}.

2.3.2. X-Ray Diffractometry (XRD)

X-ray diffraction data were collected by applying CuKα radiation of a wavelength of 0.154 nm in a Philips X'Pert Pro diffractometer (Eindhoven, the Netherlands). The operation setting for the diffractometer was 30 mA and 40 kV. The spectra over the range of 5.0–60.0° 2θ were recorded at a scan rate of 0.02° 2θ/s. All samples were conditioned for 7 days in a desiccator over P_2O_5 and dried in compressed air before starting the tests. A crystallinity index (CrI) was calculated using the following equation (Ma et al., 2016):

$$CrI = (I_9 - I_{14})/I_9 \times 100\%$$

where I_9 is the maximum intensity of crystal lattice diffraction with 2θ at around 9° and I_{14} is the minimum diffraction intensity with 2θ at around 14°. In general, the higher CrI value indicates higher crystallinity of the samples.

2.3.3. Thermogravimetric Analysis (TGA)

TGA was performed with a TGA 2, STAR® System (Mettler Toledo, Switzerland). The 1.5-3.0 mg samples were heated in the open corundum crucibles at the rate of 10°C /min in the temperature range of 30-850°C. An inert gas atmosphere (N_2) was used during the test. All samples were conditioned before their analysis for 7 days in a desiccator over P_2O_5.

2.3.3. Differential Scanning Calorimetry (DSC)

The DSC thermograms of keratin preparations were collected on a calorimeter DSC 3, STAR® System (Mettler Toledo, Switzerland), with Simple FlexCal® calibration. Approx. 1.2-1.6 mg of each sample was heated at a rate of 20°C/min from 30 to 105°C (the first scan), held at 105°C for 5 minutes, and then cooled at a rate of 20°C/min to -60°C and reheated at a rate of 10°C/min to 300°C (the second scan). An empty pan was used as a reference. Prior to the analysis, the samples were conditioned in a desiccator over P_2O_5 for 7 days.

3. Results and discussion

3.1. Effect of L-Cys treatment on the feather keratin characteristics

3.1.1. Characterization of keratin by ATR FT-IR Spectroscopy

Table 1 lists the band assignment in the ATR FT-IR spectra of feathers and resulting KI (Alahyaribeik & Ullah, 2020; Cardamone, 2008; Kristoffersen et al., 2019, 2020; Ma et al., 2016; Martínez-Hernández et al., 2005; Pourjavaheri et al., 2015, 2019; Ramirez et al., 2021; Taraszkiewicz et al., 2025). The spectra revealed that the KI had a chemical structure as much as that of the keratin in feathers (Fig. 1). However, analysis of the difference band patterns disclosed groups involved in some conformational changes caused by the L-Cys extraction process.

The first effect seen in the spectra because of KI extraction was a change in the absorption bands in the region 3700–2700 cm^{-1} (Fig. 1A). An increase of absorption in the original spectrum and a positive peak in the difference spectrum at 3423 cm^{-1} as well as a decrease of absorption in the original spectrum and two negative peaks in the difference spectrum at 2920 and 2851 cm^{-1} were observed. The former peak in the difference spectrum was due to the asymmetric O–H stretching vibrations (ν_{OH}) and ν_{NH} in free and in amide form, while the latter ones owing to asymmetric and symmetric ν_{CH_2} side chain of protein, respectively. The broadening of the band with the maximum at 3280 cm^{-1} in the original spectrum of KI, with the appearance of the peak at a higher wave number of 3423 cm^{-1} in the difference spectrum, showing a decrease in the H-bond strength of ν_{OH} and ν_{NH}, indicates that some non-helical material in the KI occurred, as these groups in the non-helical phase are less strongly H-bonded than in the helix (Bendit, 1966). The drop in the intensity of the bands at 2920 and 2851 cm^{-1} in the original KI spectrum suggests a decrease in the molecular weight of the keratin obtained due to the breakdown of peptide bonds (Fortunati et al., 2015). However, the cleavage of these bonds was rather small, as indicated by the results of size exclusion chromatography, degree of hydrolysis (DH) determination, and free amino acid analysis, as we reported previously (Taraszkiewicz et al., 2025). The KI was composed primarily of peptides of above 10 kDa with a DH below 1% and the total free amino acids content (except L-Cys) of 1.44 mg/g.

The second effect occurring in the spectra because of the extraction process was a change in the absorption bands in the 1800–400 cm^{-1} region (Fig. 1B). A decrease of absorption in the original spectrum of KI was accompanied by three negative peaks at 1616, 1493, and 1207 cm^{-1} in the difference spectrum. All these peaks confirm the decrease in the intensity of the amide I, amide II, and amide III bands in the original spectrum of KI, and

their slight shift towards higher wave numbers suggests the formation of random coils in the KI at the expense of α-helix and β-sheet present in the feathers (Khosa et al., 2013).

Moreover, the band at 517 cm^{-1} in the original spectrum of feathers was shifted by 20 cm^{-1} toward higher wave numbers in the original spectrum of KI. Likely, the energy of the cross-linking S–S covalent bond decreases due to the formation of conformational changes in feather keratin to get it a water-soluble form, i.e., KI. However, as the band attributed to the ν_{S-S} usually appears in both L-cystine and L-cysteine spectra, a band in the region of 2500–2100 cm^{-1} was further analyzed. According to Su et al. (2022), that band can be used to distinguish between the S–H bending vibrations, which form in L-cysteine only, and the S–S stretching vibrations, which occur in L-cystine only. Two small bands at 2323 and 2291 cm^{-1}, assigned to the δ_{SH} developing in the L-cysteine in the original spectrum of KI, and two positive peaks in the difference spectrum could be recognized (Fig. 1C).

As the amide I is the most sensitive band of the protein backbone and provides the most information on the protein's secondary structure, the second-derivative procedure was employed to resolve a number of overlapping peaks into their components across the 1800–1200 cm^{-1} region. The spectra of feathers and KI showed three peaks in the amide I band (Fig. 1D). The first low-intensity component peak observed at 1696 cm^{-1} in both spectra was assigned to the β-sheet (anti-parallel β-strand), while the second one seen at 1659 cm^{-1} in the feathers-only spectrum was assigned to the α-helix, and that observed at 1682 cm^{-1} in the KI-only spectrum was assigned to the β-sheet (parallel β-strand) (Dong et al., 1990; Kong & Yu, 2007; Lin et al., 2022; Ramirez et al, 2021, 2022; Yang et al., 2015). Apparently, the share of α-helix in the KI structure decreases in favor of the β-sheet due to the L-Cys extraction process. The third, high-intensity component peak appearing at 1633 cm^{-1} in the spectrum of feathers was assigned to the β-sheet (parallel β-strand). A slight shift of this peak toward

247 higher wave numbers in the spectrum of KI (to 1637 cm^{-1}) indicated a less ordered structure
248 (Dong et al, 1990; Matheus et al., 2006; Ramirez et al, 2021, 2022).
249 In the region of amide II band, one well-developed peak at 1514 cm^{-1} with a well-
250 defined shoulder at 1539 cm^{-1} in both feathers and KI spectra was recognized (Fig. 1D).
251 According to Pelton and McLean (2000), proteins and peptides with an anti-parallel β-sheet
252 structure have strong amide II bands in the region of 1510–1530 cm^{-1}, and those with parallel
253 β-sheet structure in the region of 1530–1550 cm^{-1}. In the amide III band region, a low-
254 intensity peak at 1236 cm^{-1} in both feather and KI spectra was indicative of the β-sheet
255 structure (Khosa et al., 2013).
256 Thus, the ATR FT-IR spectra analysis showed that the L-Cys extraction resulted
257 primarily in the change in H-bond interactions as well as cleavage of disulfide bonds and, to
258 some extent, polypeptide linkages in the keratin. Hence, the α-helix and the β-sheet
259 crystalline structures in the feather keratin tended to transform into an unfolded and
260 disordered structure in the KI. To analyze how this change in the ordered secondary structure
261 of keratin after L-Cys extraction affected its crystallinity, XRD diffraction patterns were
262 studied.
263
264 *3.1.2. Characterization of keratin by XRD*
265 Bird feathers are known to have a semi-crystalline structure (Vineis et al., 2019). As shown in
266 Fig. 1E, feathers displayed a typical diffraction pattern of β-keratin. Two prominent 2θ
267 peaks, the first at 9.4° corresponding to the α-helix and β-sheet and the second at 19.1°
268 related to β-sheet, as well as with a peak valley at 14° assigned to the amorphous region were
269 recognized (Irdis et al., 2013; Long et al., 2013; Nuutinen et a., 2019; Qin et al, 2023; Rao &
270 Gupta, 1992). The first peak was less intense in the KI diffraction pattern than that in the

feathers, while the second one was broadened and more intense. The shift of the latter from 19.1° to 20.1° in the patterns of KI was also observed.

The decreased intensity of the peak at 9.4° in the KI diffraction pattern compared to that in the feather's diffraction pattern indicated that the feather treatment with L-Cys deconstructed the α-helix and β-sheet, and the increase in intensity at 19.1° suggested the rise in the β-sheet content (Ma et al. 2016; Khosa et al., 2013). However, as reported by many authors, the broadening of the diffraction band at 19° could prevent unambiguous differentiation between the peak at 19.1° and the peak at 17.8° indexed for the β-sheet the α-helix structure, respectively, due to their overlap (Ma et al., 2016; Nuutinen et a., 2019; Qin et al, 2023; Rao & Gupta, 1992). On the other hand, according to Xu and Yang (2014), unlike the narrower 2θ peak, the broader one implies smaller crystals in the proteins. Hence, the KI with the broader 2θ peak at 19.1° could have relatively smaller crystals than the feather keratin with a narrower peak in its diffraction pattern. Moreover, the shift of the peak at 19.1° to a higher value of 2θ demonstrates the decrease of interplanar spacing in the KI, i.e., tightening of the packed crystals (Qin et al, 2023).

Thus, the extraction of keratin from feathers using L-Cys led primarily to the breaking out of available H-bonds and hence to the movement and interaction of the keratin polypeptide chains. As a result, these chains were rearranged, converting the α-helix and the β-sheet crystal structures into less ordered ones. However, the crystallinity of keratin in the KI was retained, as evidenced by the intensity of diffraction peaks. To assess the extent to which the keratin crystallinity was changed, the CrI was calculated, with the higher CrI value indicating a higher crystallinity. These results showed that the crystallinity decreased by about 44% after the extraction of keratin from feathers (Fig. 1E). So, despite the reorganization of the keratin structure by the feather treatment with L-Cys, the crystallinity of keratin was retained, although to a lesser extent.

296

297 *3.1.3. Characterization of keratin by TGA*

298 TGA thermograms showed the two-step decomposition of feathers (Fig. 2A, B). The first one
299 occurring below 150°C was associated with the loss of freely and highly bonded water, while
300 the second one between 150 and 460°C was related to the decomposition of the keratin
301 structure. The L-Cys extraction decreased the keratin decomposition temperature as the shift
302 of the main minimum on the DTG line towards lower temperatures indicated (Fig. 2B).
303 Although the decomposition pattern of the TGA thermogram remained essentially the same, a
304 higher rate of the KI decomposition was also noted between 460-850°C as showed by a more
305 rapid fall of the TG line in this temperature range (Fig. 2A). Moreover, a small minimum on
306 the DTG line at 48°C in the TGA thermogram of feather did not occur in the thermogram of
307 KI (Fig. 2B). An insight in Table 2 disclosed that after the L-Cys extraction, the thermal
308 stability of the keratin was essentially preserved, although the decomposition temperature
309 decreased by 15°C, and the total weight loss increased by about 12%. In fact, the KI was
310 slightly more stable than feathers up to 460°C, but less stable above that temperature, as the
311 weight loss of the KI decreased by about 10% in the temperature range of 30-460°C but
312 increased by about 20% in the that of 460-850°C. The weight loss in the first temperature
313 range was related to the dehydration and denaturation process, i.e., the transformation of the
314 protein from an ordered (native) to a less ordered state because of H-bond rearrangements
315 (Tesfaye et al., 2018). This was promoted by the weakening of disulfide bonds, and even their
316 destruction, with some elimination of sulfur dioxide and hydrogen sulfide. Water that forms a
317 3D network of interactions in the keratin structure and together with cystine disulfide bonds
318 contributes to the conformational stability of the protein, also plays a role in this process, as
319 evidenced by the disappearance of the minimum at temperatures below 100°C on the DTG
320 line of the KI thermogram. The relaxation process of the keratin's secondary structure was

followed by thermal pyrolysis of peptide linkages resulting in depolymerization and degradation of the protein backbone due to the breaking of covalent peptide bonds (Martínez-Hernández et al., 2005; Qin et al., 2023). According to Brebu and Spiridon (2011), in the temperature range of 170–300°C, inorganic gases such as NH_3, CO_2, SCS, SCO, H_2S, SO_2, and thiols are released. The higher weight loss in the 460-850°C temperature range in the case of the KI indicated that, due to the weakening of KI's secondary structure, its further decomposition was already easier. As demonstrated by Brebu and Spiridon (2011), the most important degradation compounds released above 300°C include nitriles, pyrroles, pyridines, amides, sulfides, thiols, thiazoles and thiophenes. These compounds are formed from the initial amino acids that make up keratin. Thus, the ordered secondary structure of keratin in feathers makes it thermally stable up to 327°C but decomposes almost entirely up to 460°C. Disruption of this structure due to the L-Cys extraction makes it decompose more slowly but gradually, with the lower weight loss up to 460°C, but higher once this temperature is exceeded and with higher total weight loss up to 850°C.

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3.1.4. Characterization of keratin by DSC

The transition of keratin crystallites into less ordered structures after the L-Cys extraction was also confirmed by DSC thermograms (Fig. 2C, D). There was a broad peak between 30-105°C noted in the DSC thermograms of both feathers and KI during the first scan (Fig. 3C). This peak is referred to as the ‘‘denaturation’’ temperature and shows the removal of water which is bonded to keratin via H-bonded bridges stabilizing the protein’s secondary structure (Tesfaye et al., 2018). The shift of the maximum of this peak towards lower temperatures in the DSC thermogram of KI indicated a rearrangement of H-bonds in the keratin structure after L-Cys extraction that facilitated the elimination of bound water. During the second scan, the first sharp endothermic peak recorded at about 238°C in the DSC thermogram of feathers,

generally related to the α-helix decomposition and disordering (Tesfaye et al., 2018), was milder, broadened, and shifted by 13°C towards lower temperature in the DSC thermogram of KI (Fig. 2D). These changes confirmed some loss of α-helix crystal structure in keratin due to the extraction process (Tesfaye et al., 2018). The second, endothermic peak between 245 and 275°C in the DSC thermogram of both feathers and KI indicated a further keratin degradation and could be assigned either to melting (Haly & Snaith, 1970; Menefee & Yee, 1965; Wortmann et al., 2006) or denaturation (Haly & Snaith, 1970; Wortmann et al., 2006) of crystalline phases of keratin. The third, endothermic peak around 280–320°C in the DSC thermogram of feathers and KI, according to Monteiro et al. (2005), could be attributed to thermal degradation of disulfide bonds and denaturation of helical structures. Haly and Snaith (1970), and Schmidt and Jayasundera (2004) believe that this peak is related to the total decomposition of chicken feather keratin. The temperature range at which that peak occurred is consistent with the highest weight loss recorded during TGA (Table 2).

3.2. Effect of enzyme treatment on the hydrolysate characteristics

3.2.1. Characterization of hydrolysates by ATR FT-IR Spectroscopy

When comparing the ATR FT-IR spectrum of KI with the spectra of hydrolysates (Fig. 3) it could be seen that the features of the amide A, amide I, amide II, and amide III were preserved, with pronounced changes observed in the KI-S spectrum (Fig. 3J-L).

In fact, the original KI-T spectrum resembled that of KI (Fig. 3A-C). When comparing the original spectrum of KI with the original spectra of other hydrolysates it could be seen that the amide A band was shifted towards lower wave numbers (Fig. 3D, G, J). Also, the right shoulder of that band was developed, with a negative and positive peak in the difference spectrum at about 3285 and 3141 cm^{-1}, respectively. The former was due to ν_{NH} and ν_{OH}, and the latter was owing to the symmetric ν_{OH}. Typically, a shift of the amide A band towards the

372 lower wave numbers is described as indicative of the scission of H-bonds necessary to
373 maintain the protein helical structure (Staroszczyk et al., 2012). According to Silva et al.
374 (2008) and Yakimets et al. (2007), these bonds are mediated by water molecules. Apparently,
375 the KI-S had the highest number of H-bonds, as shown by changes in its spectrum in the
376 amide A band (Fig. 3J).

377 Changes in the 3700–2700 cm^{-1} region were accompanied by some changes in that of
378 1800–400 cm^{-1} (Fig. 3 E, H, K). There was a slight shift of the amide I band towards higher
379 wave numbers in the original spectra of KI-C (Fig. 3E) and KI-P (Fig. 3H), as confirmed by a
380 positive peak at 1728 cm^{-1} and a negative peak at 1690 cm^{-1} in their difference spectra. Such a
381 shift is indicative of hydration of side chains-backbone (Mukherjee et al., 2007).

382 Apart from the KI-S spectrum, no other significant changes in the amide I, amide II
383 and amide III bands were observed in the spectra of the other hydrolysates. In the original KI-
384 S spectrum, the amide I band decreased in intensity, and it was split into two components, at
385 1633 and 1596 cm^{-1}. Moreover, the increase in intensity of the band at about 1400 cm^{-1} was
386 significant (Fig. 3K). These changes were accompanied by two positive peaks in the
387 difference spectrum at 1587 and 1394 cm^{-1}. The appearance of the band at 1600-1590 cm^{-1}
388 and at about 1400 cm^{-1}, related to carboxylate ions (Silverstein et al., 2007), was indicative of
389 hydrolysis of peptide bonds in KI-S and the formation of sodium carboxylate (Doofan et al.,
390 2021; Max and Chapados, 2002). Although the complete spectral characterization of metal
391 carboxylates, with exception of acetates, is neglected, resulting in a deficiency within the
392 literature on these compounds, it is known that the release of a proton from a carboxylic acid
393 leads to the formation of a carboxylate anion, which is stabilized by delocalization of the
394 negative charge between two oxygen atoms (resonance). As shown by Max and Chapados
395 (2002) and Doofan et al (2021), upon binding with a metal center (e.g., sodium), there is a
396 shift in the band characteristic of the ν_{COO-} (1700-1600 cm^{-1}) toward lower wave numbers and

an increase in the band intensity at about 1400 cm^{-1}. Therefore, the appearance of additional bands at 1596 and 1400 cm^{-1} only in the original spectrum of KI-S, confirmed by positive peaks in the difference spectrum at 1587 and 1394 cm^{-1}, indicates precisely the formation of sodium carboxylate, which was obtained under the most alkaline hydrolysis conditions (pH 9). In contrast, the lack of clear changes in the band at 1600-1590 cm^{-1} and the disappearance of the band at about 1400 cm^{-1} in the original KI-P spectrum (Fig. 3H) was due to pH below pI (pH 2) during hydrolysis, resulting in the protonation of the carboxyl group in this hydrolysate, and the lack of clear changes in both bands in the original spectra of the KI-T and KI-C (Fig. 3B, E) obtained using smaller amounts of NaOH than KI-S, indicated only the deprotonation of the carboxyl group.

Further, according to Alahyaribeik and Ullah (2020), in the keratin extraction process, the reaction of sulfites and cysteine forms the $\nu_{S=O}$ (cysteine-S-sulfonated residues). As could be seen, there was an increase in absorbance in the 1100-1000 cm^{-1} range in the original spectra of all hydrolysates compared to that of KI, with a positive peak at about 1040 cm^{-1} in the difference spectra (Fig. 3B, E, H, K), suggesting the presence of cysteine-S-sulfonated residues. These results followed our former findings: both KI and its hydrolysates contained cysteic acid, an oxidized cysteine derivative bearing S=O bond, with its content amounting to 0.97, 4.98 and 8.76 in KI, KI-T and KI-C, respectively, and 11.69 and 11.23 in KI-P and KI-S (Taraszkiewicz et al. 2025). Since, unlike the KI-T and KI-C, two negative peaks at 2323 and 2291 cm^{-1} were observed in the differential spectra of KI-P and KI-S (Figure 3C, F, I, L), this confirms the reduction of free thiol groups due to their oxidation to S=O in those hydrolysates, in which cysteic acid content was the highest.

Unlike the second-derivative spectrum of KI-S, the second-derivative spectra of other hydrolysates did not differ significantly from that of KI (Fig. 3M). As the second-derivative spectrum of KI, those of KI-T, KI-C, and KI-P showed three peaks in the amide I band: two

low-intensity peaks at 1696, 1682 cm^{-1} and one high-intensity peak at about 1633 cm^{-1}, one peak in the amide II band at 1514 cm^{-1} with the shoulder at 1539 cm^{-1}, and one low-intensity peak at the amide III band, at 1241 cm^{-1}. These features indicated the β-sheet (antiparallel and parallel β-strand), showing that the enzymatic hydrolysis with trypsin, chymotrypsin, and pepsin did not cause changes in the KI's secondary structure. Although the second-derivative spectrum of KI-S included all these peaks, splitting the high-intensity peak in the amide I band into two medium-intensity peaks at 1642 and 1600 cm^{-1} was evident. According to Lin et al. (2022), the former points out the unordered structure, and the latter β-turns. Thus, when the KI converts into short peptide fragments during hydrolysis with subtilisin, it loses its secondary structure completely. Nevertheless, a clear splitting of the single peak in the amide II band into two components at 1522 and 1514 cm^{-1}, and an increased intensity of a peak at 1400 cm^{-1} in the order of KI, KI-T, KI-C, KI-S in the second-derivative spectra of these hydrolysates were also observed (Fig. 3M). As reported by Böcker et al. (2017), Wubshed et al. (2017), and Måge et al. (2021), the band at 1520–1512 cm^{-1} is assigned to the NH_3^+ scissoring vibrational mode. According to Güler et al. (2016), who studied the process of enzymatic hydrolysis of β-lactoglobulin and β-casein, the bands at 1594 and 1406 cm^{-1} are related to antisymmetric and symmetric ν_{COO}-, respectively. Böcker et al. (2017) showed similar bands in the second-derivative spectra of enzymatic digestion products of chicken-based substrates, and Måge et al. (2021) enzymatic digestion products of poultry by-products. Thus, the splitting of the amide II band and the appearance of the second peak at about 1522 cm^{-1}, and the increasing intensity of the band at about 1400 cm^{-1} indicated the hydrolysis of the peptide bond and the formation of the C-terminal carboxylate group (COO^-) and the N-terminal amino group (NH_3^+). As the pH of KI-P was below the pI, causing protonation of the –COOH group, the second-derivative spectrum of KI-P did not show the peak at about 1400 cm^{-1}.

Thus, the least pronounced changes in the spectra of hydrolysates relative to that of KI were observed in the KI-T, more pronounced in the KI-C and KI-P, and the most pronounced in the KI-S. These findings are reflected in the DH and average molecular weight changes described in our previous work (Taraszkiewicz et al. 2025). As we reported, the DH determined by the OPA method (DH_{OPA}) was 0.8 % for KI, 5.9% for KI-T, 6.9% for KI-C, 7.6% for KI-P, and 20.5% for KI-S, indicating increasing protein fragmentation. A similar trend was observed for the DH determined by the pH-stat method ($DH_{pH\text{-}stat}$): 8.4% for KI-T, 12.8% for KI-C, and 36.2% for KI-S. The average molecular weight determined by the size exclusion chromatography decreased at once from 9.04 kDa for KI, through 5.96 kDa for KI-T, 5.79 kDa for KI-C, 5.23 kDa for KI-P, to 2.10 kDa for KI-S, confirming the progressive fragmentation of keratin after enzymatic hydrolysis.

The observed changes in the keratin structure are consistent with the specificity characteristics of the enzymes used. Trypsin is a highly specific protease, chymotrypsin is slightly less specific, and pepsin and subtilisin exhibit low specificity (Minkiewicz et al., 2019). Precisely, trypsin cuts only peptides at the polar Lys and Arg residues, which results in a limited number of cutting sites and relatively low DH of the protein. Chymotrypsin cut on the carboxyl side of peptide bonds with aromatic and large hydrophobic residues such as Tyr, Top, and Phe, which leads to moderate DH, while pepsin and subtilisin exhibit the ability to cut a wide variety of peptide bonds including those formed by aromatic (Phe, Trp, Tyr), aliphatic (Leu, Ala), acidic (Glu), sulfur (Met), hydroxyl (Ser) and basic (Lys) amino acid residues, which results in intensive hydrolysis, the substantial reduction in the molecular weight and pronounced changes in the protein's secondary structure. This was particularly evident in the case of KI-S.

3.2.2. Characterization of hydrolysates by XRD

The hydrolysis of KI with enzymes did not cause any significant changes in the diffraction patterns, although an increase and a broadening of the second peak (at 20.1°) in the diffraction patterns of all hydrolysates except KI-S was even more evident (Fig. 3N). A decrease in the intensity of both peaks (at 9.1 and 20.1°) in the diffraction pattern of KI-S compared to that of KI was also observed.

Thus, in addition to the changes caused by the L-Cys extraction of feather keratin, the hydrolysis of KI with enzymes induced further changes in the deconstruction of the α-helix and β-sheet structures as indicated by the increased intensity of the broadened 2θ peak at 20.1° in the diffraction patterns of KI-T, KI-C, and KI-P hydrolysates. These hydrolysates had a less ordered crystal structure and smaller crystals than KI. The decreased intensity of both peaks (at 9.1 and 20.1) in the KI-S diffraction pattern confirmed that treatment of KI with subtilisin deepens the destruction effect of the protein's secondary structure. Nevertheless, the crystallinity of KI after enzymatic hydrolysis was also preserved, although the CrI of the hydrolysates was slightly lower than that of KI in most cases (Fig. 3N). The CrI of KI and its hydrolysates exhibited a moderate negative correlation with DH (R^2>0.71) and a strong positive correlation with average molecular weight (R^2=0.90), which we reported previously (Taraszkiewicz et al., 2025). These findings indicate that peptide fragmentation reduced the ability of keratin molecules to align, and form ordered structures, while longer peptide chains contribute to maintaining intermolecular organization and preserving crystallinity.

3.2.3. Characterization of hydrolysates by TGA

The TGA thermograms of the enzymatic hydrolysates reflected stepwise weight loss associated with moisture release - up to 150°C, protein decomposition - up to 460°C, and thermal degradation of the residue - up to 850°C (Fig. 5A, B). In detail, the onset of thermal decomposition of hydrolysates began earlier than that of KI, with intense weight loss at a

lower temperature than KI for KI-T and KI-C, and at approximately the same temperature for KI-P. The splitting of the DTG minimum in the KI-S thermogram indicated that there were additional thermal effects in this case. Likely, this resulted from a degradation of the KI by the proteolytic activity of subtilisin into short peptide linkages. In addition, the composition of the KI after subtilisin treatment could be in the balance between depolymerization of peptide chains and the further cross-linking, as indicated by the higher decomposition temperatures than KI (Table 2). However, the formation of sodium carboxylate in KI-S, as suggested by analysis of ATR FT-IR spectra, seems more likely to be the reason for the increased thermal stability of this hydrolysate compared to KI-T and KI-C which contained the deprotonated counterparts. Despite the earlier initiated decomposition process, the KI-T showed the total weight loss like that noted for the KI, and the total weight loss of other hydrolysates was only a few percent lower than that of KI. In fact, the weight loss of all hydrolysates in the 30-460°C temperature range was almost the same as that of KI, and lower than that of KI in the 460-850°C temperature range. These results indicated that the enzymatic hydrolysates were less thermally stable than KI, but their thermal decomposition was more complex. Likely, the disruption of their secondary structure facilitated the decomposition process resulting in products with shorter particle sizes that started to decompose earlier (i.e., at lower temperatures). Comparing the decomposition data obtained with the proximate composition results reported in our previous study (Taraszkiewicz et al., 2025), some differences become apparent. These differences can be attributed to different methodologies: the TGA reflects the thermal decomposition profile in an inert nitrogen atmosphere, including water and non-volatile residues, while the proximate analysis was performed in air. In the latter, the particularly high ash content observed in the KI-S supported the presence of residual NaOH, which remains stable under TGA conditions and contributes to the lower total mass loss.

3.2.4. Characterization of hydrolysates by DSC

It is well known that thermal transitions in proteins are strongly affected by water and depend on the amount of water. Unlike the DSC thermogram of KI, KI-C and KI-P, the DSC thermograms of KI-T and KI-S showed no endothermic water loss on the first scan (Fig. 4C). However, unlike KI-T, the KI-S also did not show any crystalline melting during the second scan (Fig. 4D). It could be an additional argument in favor of complete secondary structure loss in the latter hydrolysate. Although the hydrolysis with subtilisin led to the shortest peptides, with an average molecular weight of 2.10 kDa, among all the hydrolysates obtained (Taraszkiewicz et al., 2025), the decomposition temperature of KI-S was higher than that of KI and the other hydrolysates (Table 2). Thus, the KI-S structure blocked the melting process and contributed to the formation of a more structurally stable hydrolysate. The KI-T and KI-C showed endothermic peaks on the DSC thermograms during the second scan, one at around 220°C and the other at 300°C (Fig. 4D). The shift of the former, much milder than that in the DSC thermogram of KI, towards a lower temperature range indicated the loss of α-helix crystal structure, while the latter pointed out the hydrolysate decomposition. These both hydrolysates did not show the endothermic peak between 245– 275°C that was characteristic of feathers and KI. Thus, either the melting/denaturation of crystalline phases in KI-T and KI-C coincided with their decomposition process or did not occur at all. In turn, the DSC thermogram of KI-P did not show the endothermic peak at about 220°C relating to the α-helix decomposition, while that indicating the melting/denaturation process was evident.

4. Conclusions

After the L-Cys extraction of keratin from feathers, the available hydrogen and disulfide bonds are broken down, leading to the rearrangement of the protein chains. Hence, the share of the α-helix decreases in favor of the β-sheet, and both crystal structures tend to transform

into less ordered ones resulting in decreased keratin crystallinity, as the ATR FT-IR spectra and the XRD diffractograms, respectively, revealed. The transition of crystallites into disordered structures leads to a decrease in the thermal stability of keratin, which decomposes more slowly but gradually, as the TGA and DSC thermograms confirmed. The changes taking place in the feather keratin structure because of L-Cys extraction occurred to convert protein into a soluble form, i.e., KI.

Hydrolysis of the resulting KI with trypsin, chymotrypsin, pepsin, and subtilisin further modifies the protein structure, with subtilisin causing the most profound changes. Due to the cleavage of peptide bonds, the C-terminal carboxylate group (COO^-) and the N-terminal amino group (NH_3^+) are formed. The KI-T and KI-C show a similar profile of physicochemical properties, i.e., slight changes in the secondary structure and good thermal stability, while KI-S, although it loses the secondary structure entirely, also shows the highest thermal stability. All hydrolysates obtained have smaller crystals and lower crystallinity, and their thermal decomposition starts earlier, although the KI-S shows the highest resistance to decomposition above 300°C due to the formation of thermally stable sodium carboxylate.

In order to check how the modified structure of enzymatic hydrolysates affects their biological activity, the next step is to test the antioxidant activity, cytotoxicity and mutagenicity of the hydrolysates obtained. These studies have already been undertaken.

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CRediT authorship contribution statement

Hanna Staroszczyk: Conceptualization, Visualization, Methodology, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition, Project administration. **Agata Sommer:** Investigation, Formal analysis, Software, Data curation. **Antoni Taraszkiewicz:** Investigation, Formal analysis, Writing – review and editing, Funding acquisition, Project administration. **Marek Michalec:** Investigation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Table 1

ATR FT-IR spectral characteristic of feathers, keratin isolate (KI), and keratin hydrolysates obtained by reaction of KI with trypsin, chymotrypsin, pepsin, and subtilisin (KI-T, KI-C, KI-P, KI-S, respectively).

Region	Position in cm^{-1}						Band assignment
	Feathers	KI	KI-T	KI-C	KI-P	KI-S	
Amide A	3280	3280	3280	3278	3278	3278	ν_{NH}, ν_{OH}
Amide B	3064	3064	3064	3064	3064	3064	ν_{NH}
	2960	2960	2960	2960	2960	2960	asym ν_{CH3}
	2922	2933	2933	2933	2933	2929	asym ν_{CH2}
	2874	2876	2876	2878	2878	2874	sym ν_{CH3}
	2851	–	–	–	–	–	sym ν_{CH2}
	2660	2653	2653	2653	2653	2660	ν_{SH}
Amide I	1629	1633	1633	1636	1638	1633	$\nu_{C=O}$, ν_{NH}
	–	–	–	–	–	1596	sym ν_{COO-}
Amide II	1513	1517	1525	1525	1517	1517	ν_{C-N}, δ_{NH}, ν_{C-C}
	1448	1448	1448	1448	1448	1448	δ_{CH2}, δ_{CH3}
	1386	1389	1392	1393	–	1392	δ_{CH2}, ν_{COO-}
Amide III	1230	1236	1236	1236	1236	1239	ν_{C-N}, δ_{NH}
	1059	1059	1052	1046	1056	1046	skeletal ν_{C-O}, ν_{C-C}
	517	537	537	537	537	537	ν_{SS}

819 Table 2

820 Thermal characteristics of feathers, keratin isolate (KI), and keratin hydrolysates obtained by
821 reaction of KI with trypsin, chymotrypsin, pepsin, and subtilisin (KI-T, KI-C, KI-P, KI-S,
822 respectively).

Sample	Temperature range (°C)	Weight loss (%)	DTG peak temperature (°C)[a]
Feathers	30 – 150	7.40	48
	150 – 460	69.18	327
	460 – 850	8.78	
	Total	85.36	
KI	30 – 150	4.35	
	150 – 460	62.65	312
	460 – 850	30.65	
	Total	97.65	
KI-T	30 – 150	8.65	63
	150 – 460	60.00	298, 330sh
	460 – 850	27.97	
	Total	96.62	
KI-C	30 – 150	4.58	
	150 – 460	67.80	289sh, 301
	460 – 850	19.28	
	Total	87.08	
KI-P	30 – 150	2.86	
	150 – 460	69.30	314
	460 – 850	8.80	
	Total	80.96	
KI-S	30 – 150	2.39	
	150 – 460	66.69	299, 330, 362
	460 – 850	17.31	
	Total	86.39	

823 [a] sh denotes a shoulder.

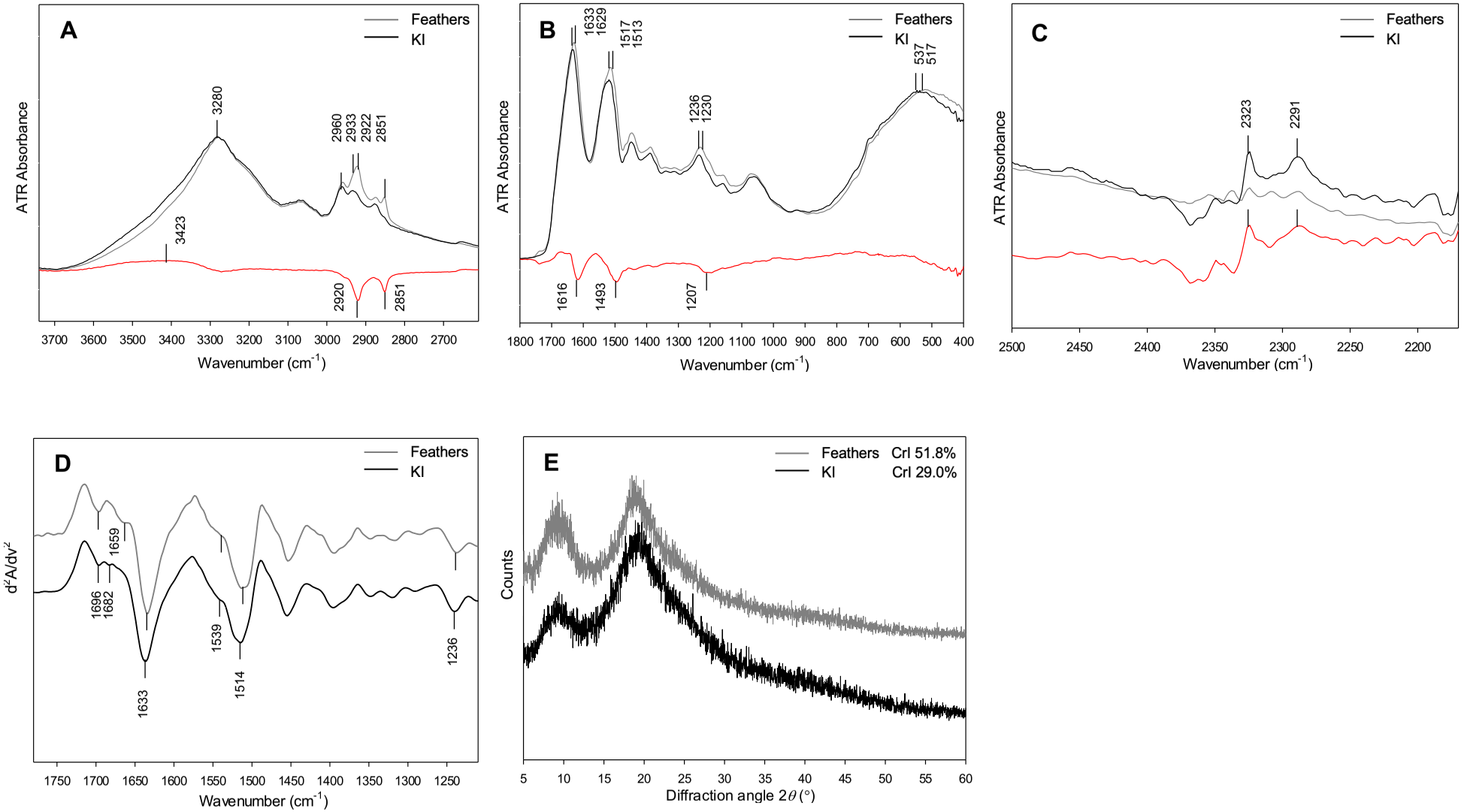


Fig. 1

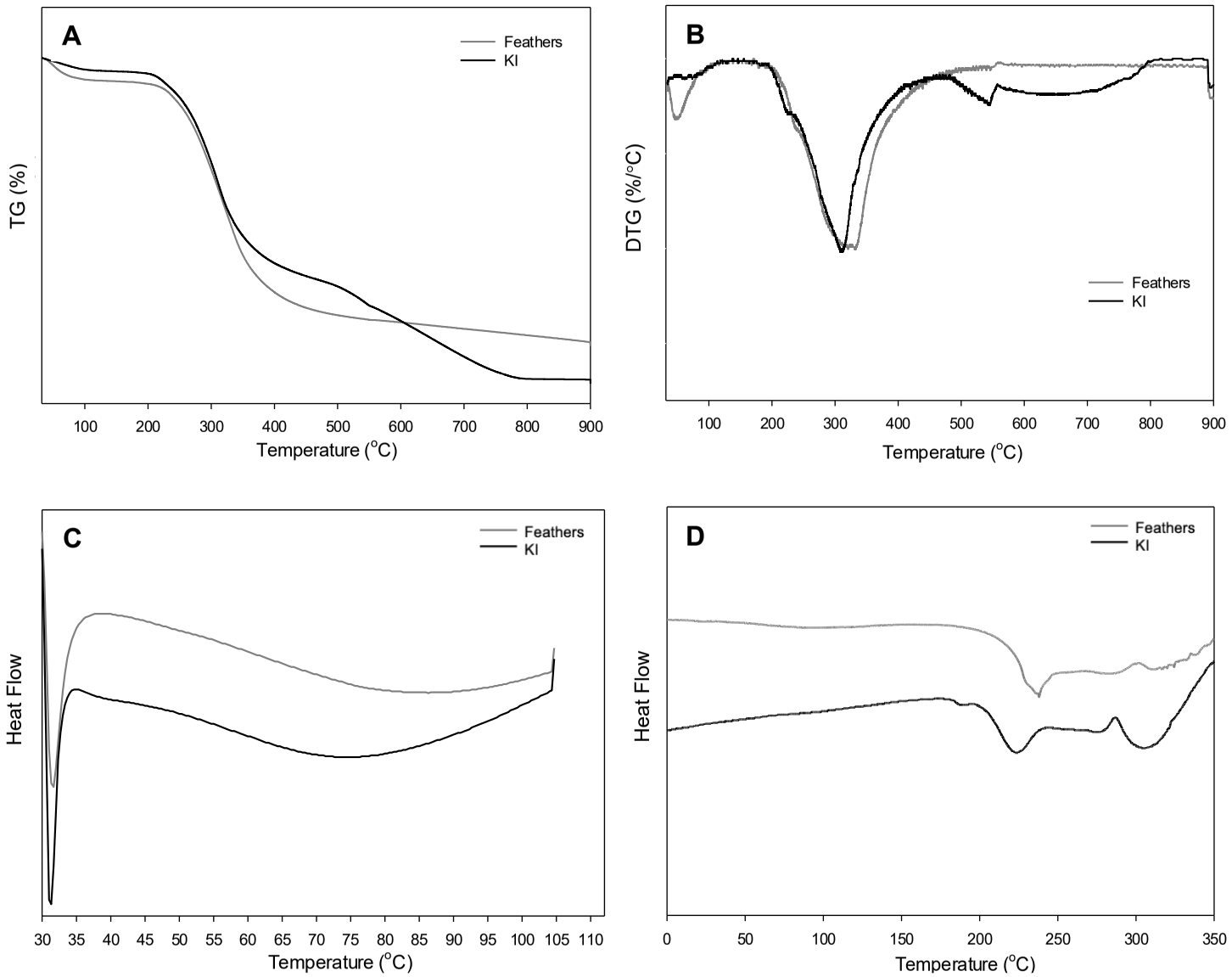
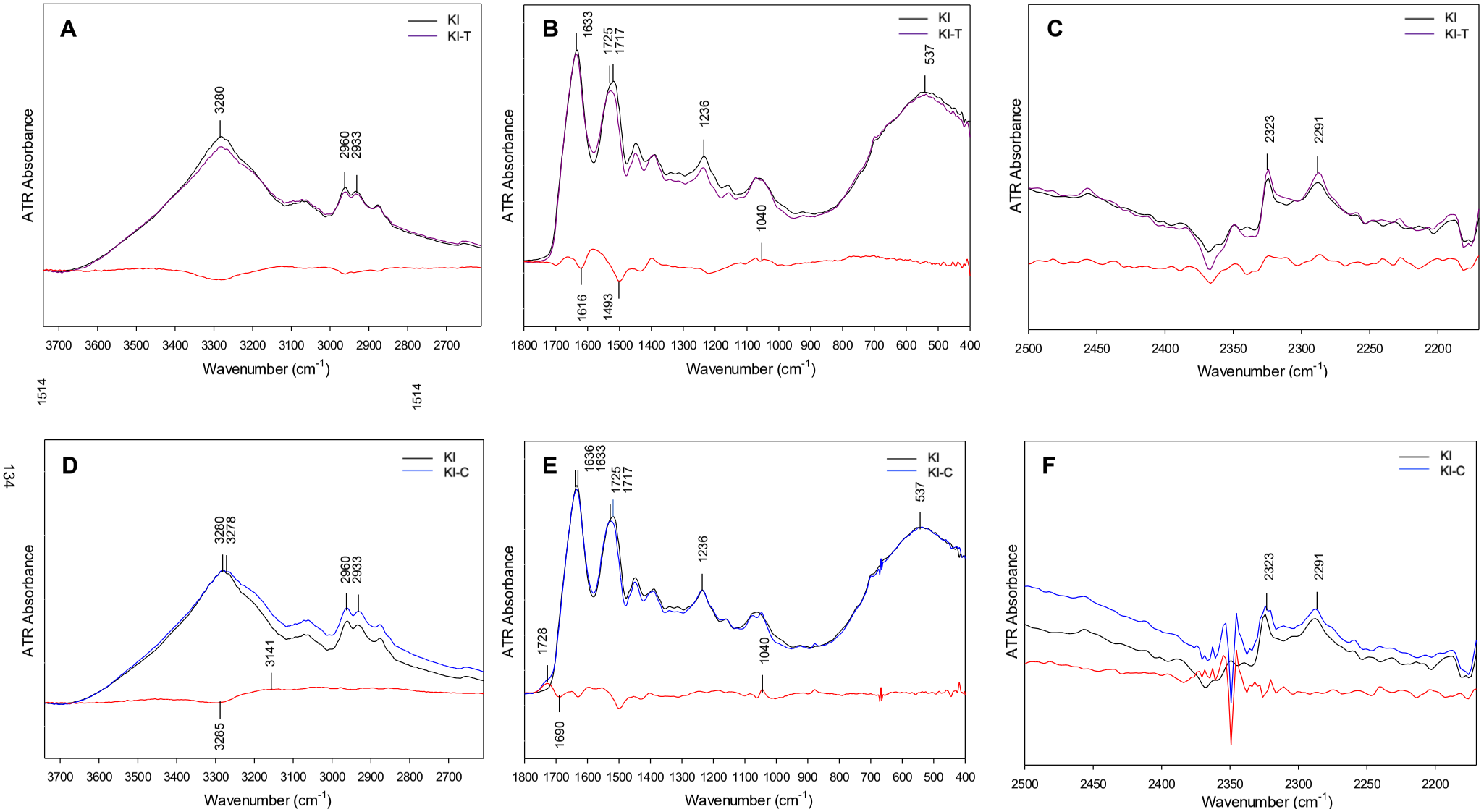
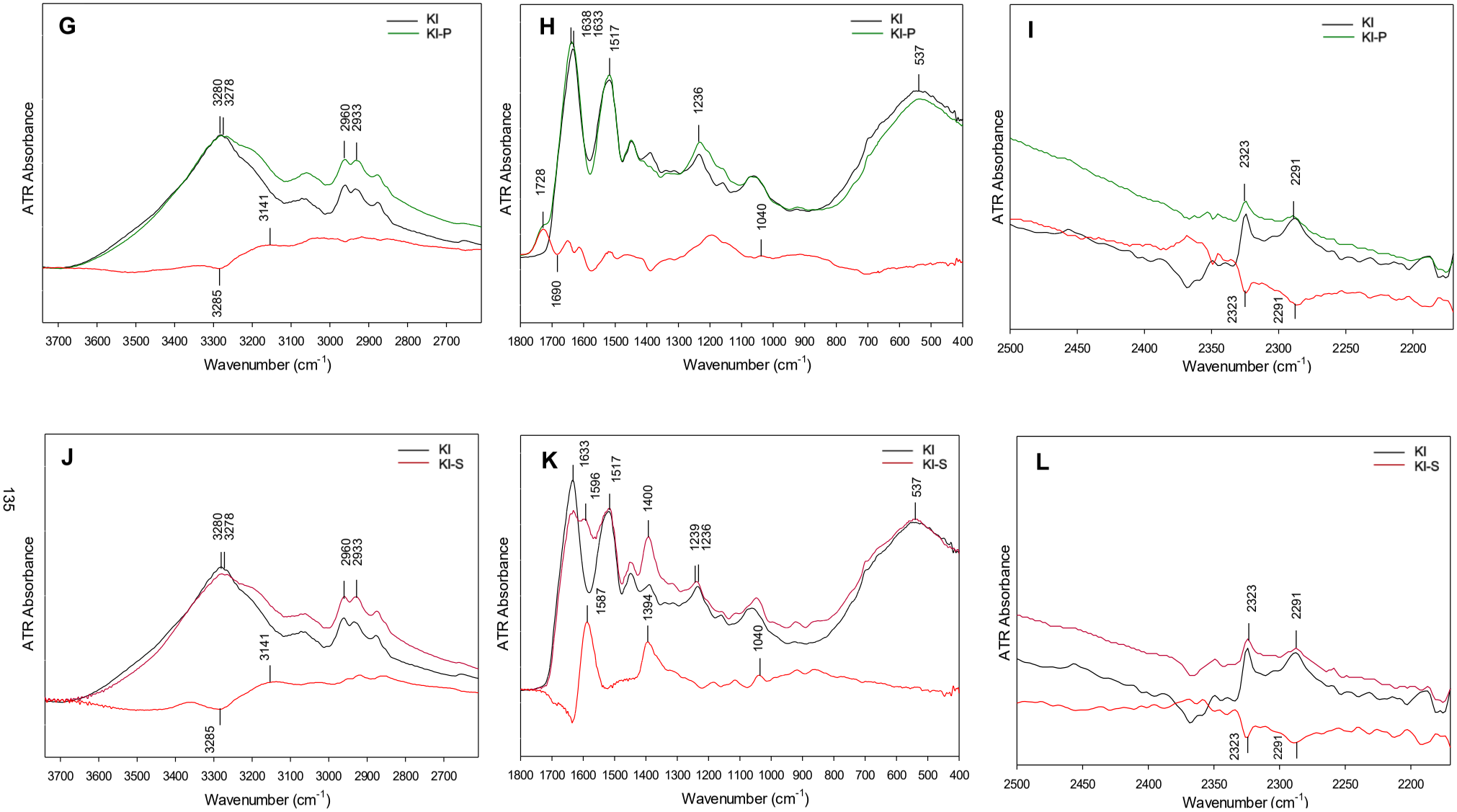


Fig. 2





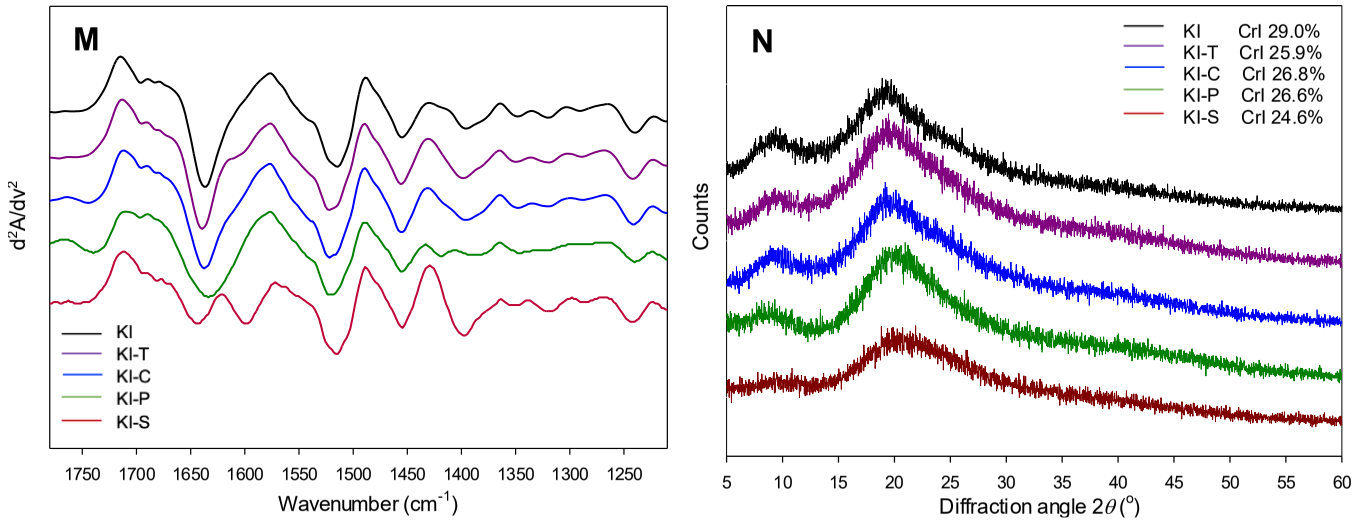


Fig. 3

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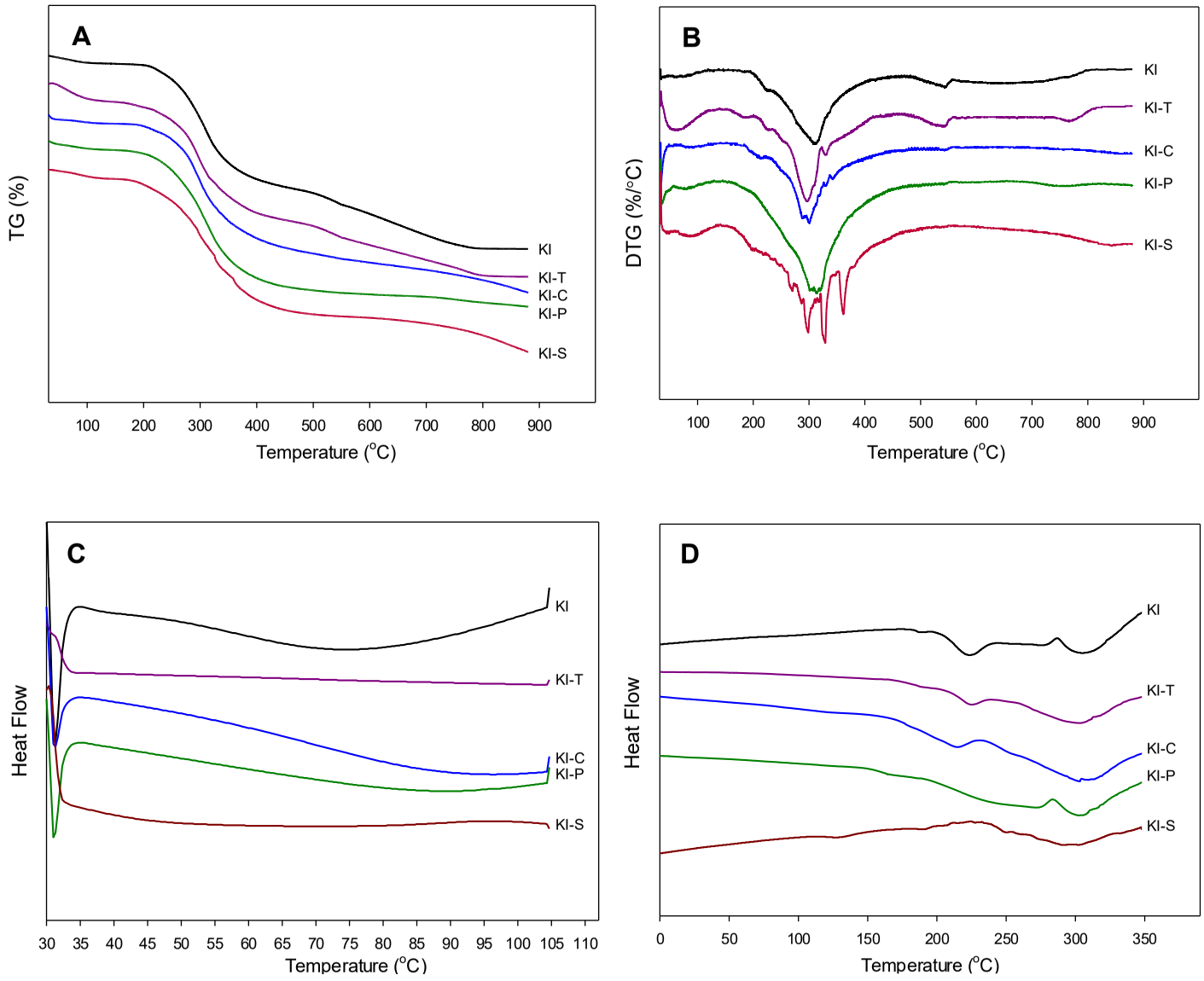

Fig. 4

Figure captions

Fig. 1. Effect of L-Cys treatment of feathers on the chemical and crystalline structure of keratin: ATR FT-IR spectra of feathers and keratin isolate (KI) and the difference spectrum (—) of KI from which spectrum of feathers was subtracted (A, B, C); second derivative spectra (D); XRD diffractograms (E).

Fig. 2. Effect of L-Cys treatment of feathers on the thermal characteristics of keratin: TGA thermograms (A, B); DSC thermograms (C, D).

Fig. 3. Effect of enzyme treatment on the chemical and crystalline structure of keratin isolate (KI): ATR FT-IR spectra of KI and its hydrolysates with trypsin (KI-T) (A, B, C), chymotrypsin (KI-C) (D, E, F), pepsin (KI-P) (G, H, I), subtilisin (KI-S) (J, K, L), and the difference spectrum (—) of hydrolysate from which spectrum of KI was subtracted (A, B, C); second derivative spectra (D); XRD diffractograms (E).

Fig. 4. Effect of enzyme treatment on the thermal characteristics of resulting hydrolysates: TGA thermograms (A, B) and DSC thermograms

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Valorising feather keratin: antioxidant activity, cytotoxicity and mutagenicity during processing and gastrointestinal digestion

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ABSTRACT

Chicken feathers are an underutilized resource with high potential for bioactive peptide production. In this study, the valorisation of feather keratin through a three-step processing approach, i.e., keratin extraction with L-cysteine, followed by its enzymatic hydrolysis with pepsin, trypsin, chymotrypsin or subtilisin, and finally Maillard reaction with glucose or xylose of resulting hydrolysates was proposed. Representative preparations were subjected to simulated gastrointestinal digestion to evaluate their behaviour under physiological-like conditions. Subtilisin hydrolysate showed the strongest radical scavenging, iron-chelating and reducing activity. Its glycation with xylose yielded a preparation with enhanced radical scavenging and reducing activity, diminished iron chelation pre-digestion but the highest overall antioxidant capacity post-digestion. Neither processing nor digestion compromised the safety of the keratin preparation, as the absence of acute cytotoxicity or mutagenicity indicated. All digests showed high digestibility and retained antioxidant properties. These findings support the potential of feather keratin preparations as antioxidant ingredients in nutraceutical applications.

1. Introduction

Bioactive peptides are increasingly recognized for their numerous health-promoting properties. Typically ranging between 0.2 and 3.0 kDa in molecular weight (MW), they serve not only as valuable nutritional components but also demonstrate various biological activities, e.g. antioxidant, antimicrobial, hypoglycaemic, hypotensive, immunomodulatory and procognitive (Hayes, 2021; Sun et al., 2024). Owing to their multifunctionality and safety profile, bioactive peptides are emerging as promising components in food and pharmaceutical applications, with high potential as nutraceuticals, functional food ingredients and natural bioactive additives (Wen et al., 2023). Most bioactive peptides have been obtained from well-studied food proteins, such as milk, soy, fish and eggs, and some have been validated in human studies (Hayes, 2021; Iwaniak et al., 2018). However, there is a growing need to explore more sustainable protein sources, such as those from protein-rich agricultural by-products. Valorising these by-products not only offers a cost-effective alternative to conventional food proteins but also contributes to the circular economy by transforming environmentally noxious waste into high value bioproducts (Ossai et al., 2022; Talha et al., 2024).

Among agricultural by-products, chicken feathers emerge as a particularly promising resource. The poultry industry produces 8.5

Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); DAD, diode array detector; DG, degree of glycation; DH, degree of hydrolysis; EDTA Na_2, ethylenediaminetetraacetic acid disodium salt; EE, EDTA equivalent; FereneNa_2, 3-(2-pyridyl)-5,6-di(2-furyl)-1,2,4-triazine-5′,5″-disulfonic acid disodium salt; FCR, Folin-Ciocâlteu reagent; GAE, gallic acid equivalent; HP-SEC, high performance-size exclusion chromatography; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MRPs, Maillard reaction products; MW, molecular weight; MWD, multiple-wavelength detector; PBS, phosphate-buffered saline; SGID, simulated gastrointestinal digestion; SIF, simulated intestinal fluid; TCA, trichloroacetic acid; TE, Trolox equivalent; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.

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billion tons of feather waste annually (Jagadeesan et al., 2023). Chicken feathers are usually underutilized, despite comprising nearly 10 % of the birds' body mass and containing up to 90 % of a fibrous protein called keratin (Sharma & Kumar, 2019). The chicken feather keratin, although deficient in certain essential amino acids (His, Lys, Met and Trp), holds promise as a source of potent bioactive peptides, (Callegaro et al., 2019; Sinkiewicz et al., 2018). This potential arises from its unique amino acid composition. The protein contains about 10 % Pro residues (Sharma & Kumar, 2019), which provide resistance to gastrointestinal enzymes and improve the chances of keratin-derived peptides crossing the gut barrier and becoming bioavailable (Udenigwe et al., 2021). It has a high, approx. 55 %, content of hydrophobic amino acid residues (Sinkiewicz et al., 2018) which promote interactions with nonpolar biological structures (cell membranes, receptors and enzyme active sites), facilitate transport across enterocytes, and enhance various bioactivities including antihypertensive, antidiabetic, antimicrobial and antioxidant effects (Acquah et al., 2018). The antioxidant potential of keratin-derived peptides is supported by the particularly high content of Cys, Glu, Gly and Ser residues (Taraszkiewicz et al., 2025), which are involved in the biosynthesis of glutathione, the most important endogenous antioxidant (Asantewaa & Harris, 2021; Maugard et al., 2021). Moreover, Cys and Cys-containing peptides further enhance antioxidant properties by acting directly as reducing agents (Pan et al., 2020; Ulrich & Jakob, 2019).

Despite its promising potential, the use of feather keratin in food or pharmaceutical formulations requires keratin's bioactive peptides to be released, which is limited by the challenges associated with the protein solubilization/extraction. The highly cross-linked structure of native keratin, resulting from dense disulfide and hydrogen bonds, makes it exceptionally stable, insoluble in conventional solvents and highly resistant to the majority of proteases (Shavandi et al., 2017; Sinkiewicz et al., 2018). Thus, even though enzymatic hydrolysis is the preferred method for producing bioactive peptides due to its repeatability and ease of reaction control (Lorenzo et al., 2018; Sun et al., 2024), in the case of keratin it is practically impossible without first solubilizing the protein (Lasekan et al., 2013; Sinkiewicz et al., 2018). Numerous methods to solubilize keratin have been developed so far but most rely on harsh conditions, e.g., high temperature, pressure or hazardous chemicals. These treatments usually either compromise the integrity of the extracted protein or result in the formation of sulphurous, odorous by-products, as well as toxic residues, limiting their suitability for human consumption (Crum et al., 2018; Shavandi et al., 2017; Sinkiewicz et al., 2018). One of the most sustainable and efficient methods for solubilizing feather keratin and obtaining of high-purity protein is L-Cys reduction (Ghaffari-Bohlouli et al., 2023; Taraszkiewicz et al., 2025; Xu & Yang, 2014). Such a unique two-step peptide release process, i.e., chemical reduction with L-Cys followed by enzymatic hydrolysis, was recently proposed as a feasible strategy for feather keratin valorisation (Taraszkiewicz et al., 2025) but has not yet been widely explored.

Among the various methods capable of modifying bioactive peptides, the Maillard reaction stands out as a promising approach to enhance their bioactive and functional properties. The Maillard reaction refers to the complex process of non-enzymatic browning initiated by the reaction of the free carbonyl group of saccharides with the free amino group of amino acids, peptides or proteins, especially at increased temperature. Although Maillard reaction leads to the formation of aroma-enhancing volatiles in heated foods, it is usually regarded as detrimental to the nutritional value of proteins. This is due to the potential formation of non-utilizable amino acid derivatives, toxic and mutagenic compounds (e.g. acrylamide, furfural and hydroxymethylfurfural) and induction of cross-linking or aggregation that impairs digestibility (Staroszczyk, 2023). However, emerging evidence indicates that a well-controlled Maillard reaction can effectively enhance biopeptide activity while remaining nutritionally and economically viable (Arihara et al., 2017; Fu et al., 2020; Wu et al., 2021). For instance, Chen et al. (2020) demonstrated that snapper peptides glycated with Xyl at 100 °C for 4 h showed improved antioxidant and hepatoprotective effects in mice, compared to unmodified peptides. Gómez-Estaca et al. (2021) reported a notable increase in the antioxidant activity of shrimp protein hydrolysate after heating with Glc at 100 °C for 40–180 min. According to Dittrich et al. (2009), a diet rich in Maillard reaction products (MRPs) increased the plasma oxidative resistance of low-density lipoprotein by 36 % in human volunteers, compared to a low-MRP diet. To the best of our knowledge, the bioactivities of MRPs derived from feather keratin have not yet been explored, despite specific advantages of using it in the Maillard reaction. Firstly, the Cys residues in keratin contribute to the formation of MRPs with a pleasant, meat-like aroma (Lasekan et al., 2013; Staroszczyk, 2023), and greatly inhibit the formation of acrylamide (Augustine & Bent, 2022). Secondly, the low Lys content in keratin limits the typical loss of this amino acid during the Maillard reaction, a common problem with Lys-rich proteins like milk (Staroszczyk, 2023; van Lieshout et al., 2025).

Orally administered peptides are susceptible to proteolytic cleavage in the stomach, intestines, brush border and plasma, which significantly affects their bioactivity and bioavailability. The initial stages of nutrient breakdown can be mimicked using the simulated gastrointestinal digestion (SGID) methods, among which the INFOGEST 2.0 protocol is the internationally recognized gold standard (Brodkorb et al., 2019). This protocol enables accurate prediction of molecular transformations in food components occurring during upper gastrointestinal transit. It has been validated in human studies, particularly for protein digestion (Kondrashina et al., 2024), yet to date it has not been applied in the context of feather keratin. In our earlier work, we predicted the release of bioactive peptides with 15 different bioactivities from chicken feather keratin using low-cost conventional proteases, i.e., trypsin, chymotrypsin, pepsin and subtilisin (Taraszkiewicz et al., 2022). Then, keratin hydrolysates by the same enzymes were produced from high-purity keratin isolate (KI) and examined for their chemical composition and techno-functional properties (Taraszkiewicz et al., 2025). This study aimed to investigate whether a novel three-step keratin processing strategy, involving L-Cys extraction, followed by enzymatic hydrolysis and mild Maillard reaction, could act synergistically to enhance the antioxidant potential of keratin while maintaining its safety, with low cytotoxic and mutagenic risk. This integrated approach was designed to overcome keratin's natural resistance to digestion and unlock its biofunctional potential, which was further verified through the SGID protocol assessment of bioaccessibility and digestibility under physiologically relevant conditions. Collectively, these findings provide novel insights into the potential use of feather keratin-derived peptides and MRPs as effective ingredients in functional foods and nutraceuticals.

2. Materials and methods

2.1. Materials

White chicken feathers were sourced from Drobful, Poland. Feather washing involved the commercial detergent Ludwik® (Grupa Inco S.A., Poland). L-Cys, urea (Sigma-Aldrich, USA) and NaOH (POCH, Poland) were used for the extraction of keratin. Enzymatic hydrolysis of keratin involved porcine trypsin (cat. no. 93615), bovine α-chymotrypsin (cat. no. C4129), porcine pepsin (cat. no. P7012), subtilisin A from *Bacillus licheniformis* (Alcalase® 2.4 L, cat. no. P4860), HCl (Sigma-Aldrich, USA) and NaOH (POCH, Poland). The MRPs were obtained using D-Glc and D-Xyl (Sigma-Aldrich, USA). SGID was performed using porcine pepsin (cat. no. P7012), porcine pancreatin (cat. no. P7545), bovine bile (cat. no. B3883), cell culture-grade inorganic salts, HCl and NaOH (Sigma-Aldrich, USA). Antioxidant activity assays involved the use of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), $K_2S_2O_8$, phosphate-buffered saline (PBS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), $FeSO_4$, 3-(2-pyridyl)-5,6-di(2-furyl)-1,2,4-triazine-5′,5″-disulfonic acid disodium salt (FereneNa_2), ethanol (absolute), ethylenediaminetetraacetic acid disodium salt (EDTA Na_2), commercial

Folin-Ciocâlteu reagent (FCR), Na_2CO_3 and gallic acid monohydrate (Sigma-Aldrich, USA). The degree of glycation (DG) was measured using dithiothreitol, L-leucine, o-phthaldialdehyde, sodium dodecyl sulphate and sodium tetraborate (Sigma-Aldrich, USA). Chromatographic analyses were performed using HPLC-grade solvents, protein and amino acid standards and trichloroacetic acid (TCA) (Sigma-Aldrich, USA). For cellular experiments, Dulbecco's modified Eagle's medium (cat. no. 5796), dimethyl sulfoxide, fetal bovine serum, penicillin-streptomycin solution and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, USA) were used. The mutagenicity assay was conducted using the Ames test kit (Ames MPF 98/100, Xenometrix, Switzerland).

2.2. Workflow

Keratin isolate (KI) was obtained from pretreated feathers through extraction with L-Cys and then hydrolysed with either trypsin (KI-T), chymotrypsin (KI-C), pepsin (KI-P) or subtilisin (KI-S). The MW distribution, antioxidant activity, cytotoxicity and direct mutagenic capacity of KI and its hydrolysates were determined (Fig. 1A). The antioxidant potential was measured using ABTS scavenging, Fe^{2+} chelating and FCR reducing activity assays, allowing the selection of KI-S for the next processing step. KI-S was then thermally treated with either Glc or Xyl (Maillard reaction) under different temperatures and times, obtaining 9 samples for each saccharide (Table 1). The resulting MRPs were described in terms of browning intensity, DG, MW distribution, antioxidant activity, cytotoxicity and direct mutagenic capacity (Fig. 1B).

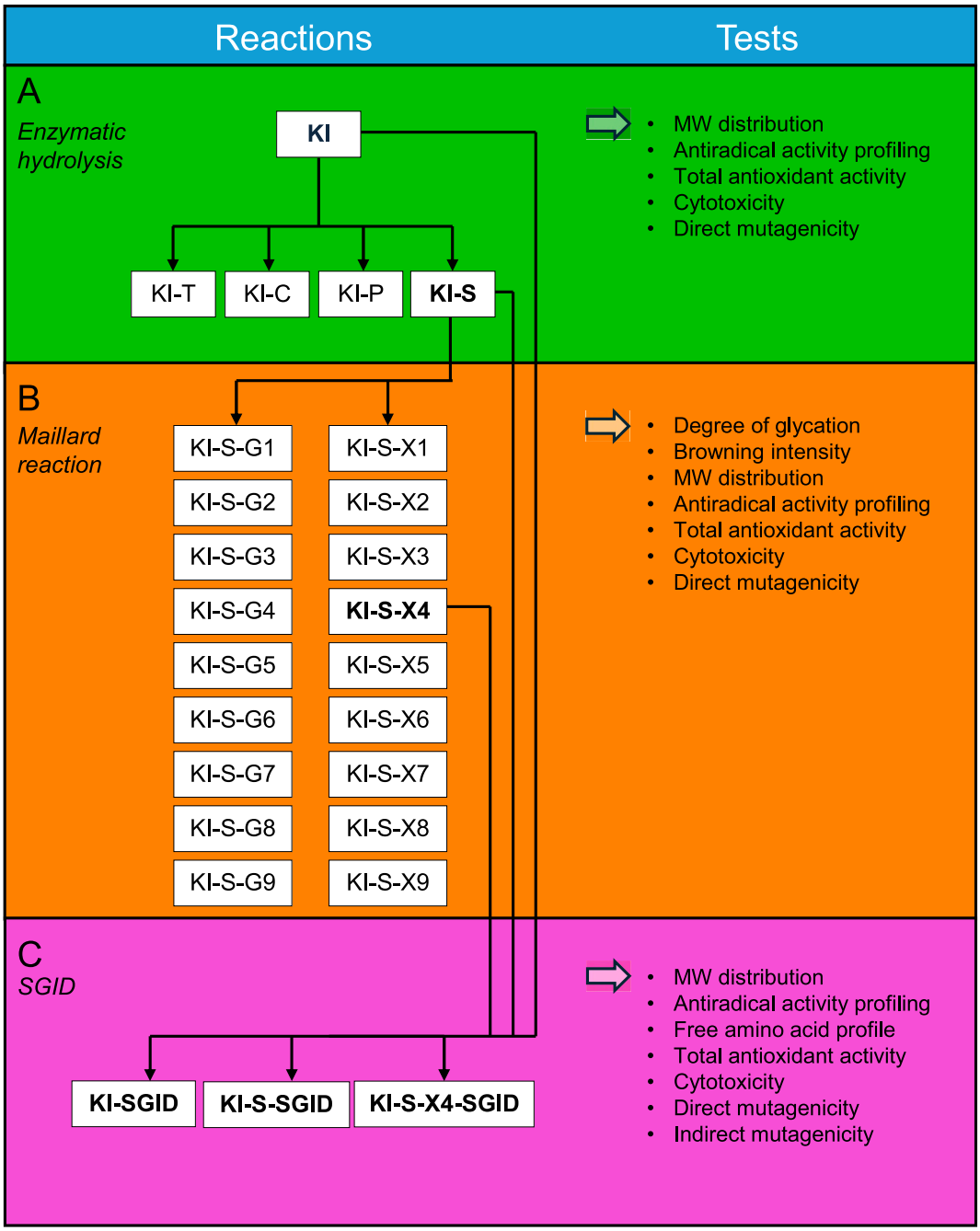

Fig. 1. Study workflow. KI – keratin isolate; KI-T, KI-C, KI-P and KI-S – KI hydrolysate by trypsin, chymotrypsin, pepsin and subtilisin, respectively; KI-S-G and KI-S-X – mixtures of KI-S with Glc and Xyl, respectively (see Table 1); MW – molecular weight; SGID – simulated gastrointestinal digestion.

Table 1
Experimental conditions of the Maillard reaction applied to keratin isolate hydrolysate by subtilisin with glucose (KI-S-G) or xylose (KI-S-X).

Name	Saccharide	Temp. [°C]	Time [h]
KI-S-G1	Glc	90	1
KI-S-G2	Glc	90	2
KI-S-G3	Glc	90	3
KI-S-G4	Glc	105	1
KI-S-G5	Glc	105	2
KI-S-G6	Glc	105	3
KI-S-G7	Glc	120	1
KI-S-G8	Glc	120	2
KI-S-G9	Glc	120	3
KI-S-X1	Xyl	90	1
KI-S-X2	Xyl	90	2
KI-S-X3	Xyl	90	3
KI-S-X4	Xyl	105	1
KI-S-X5	Xyl	105	2
KI-S-X6	Xyl	105	3
KI-S-X7	Xyl	120	1
KI-S-X8	Xyl	120	2
KI-S-X9	Xyl	120	3

The antioxidant assessment selected KI-S-X4. Finally, representatives of each processing step i.e. KI, KI-S and KI-S-X4 were subjected to SGID and then characterised in terms of free amino acids profile, MW distribution, antioxidant activity, cytotoxicity, direct and indirect mutagenicity (Fig. 1C).

2.3. Obtaining keratin preparations

2.3.1. Pretreatment of feathers and keratin extraction

The feather pretreatment and keratin extraction were performed as previously described (Taraszkiewicz et al., 2025). The feathers were washed in warm tap water with commercial detergent, followed by a rinse with distilled water. The feathers were dried overnight at 50 °C, cut into filaments of 2–3 cm and milled into 0.75 mm pieces. KI was prepared by mixing the pretreated feathers at a ratio of 1:20 (*w/v*) with the extraction solution consisting of 1.5 % (w/v) L-Cys and 8 M urea, pH 10.5. The mixture was shaken (200 rpm, 30 °C, 1 h), then centrifuged (5000 rpm, 10 min) and filtered to separate soluble keratin from the undissolved feather residue. The filtrate was purified against distilled water using Spectra/Por dialysis membranes of regenerated cellulose (molecular weight cut-off 3.5 kDa) at 4 °C for 3 days and lyophilized.

2.3.2. Enzymatic hydrolysis of KI

The enzymatic hydrolysates of KI were prepared as described previously (Taraszkiewicz et al., 2025). Briefly, hydrolysis was performed for 1 h under pH-stat conditions at enzyme/substrate ratio of 1:20 (*w*/w) using either trypsin (KI-T, pH 8 at 37 °C), chymotrypsin (KI-C, pH 8 at 37 °C), pepsin (KI-P, pH 2 at 37 °C) or subtilisin (KI-S, pH 9 at 60 °C), followed by enzyme inactivation (85 °C, 15 min) and lyophilization.

2.3.3. The Maillard reaction

The MRPs were obtained based on methods reported previously (K. Chen et al., 2019; W. Jiang et al., 2018; Z. Jiang et al., 2013). A mixture of KI-S (20 mg/mL) and either Glc or Xyl (40 mg/mL) was prepared in a total volume of 10 mL. The initial unadjusted pH was 9. The mixtures were heated at 90, 105 or 120 °C for 1, 2 or 3 h (Table 1) in triplicate, then cooled down to room temperature. Triplicates were pooled, lyophilized and stored at 4 °C. Control samples were prepared with KI-S (20 mg/mL) heated without any saccharide.

2.3.4. SGID

Three keratin preparations (KI, KI-S and KI-S-X4) were subjected to the SGID procedure following the static INFOGEST 2.0 protocol (Brodkorb et al., 2019), with each sample digested in triplicate. KI and KI-S were weighed to ensure that each contained 500 mg of protein, while KI-S-X4 was weighed to ensure 500 mg of MRPs content. All preparations were dissolved in distilled water to a final volume of 5 mL. For the gastric digestion, 4 mL of simulated gastric fluid and 5 μL of 0.15 M $CaCl_2$ were added and adjusted to pH 3 with 2 M HCl. Then 0.25 mL of pepsin solution in distilled water (pepsin activity: 2233 U/mg) was added to reach 2000 U/mL in the final mixture and the total reaction volume was adjusted to 10 mL with distilled water. The sample was incubated using a rotating wheel (15 rpm, 37°, 2 h). For the intestinal digestion, 4.25 mL of simulated intestinal fluid (SIF) was added, followed by 40 μL of 0.15 M $CaCl_2$ and 1.25 mL of bile solution in SIF (bile salt concentration: 440 mg/mmol) to reach 10 mM in the final mixture. The pH of the mixture was adjusted to 7 with 2 M NaOH, 2.5 mL of pancreatin suspension in SIF (trypsin activity: 8.9 U/mg) was added to reach 100 U/mL in the final mixture and the total volume was made up to 20 mL with distilled water. The sample was incubated using a rotating wheel (15 rpm, 37°, 2 h). After the intestinal digestion, the sample was heated at 90 °C for 10 min to stop the enzyme reaction, triplicates pooled and stored at −20 °C. A control sample with the keratin preparations replaced by an equal volume of distilled water (H_2O-SGID) was also prepared. In H_2O-SGID, the thermal enzyme inactivation was conducted before the incubation to limit the protease autolysis occurring in the absence of digestible proteins (Kondrashina et al., 2024).

2.4. Determination of physiochemical properties

2.4.1. High performance size exclusion chromatography (HP-SEC) with post-column ABTS derivatization

MW distribution analysis and antiradical activity profiling with post-column ABTS derivatization were determined using an HPLC system (1200 series, Agilent Technologies, USA) equipped with a Pinnacle PCX Derivatization Instrument (Pickering Laboratories Inc., USA), following Kusznierewicz et al. (2011a, 2011b) and Taraszkiewicz et al. (2025). Keratin preparations were dissolved in the mobile phase (acetonitrile/water/trifluoroacetic acid, 30:60.9:0.1, *v*/v) at a concentration 5 mg/mL and centrifuged at 12,000 rpm for 10 min. Thirty μL of supernatant was injected into a ReproSil 50 SEC column (300 × 8 mm, 5 μm, fractionation range 0.5–10 kDa), eluted at 0.8 mL/min and detected at 215 nm using a diode array detector (DAD). A MW calibration curve was prepared using standard proteins: aprotinin (6.5144 kDa), bovine insulin (5.7335 kDa), oxidized bovine insulin chain B (3.4959 kDa), bacitracin A (1.4227 kDa) and bradykinin (1.0602 kDa). Data are presented as the area under the curve for each MW range (<0.5, 0.5–1 kDa, 1–2 kDa, 2–5 kDa, 5–10 kDa, and > 10 kDa), expressed as a percentage of total area under the curve.

The 7 mM ABTS stock solution was prepared by dissolving ABTS in 2.45 mM $K_2S_2O_8$ and incubating in the dark for 24 h. For post-column antiradical activity profiling, a 10 % (*v*/v) aqueous solution of the ABTS stock was mixed with the eluate post-DAD at 0.1 mL/min and directed to a reaction loop (1 mL, 130 °C). The eluate was then sent to a multi-wavelength detector (MWD), where the ABTS radical scavenging was monitored at 734 nm. The relative contribution of analytes to the antiradical activity was estimated if 100 % corresponds to the total integrated negative area under the curve in ABTS-derivatized chromatograms.

2.4.2. DG measurement

DG was defined as the % decrease in free amino groups due to the Maillard reaction (Section 2.3.3), relative to their concentration before the thermal treatment. The content of free amino groups was measured according to Bavaro et al. (2021), with a few modifications. Ten μL of the sample was mixed with 200 μL of the reagent containing 0.8 mg/mL o-phthaldialdehyde, 38.1 mg/mL sodium tetraborate, 1 mg/mL sodium dodecyl sulphate and 0.88 mg/mL dithiothreitol. After 15 min incubation without light, A_{340} was measured using a microplate reader (Synergy HT, BioTek Instruments, Inc., USA). L-Leucine was used to generate a calibration curve.

2.4.3. Browning intensity measurement

The browning of KI-S before and after the Maillard reaction (Section 2.3.3) was measured using NanoDrop 2000c spectrophotometer (Thermo Scientific, USA). The absorbance of intermediate, colourless MRPs (A_{294}) and advanced, brown MRPs (A_{420}) was determined post 20- and 10-fold dilution with distilled water, respectively.

2.4.4. Free amino acid content determination

The analysis of the free amino acid profile (except Trp) in the SGID-samples followed the method of Mounier et al. (2007). An equal volume of the sample and 24 % (*w/v*) TCA were mixed, left for 10 min and then centrifuged (15,000 rpm, 10 min). The supernatant was removed, and the sample was diluted 1:2 with the internal standard, norleucine, to give a final concentration of 125 nmol/mL norleucine. Free amino acids were quantified using a Jeol JLC-500/V amino acid analyser (Jeol (UK) Ltd., Garden City, Herts, UK) fitted with a Jeol Na^+ high performance cation exchange column.

2.5. Determination of biological activities

2.5.1. Measurement of total antioxidant activity

The total antiradical capacity was determined using the ABTS assay following the previous methods (Pérez-Burillo et al., 2018; Zheng et al., 2016). Directly before measurement, the ABTS stock solution (prepared as per Section 2.4.1) was diluted with PBS to achieve A_{734} of 0.7. A mixture of 20 μL of sample and 280 μL of the diluted ABTS solution was incubated in the dark at 37 °C for 30 min and then A_{734} was recorded using the Synergy HT microplate reader.

The total Fe^{2+} chelating capacity was determined based on the methods of Fontoura et al. (2019) and Tel et al. (2012). A mixture of 200 μL of sample and 25 μL of 2 mM $FeSO_4$ was incubated at 37 °C for 10 min, followed by addition of 100 μL of 5 mM $FereneNa_2$. After another 10 min incubation, 1.7 mL of ethanol was added to terminate the reaction. Two hundred μL of the final solution was transferred to a 96-well plate and then A_{593} was measured using the Synergy HT microplate reader.

The total reducing activity was assessed using the FCR method according to Everette et al. (2010). A mixture of 20 μL of sample, 1.58 mL of distilled water and 100 μL of commercial FCR was incubated at room temperature for 5 min, followed by addition of 300 μL of 20 % Na_2CO_3. After another incubation at 37 °C for 1 h, A_{765} was measured using the NanoDrop 2000c spectrophotometer.

The results were expressed as μmol equivalents of Trolox (TE), EDTA Na_2 (EE) and gallic acid (GAE), respectively, and normalized both per g of protein and per g of lyophilizate. This allowed for separate evaluation of the antioxidant capacity of keratin (per g of protein) and the overall antioxidant potential of the powders (per g of lyophilizate). Since Glc and Xyl do not exhibit reactivity in these assays, this approach ensured that differences in activity could be attributed to the protein substrate only or the total formulation, respectively.

2.5.2. Cytotoxicity assay by the MTT test

Caco-2 (human colon adenocarcinoma) cells purchased from the American Tissue Culture Collection were incubated at 37 °C under 5 % CO_2. The cells were cultured in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum (10 %, *v*/v), penicillin (100 U/mL) and streptomycin (100 μg/mL). The viability of Caco-2 cells treated with the keratin preparations was assessed using the standard MTT assay, according to previous methods (Bavaro et al., 2021; Corrochano et al., 2019; Parchem et al., 2023). Caco-2 cells (passage 20–40) were seeded in 96-well cell culture plates (5×10^3 cells/well in 180 μL of culture media) and allowed to attach for 24 h. The keratin preparations were dissolved in distilled water and sterilized with syringe-driven filters (0.22 μm). The cells were then treated with 20 μL of keratin preparations to reach concentration of 10, 50 and 250 μg/cm^2 for 24, 48, and 72 h. These concentrations were selected considering the total surface area of the small intestine (200 m^2) and (a) the recommended intake of a milk protein-based sport product (21 g protein/day) equivalent to 10 μg/cm^2, (b) the average daily high protein intake in a Western diet (100 g/day, 50 μg/cm^2) and (c) a 5-fold increase to account for the in vitro absence of microclimate (250 μg/cm^2) (Corrochano et al., 2019). In the case of shorter exposure, the media were aspirated from the wells and replaced with 200 μL of fresh media. For the 72 h exposure time, the media was replaced at the endpoint. After 72 h, 50 μL of MTT solution (4 mg/mL) was added, and the cells were incubated for an additional 4 h. After incubation, the culture media was aspirated, and the formazan crystals formed by viable cells were dissolved in 100 μL of dimethyl sulfoxide. Formazan specific absorbance (A_{540}) was measured and corrected by subtracting background absorbance (A_{690}) using the Synergy HT microplate reader. The cytotoxic effect was expressed as % of cell viability relative to control cells treated with 20 μL of distilled water in culture media.

2.5.3. Mutagenicity assay by the Ames test

The mutagenic potential of the keratin preparations was evaluated using the microplate version of the Ames test with *Salmonella Typhimurium* strains TA98 and TA100. All samples were tested for the direct mutagenic capacity and selected samples were additionally tested for indirect mutagenic capacity, using the S9 fraction for metabolic activation. The keratin preparations (0.22 μm-filtered) were tested at concentrations equivalent to those used in the MTT test (Section 2.5.2), i.e., 10, 50 and 250 μg/cm^2. The procedure was performed in accordance with the manufacturer's instructions.

2.6. Statistical analysis

All experiments were conducted using the pooled keratin preparations obtained in at least three independent reactions. For each analytical method, the data represent the average ± SD of triplicate measurements unless otherwise specified. The results of total antioxidant activity and mutagenicity assays were processed using SigmaPlot 11.0 (SYSTAT Software, Germany) through one-way analysis of variance (ANOVA) with a significance level of $p < 0.05$.

3. Results

3.1. Characteristics of KI and its enzymatic hydrolysates

3.1.1. MW distribution and antiradical activity profiling

The MW profiles of peptides present in KI and its enzymatic hydrolysates, obtained using SDS-PAGE and HP-SEC (detected at 215 nm, Fig. 2A), have been described previously (Taraszkiewicz et al., 2025). In this study, the HP-SEC analysis was expanded to include post-column derivatization with ABTS (detected at 734 nm, Fig. 2B), enabling profiling of antiradical activity across different peptide size fractions. Overall, the MW distribution (Fig. 2A) and antiradical activity profiles (Fig. 2B) looked similar. In KI, as expected for intact keratin, the MW fraction above 5 kDa contributed most to antiradical activity (80 %), followed by the fractions of 1–5 kDa (13 %) and below 1 kDa (8 %). The latter was likely due to free L-Cys remaining after the keratin extraction process, measured at 7 mg/g (Taraszkiewicz et al., 2025). In KI-T, KI-C and KI-P, the fractions with peptides above 5 kDa constituted 42–53 % of the content (Fig. 2A) and contributed 47–51 % to the activity (Fig. 2B). In these hydrolysates, despite differences in the proportions of peptides in the 1–5 kDa and below 1 kDa ranges (Fig. 2A), their contribution to antiradical activity was comparable (21–28 %, Fig. 2B). The most considerable difference between MW distribution and antiradical activity profiles was observed in KI-S. In this hydrolysate, the fraction below 1 kDa made up 64 % of the peptide content but accounted for only 38 % of the activity. Whereas the fraction 1–5 kDa constituted only 28 % of the peptide content, it contributed as much as 44 % to the activity.

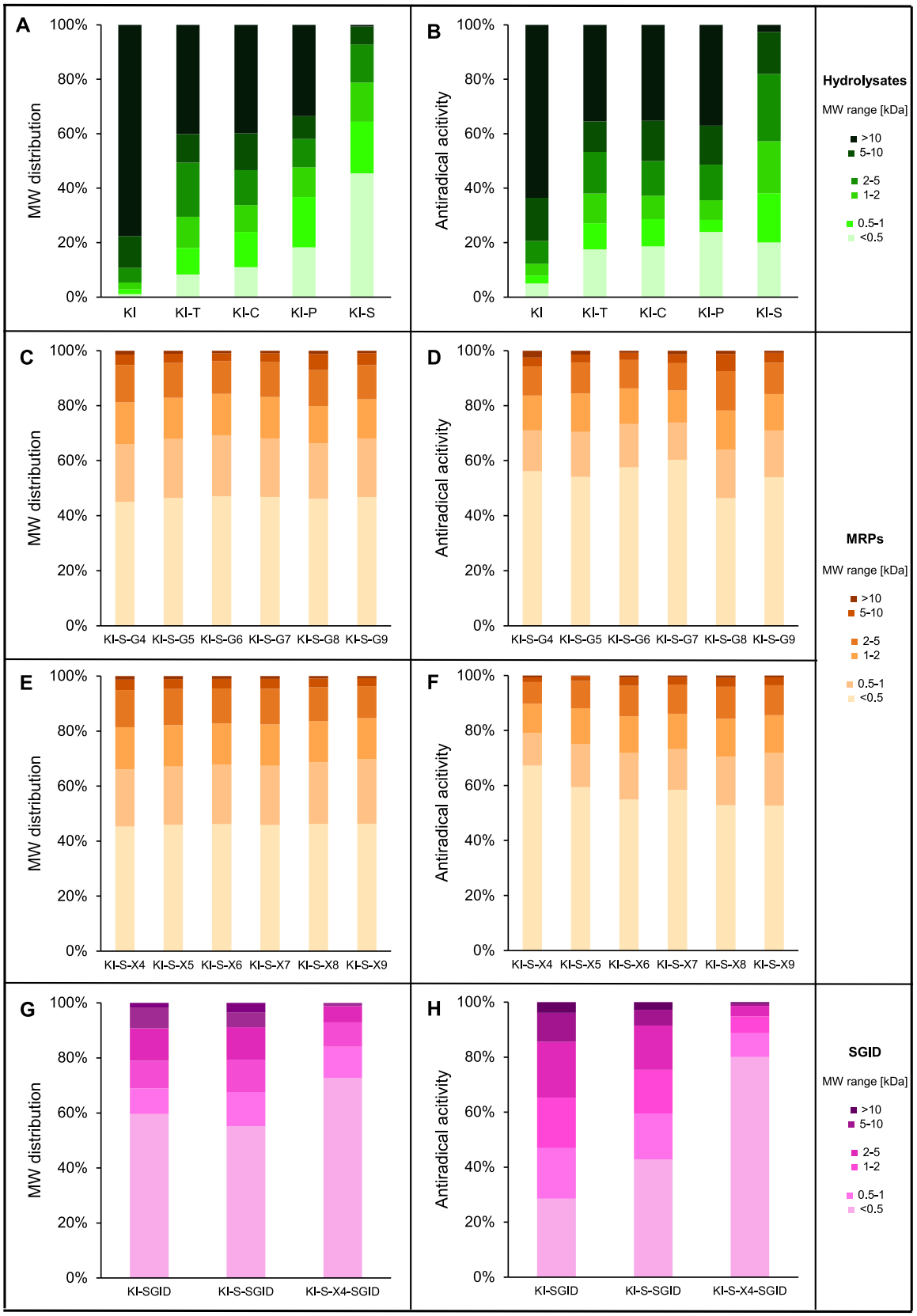

Fig. 2. Molecular weight (MW) distribution profiles (left side) and antiradical activity profiles (right side) of keratin preparations determined by size exclusion chromatography with detection at 215 nm and 734 nm (post-column ABTS derivatization), respectively. Values are based on a single chromatographic analysis of pooled samples produced in three independent reactions. (**A, B**) KI – keratin isolate; KI-T, KI-C, KI-P and KI-S – KI hydrolysates by trypsin, chymotrypsin, pepsin and subtilisin, respectively; (**C, D**) KI-S-G – KI-S and Glc-derived Maillard reaction products (MRPs, see Table 1); (**E, F**) KI-S-X – KI-S and Xyl-derived MRPs (see Table 1); (**G, H**) Keratin preparations after simulated gastrointestinal digestion (SGID), corrected with H_2O-SGID control sample.

3.1.2. Total antioxidant activity

The total antioxidant capacity was assessed using traditional colorimetric methods i.e. ABTS scavenging, Fe^{2+} chelating and FCR reducing assays and the results were expressed as μmol equivalents of the most common standard in each assay, i.e. Trolox (TE), EDTA (EE) and gallic acid (GAE), respectively. KI exhibited a baseline antioxidant capacity of 353 μmol TE/g protein and 321 μmol TE/g lyophilizate, 36 μmol EE/g protein and 33 μmol EE/g lyophilizate, and 84 μmol GAE/g protein and 77 μmol GAE/g lyophilizate (Fig. 3).

Enzymatic hydrolysis of KI generally improved antioxidant activity. In the ABTS scavenging assay, the most pronounced increase was observed in KI-S, which reached 595 μmol TE/g protein and 415 μmol TE/g lyophilizate, indicating 69 % and 29 % improvement over KI, respectively. KI-T, KI-C and KI-P demonstrated slightly enhanced scavenging activity per gram of lyophilizate, but not per gram of protein. A similar pattern was observed in the Fe^{2+} chelating assay where KI-S exhibited a markedly improved activity, reaching 459 μmol EE/g protein and 320 μmol EE/g lyophilizate – over ten times increase compared to KI. KI-C and KI-P exhibited moderate increases in chelation, regardless of data normalization, while KI-T showed slightly improved chelating activity per gram of lyophilizate, but not per gram of protein. In the FCR reducing assay, the hydrolysates also outperformed the KI. KI-S showed the highest activity, reaching 159 μmol GAE/g protein and 111 μmol GAE/g lyophilizate, KI-T and KI-C exhibited similar improvements, while KI-P showed a smaller but still statistically significant increase compared to KI.

3.1.3. Cytotoxicity and direct mutagenicity

As keratin preparations were developed for food applications, it was important to test the cytotoxic effect on human enterocytes. In the MTT test, both KI and its enzymatic hydrolysates were well tolerated at all tested concentrations and incubation times (Fig. 4A). Caco-2 cell viability with test samples consistently exceeded 90 % relative to control Caco-2, indicating the absence of acute cytotoxic effects. However, potential chronic effects warrant further investigation.

To determine the mutagenic potential of keratin prior to Mailliard reaction, the Ames test was performed using *Salmonella Typhimurium*. No direct mutagenic activity of KI and its hydrolysates was observed (Fig. 5). The revertant counts ranged from 1 to 5 for TA98 and from 2 to 7 for TA100 strains, with no significant differences compared to the negative control (C-), i.e. sterile distilled water ($p > 0.05$). Neither dose-dependency nor notable variability between replicates was observed, regardless of the strain used. The positive controls (C+), i.e. 2-nitrofluorene (2 μg/mL) for TA98 and 4-nitroquinoline N-oxide (0.1 μg/mL) for TA100, elicited strong mutagenic responses, with average revertant counts of 46 and 41, respectively, confirming the validity of the assay.

With no observed cytotoxic or mutagenic effects, the hydrolysates' antioxidant properties guided the selection for subsequent processing. As KI-S demonstrated the most potent antioxidant activity in all assays, it was selected for modification through the Maillard reaction.

3.2. Characteristics of the MRPs

3.2.1. DG and browning intensity

No considerable glycation was observed in mixtures of KI-S with Glc or Xyl treated at 90 °C, regardless of the saccharide used and the heating time, with DG values ranging between 0.04 and 0.94 % (Fig. 6). A progressive increase in DG was noted in samples treated at 105 °C and 120 °C, with values rising alongside reaction time. In Glc-derived MRPs (from KI-S-G4 to KI-S-G9), DG ranged from 4.3 to 15.6 %, with the highest glycation detected in KI-S-G9. Xyl-derived MRPs (from KI-S-X4 to KI-S-X9) exhibited more extensive glycation, with DG values ranging from 11.9 to 37.8 %, and the latter value attributed to KI-S-X9, the most glycated MRP overall.

The browning intensity followed a similar trend to DG (Fig. 7). Untreated KI-S had A_{294} of 0.45 and A_{420} of 0.17. KI-S samples heated

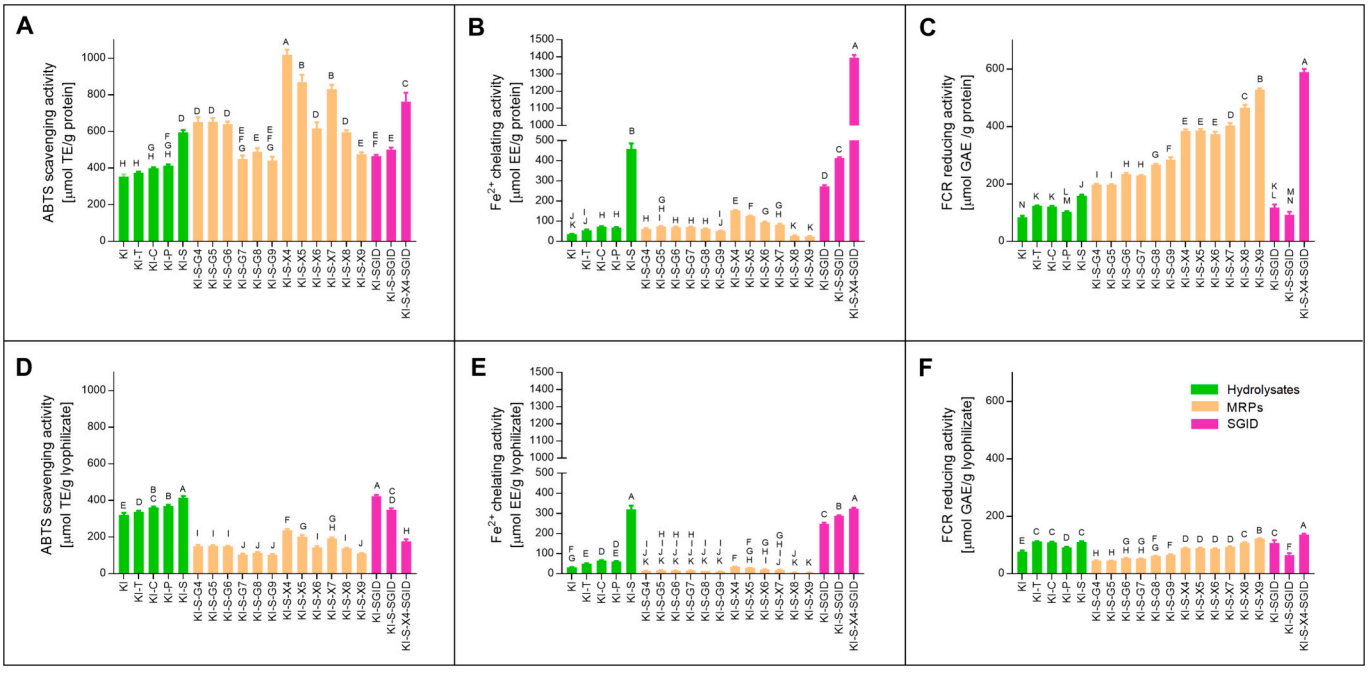

Fig. 3. Total antioxidant capacity of keratin preparations normalized per gram of protein (top row) and per gram of lyophilizate (bottom row). Values reported are means ± SD of three replicates, obtained from pooled samples produced in three independent reactions ($n = 3$). (**A, D**) ABTS scavenging activity expressed as μmol Trolox equivalents (TE). (**B, E**) Fe^{2+} chelating activity expressed as μmol EDTA Na_2 equivalents (EE). (**C, F**) Folin-Ciocâlteu reagent (FCR) reducing activity expressed as μmol gallic acid equivalents (GAE). KI – keratin isolate; KI-T, KI-C, KI-P and KI-S – KI hydrolysates by trypsin, chymotrypsin, pepsin and subtilisin, respectively; KI-S-G – KI-S and Glc-derived Maillard reaction products (see Table 1); KI-S-X – KI-S and Xyl-derived Maillard reaction products (see Table 1); MRPs – Maillard reaction products; SGID – simulated gastrointestinal digestion. Post-SGID results were corrected with H_2O-SGID control sample. Values marked with different superscript letters differ significantly ($p < 0.05$).

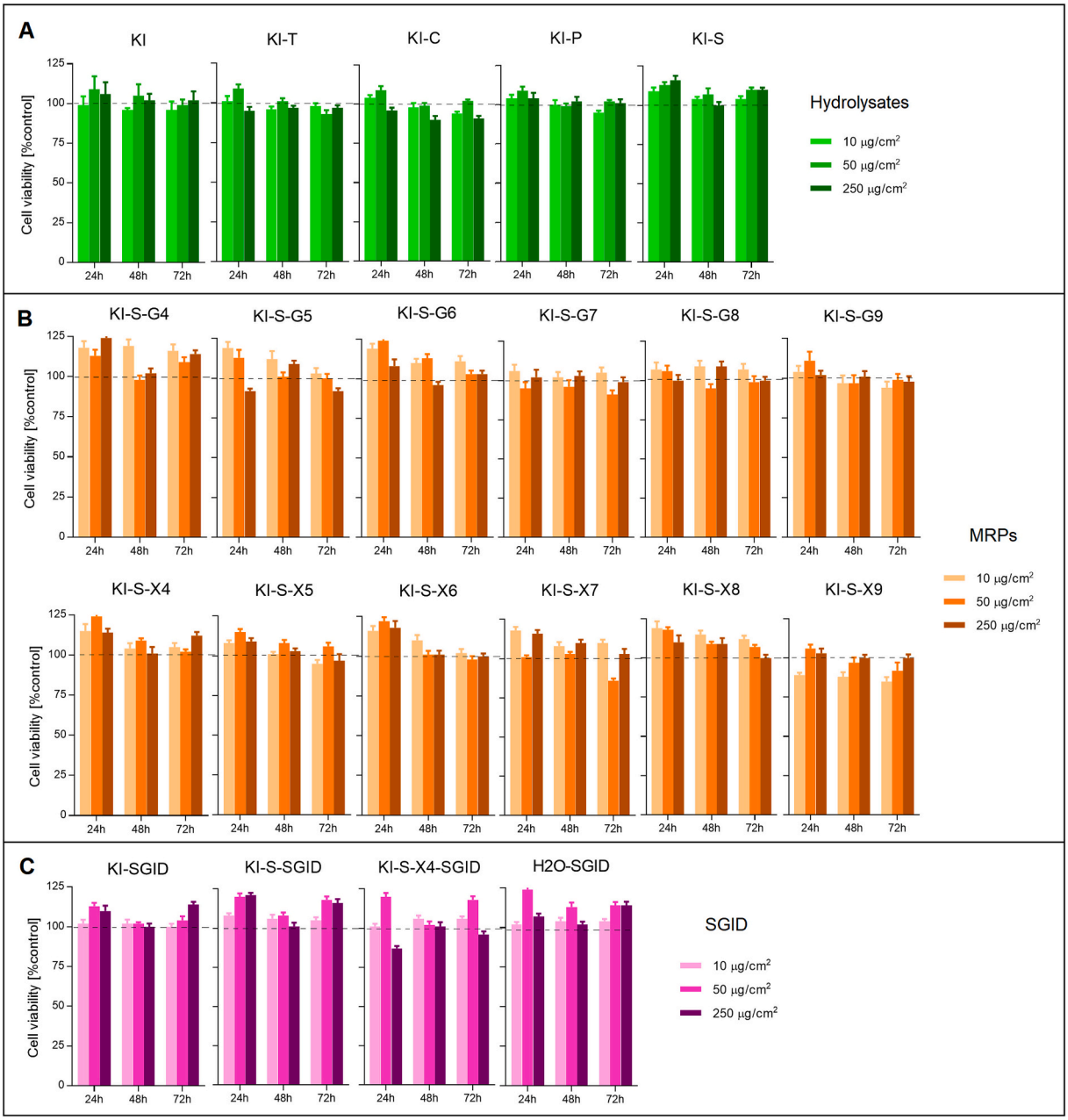

Fig. 4. Cytotoxicity of keratin preparation in Caco-2 cells determined using MTT test. Values reported are means ± SD of three technical replicates carried out on three different days ($n = 9$). (**A**) KI – keratin isolate, KI-T, KI-C, KI-P and KI-S – KI hydrolysates by trypsin, chymotrypsin, pepsin and subtilisin, respectively; (**B**) KI-S-G – KI-S and Glc-derived Maillard reaction products, KI-S-X – KI-S and Xyl-derived Maillard reaction products (see Table 1); (**C**) Keratin preparations after simulated gastrointestinal digestion (SGID), H_2O-SGID – SGID control with sample replaced by distilled water. MRPs – Maillard reaction products. Dashed line represents the viability of control Caco-2 cells treated with distilled water in culture media (assigned 100 %).

without any saccharides showed only minor increases, with A_{294} remaining below 0.56 and A_{420} reaching a maximum of 0.21, indicating minimal browning due to heat alone. Glc and Xyl solutions heated without KI-S exhibited no browning under the conditions tested (data not shown), indicating the absence of caramelization. In contrast, mixtures of KI-S with saccharides showed a marked increase in browning with both temperature and time. A_{294} values of Glc-derived MRPs ranged from 0.58 (KI-S-G4) to 0.83 (KI-S-G9), while A_{420} extended from 0.21 (KI-S-G4) to 0.27 (KI-S-G9). Xyl-derived MRPs developed substantially stronger browning as A_{294} ranged from 0.71 (KI-S-X4) to 1.72 (KI-S-X9) and A_{420} from 0.23 (KI-S-X4) to 0.43 (KI-S-X4). The strongest browning was observed in KI-S-X9. Strong correlation between A_{294} and A_{420}, A_{294} and DG, and A_{420} and DG was observed, with R^2 values of 0.98, 0.94 and 0.93, respectively.

No notable signs of glycation or browning were observed in the mixtures of KI-S and saccharides heated at 90 °C (KI-S-G1 to KI-S-G3, KI-S-X1 to KI-S-X3). Hence these preparations were excluded from further analyses.

3.2.2. *MW distribution and antiradical activity profiling*

The MW profiles of Glc- and Xyl-derived MRPs detected at 215 nm (Fig. 2C and E, respectively) were very similar to the profile of KI-S, regardless of Maillard reaction temperature and time. In all MRPs more than 93 % were below 5 kDa in size, while the content of those below 0.5 kDa was about 46 %. On the other hand, the fraction above 10 kDa remained under 1.5 %, indicating negligible content of high MW

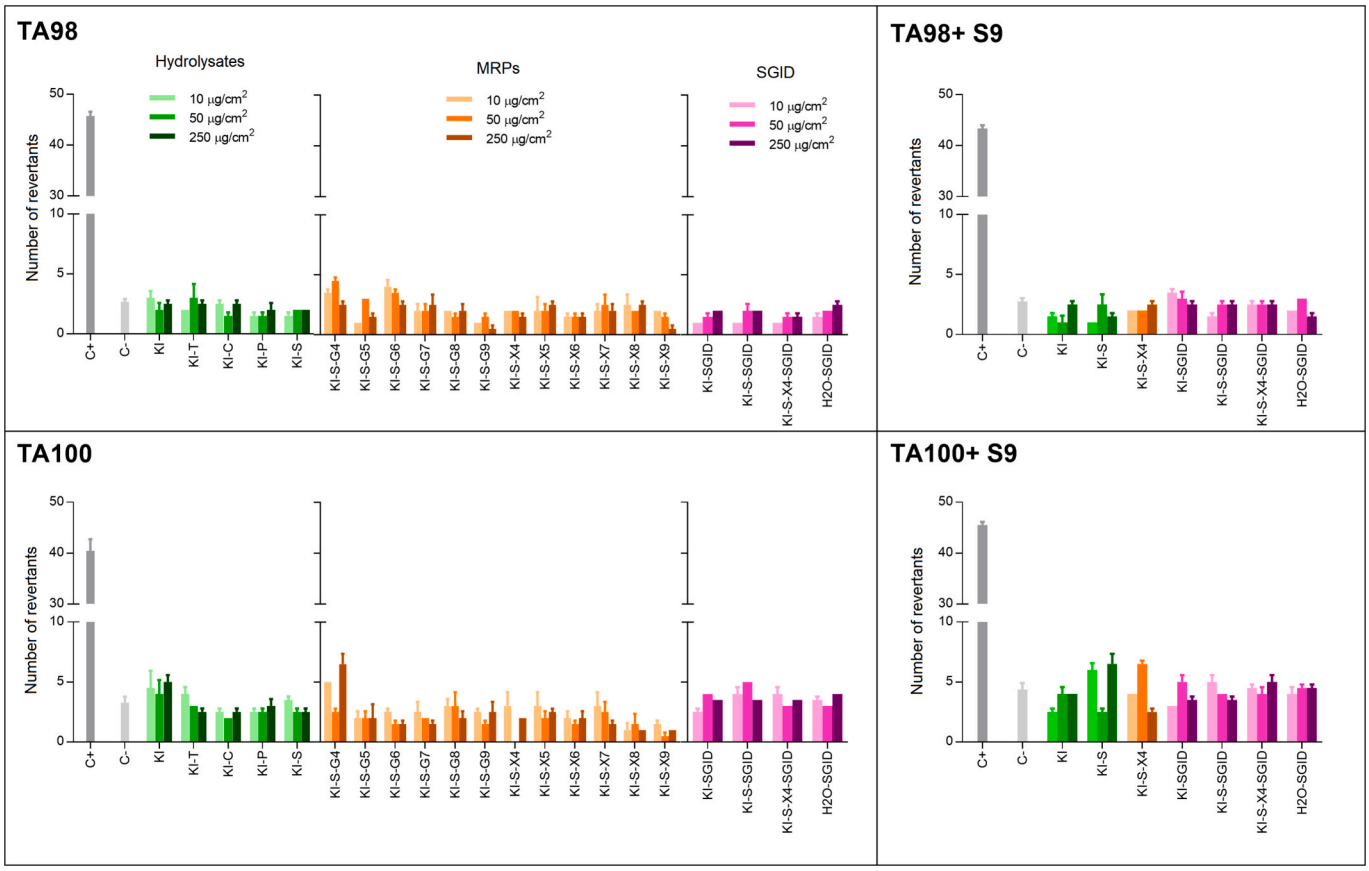

Fig. 5. Mutagenicity of keratin preparations in *Salmonella Typhimurium* determined using Ames test. Values reported are means ± SD from two independent experiments performed on different days ($n = 2$). (**TA98, TA98 + S9**) Direct and indirect mutagenicity in the strain TA98, respectively. (**TA100, TA100 + S9**) Direct and indirect mutagenicity in the strain TA100, respectively. KI – keratin isolate; KI-T, KI-C, KI-P and KI-S – KI hydrolysates by trypsin, chymotrypsin, pepsin and subtilisin, respectively; KI-S-G – KI-S and Glc-derived Maillard reaction products, KI-S-X – KI-S and Xyl-derived Maillard reaction products (see Table 1); MRPs – Maillard reaction products; SGID – simulated gastrointestinal digestion; H_2O-SGID – SGID control with sample replaced by distilled water; C+ – positive control; C- – negative control. No significant differences in the number of revertants were observed between the investigated samples and the negative control ($p > 0.05$).

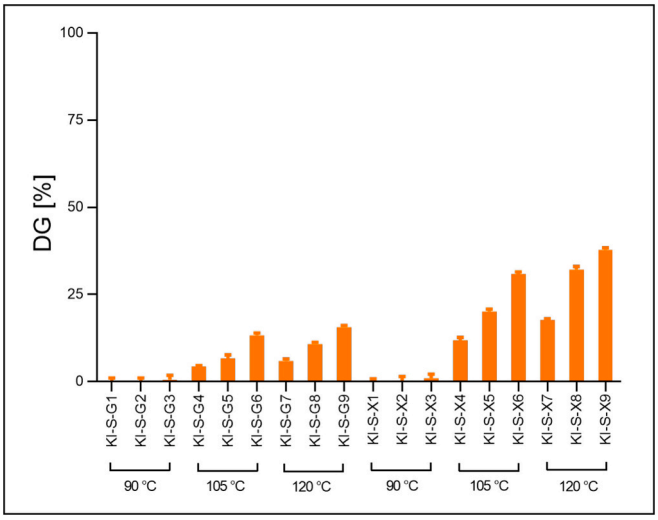

Fig. 6. Degree of glycation (DG) in mixtures of keratin isolate hydrolysate by subtilisin (KI-S) with saccharides defined as the % decrease in free amino groups due to the Maillard reaction, relative to their concentration before the thermal treatment. Values reported are means ± SD of nine replicates, obtained from pooled samples produced in three independent reactions (n = 9). KI-S-G – KI-S and Glc-derived Maillard reaction products, KI-S-X – KI-S and Xyl-derived Maillard reaction products (see Table 1).

compounds.

The antiradical activity profiles of Glc- and Xyl-derived MRPs detected at 734 nm (Fig. 2D and F, respectively) indicated bigger differences. In all MRPs, the fraction below 0.5 kDa consistently contributed the largest share of antiradical activity. In Glc-derived MRPs, this fraction accounted for 46–60 % of antiradical activity, while the fraction below 1 kDa contributed 64–74 %. Similarly, for the Xyl-derived MRPs, the fraction below 0.5 kDa contributed 53–67 % of total activity, with analytes below 1 kDa responsible for 70–79 %. The fraction above 5 kDa contributed minimally to antiradical activity, with values below 8 % in all MRPs.

3.2.3. *Total antioxidant activity*

ABTS scavenging activity of Glc-derived MRPs depended on the Maillard reaction temperature (Fig. 3A, D). The products obtained after heating at 105 °C (KI-S-G4 to KI-S-G6) exhibited slightly higher activity per g of protein than KI-S but the differences were not statistically significant, while those made at 120 °C (KI-S-G7 to KI-S-G9) showed 22 % lower antiradical activity (Fig. 3A). In the Xyl-derived MRPs, the products obtained at 105 °C (KI-S-X4 to KI-S-X6) displayed significantly higher antiradical activity than KI-S, and than the products made at 120 °C (KI-S-X7 to KI-S-X9), with KI-S-X4 being the most active of all MRPs. However, all Xyl-derived MRPs exhibited a progressive decline in ABTS scavenging capacity with increasing reaction time (Fig. 3A). The results normalized per g of lyophilizate indicated that all MRPs had worse antiradical activity compared to KI-S (Fig. 3D).

Fe^{2+} chelating activity of the MRPs was 67–95 % lower than that of

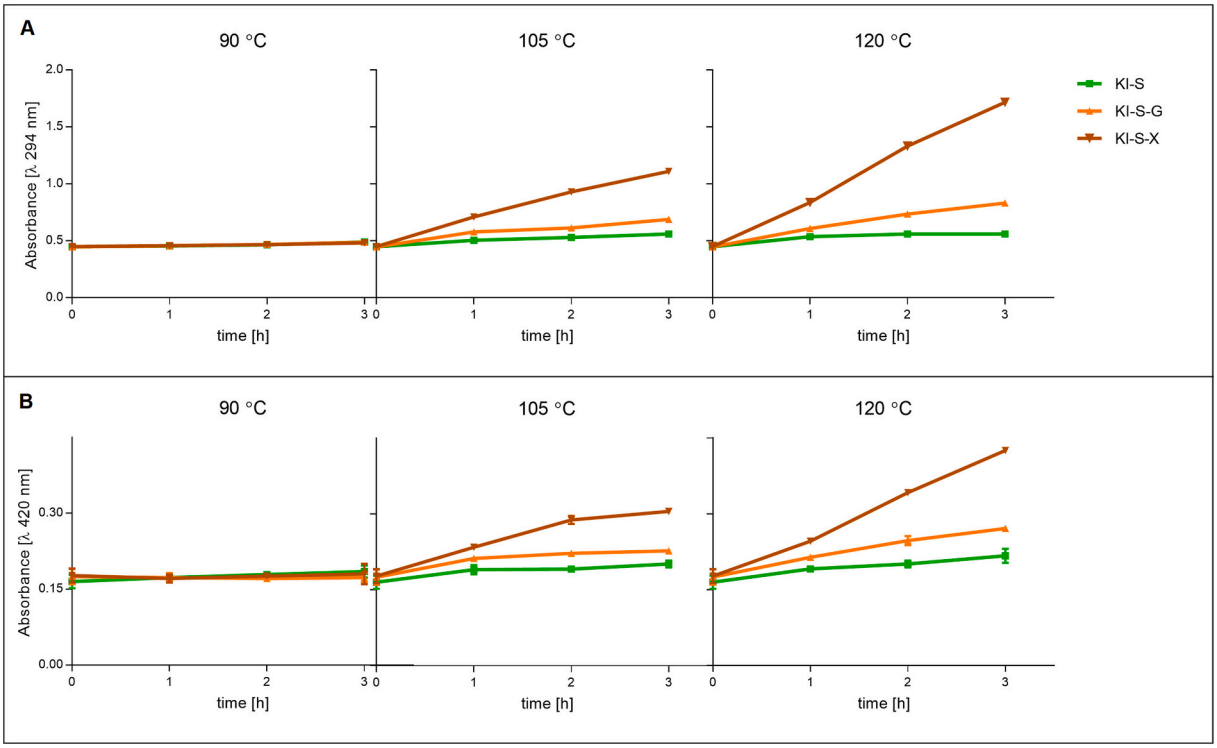

Fig. 7. Browning intensity of keratin isolate hydrolysate by subtilisin alone (KI-S), mixtures of KI-S with Glc (KI-S-G) and mixtures of KI-S with Xyl (KI-S-X) (see Table 1). Values reported are means ± SD of three replicates, obtained from pooled samples produced in three independent reactions (n = 3). The browning intensity was expressed as (**A**) A_{294} post 20-fold dilution and (**B**) A_{420} post 10-fold dilution.

KI-S and was influenced by the saccharide type, temperature and time used (Fig. 3B and E). Glc-derived MRPs (Fig. 3B) exhibited no consistent trend with respect to reaction temperature and time, while Xyl-derived MRPs demonstrated slightly higher chelating potential, with KI-S-X4 showed the highest chelating activity among MRPs, which decreased with increasing reaction temperature and time. The results normalized per g of lyophilizate indicated that all MRPs showed much lower values than KI-S (Fig. 3E).

FCR reducing assay of the MRPs (Fig. 3C and F) showed a different activity pattern compared to ABTS scavenging and Fe^{2+} chelating assays. Glc-derived MRPs exhibited lower activity per g of protein than Xyl-derived MPRs (Fig. 3C) but higher than that of KI-S. The MRPs obtained at 120 °C for 3 h (KI-S-G9 and KI-S-X9) showed the highest activity in both groups. The results normalized per g of lyophilizate indicated a similar increasing trend although the GAE values remained closer to those of KI-S (Fig. 3F).

A moderate positive correlation was observed between GAE values and Maillard reaction progression expressed as DG, A_{294} and A_{420} ($R^2 > 0.72$), whereas TE and EE showed no such trend ($R^2 < 0.20$).

3.2.4. Cytotoxicity and direct mutagenicity

Like KI and its hydrolysates, all tested MRPs were well tolerated by the Caco-2 cells, with none showing viability below 90 % relative to the control cells (Fig. 4B and C). No dose-dependent acute cytotoxicity trends were observed across the MRPs concentration or incubation time.

None of the MRPs demonstrated direct mutagenic activity in the Ames test using either *Salmonella Typhimurium* TA98 or TA100 strains (Fig. 5). Revertant counts ranged from 0 to 5 in TA98 and from 0 to 8 in TA100, with no dose-dependent patterns or significant differences relative to C- ($p > 0.05$).

Considering the absence of cytotoxicity and mutagenicity across all MRPs, their antioxidant activity guided the selection of a representative MRP for further evaluation following SGID. No MRP was deemed universally most active in all three assays. Therefore KI-S-X4, the most potent in ABTS scavenging and Fe^{2+} chelating, was chosen.

3.3. Characteristics of the keratin preparations post SGID

3.3.1. MW distribution and antiradical activity profiling

SGID resulted in a pronounced MW decrease in all keratin preparations, favouring the formation of smaller peptides (Fig. 2G). The MW fraction below 0.5 kDa increased substantially in all digests, especially in KI-S-X4-SGID, where it constituted almost 73 %. Concurrently, the fraction above 5 kDa declined to less than 10 % across all preparations, indicating effective proteolysis during SGID, regardless of prior keratin processing.

The altered MW distribution was reflected in antiradical activity profiles of the keratin preparations, as determined by HP-SEC with post-column ABTS derivatization (Fig. 2H). Initially, the antiradical activity was broadly distributed but shifted toward the MW fraction below 0.5 kDa post-digestion. The relative contribution of this fraction to antiradical activity increased progressively with each processing step, from 29 % in KI-SGID, 43 % in KI-S-SGID, up to 80 % in KI-S-X4-SGID.

3.3.2. Free amino acid profiles

SGID led to the release of substantial quantities of free amino acids from all keratin preparations (Table 2). The total content of free amino acids was the highest in KI-S-SGID (820.3 mM), followed by KI-SGID (754.4 mM), indicating that enzymatic pre-hydrolysis with subtilisin enhanced KI digestibility. KI-S-X4-SGID yielded a notably smaller total amount of free amino acids (519.9 mM), which can be attributed to the lower protein input in this preparation. While KI and KI-S contained 500 mg of keratin only, KI-S-X4 was a 500 mg keratin-Xyl mix, decreasing the content of protein available for digestion.

Among the free amino acids released, Arg (up to 137.1 mM), Leu (99.0 mM), Phe (92.2 mM) and Val (82.8 mM) were the most abundant across all keratin digests. No free Pro was detected in any SGID sample, indicating that Pro residues were retained within peptides. Asx, Cya,

Table 2
Free amino acid composition of keratin preparations post simulated gastrointestinal digestion (SGID). Values are based on a single chromatographic analysis of pooled samples produced in three independent reactions. KI-SGID – keratin isolate post-SGID, KI-S – KI hydrolysate by subtilisin post-SGID, KI-S-X4 – KI-S with Xyl heated at 105 °C for 1 h post-SGID, H_2O-SGID – SGID control with sample replaced by distilled water.

Amino acid	Free amino acid concentration [mM]			
	KI-SGID	KI-S-SGID	KI-S-X4-SGID	H_2O-SGID
Ala	41.0	57.0	37.7	27.6
Arg	117.0	137.1	60.9	46.9
Asx	8.8	11.1	10.4	17.1
Cys	14.3	13.5	5.7	6.9
Cya	6.5	6.8	5.7	4.5
Glx	24.9	29.3	26.3	35.4
Gly	40.5	50.0	40.0	57.4
His[a]	17.0	16.5	15.0	20.5
Ile[a]	36.2	30.0	21.1	15.3
Leu[a]	86.7	99.0	53.8	31.5
Lys[a]	40.2	45.5	33.2	58.6
Met[a]	9.9	8.1	5.0	5.7
Phe[a]	90.0	92.2	49.6	20.4
Pro	0.0	0.0	0.0	0.0
Ser	52.9	42.9	43.7	27.7
Thr[a]	37.8	41.6	33.3	7.1
Tyr	51.2	56.7	32.1	32.1
Val[a]	79.6	82.8	46.4	37.0
ΣEAA	397.3	415.8	257.4	196.2
ΣNEAA	357.0	404.5	262.6	255.7
ΣBCAA	202.5	211.8	121.3	83.8
TOTAL	754.4	820.3	519.9	451.9

ΣEAA, ΣNEAA, ΣBCAA – sum of essential, non-essential and branched chain amino acids, respectively.
[a] Essential amino acid.

Glx, Gly, His and Lys, were present at comparable levels in both keratin-containing digests and the H_2O-SGID control. This suggests their origin can lie in the digestive enzymes rather than the substrate. The pancreatin used was a crude suspension, poorly soluble in SIF and required vigorous vortexing to disperse. Likely, this contributed to partial autolysis of the enzymes prior to the gastric and intestinal digestion steps.

3.3.3. *Total antioxidant activity*

The total antioxidant capacity of keratin digests depended on prior processing. In the ABTS assay, KI-SGID displayed a 32 % increase in activity, while KI-S-SGID and KI-S-X4-SGID showed decreases of 16 % and 25 %, respectively, relative to their pre-digestion levels (Fig. 3A and D). Fe^{2+} chelating capacity improved significantly in KI-SGID and KI-S-X4-SGID, 6.5 and 8 times, respectively, while KI-S-SGID showed about a 10 % decrease (Fig. 3B and E). The FCR reducing potential rose by 41 % in KI-SGID and 54 % in KI-S-X4-SGID, but a decrease of 41 % was observed in KI-S-SGID (Fig. 3C and F).

3.3.4. *Cytotoxicity, direct and indirect mutagenicity*

The keratin preparations post-SGID were well tolerated by Caco-2 cells at all tested concentrations and incubation times (Fig. 4D), just like their undigested counterparts. Cell viability ranged from 87 to 124 %, indicating the absence of acute cytotoxicity. No dose- or time-dependency was observed.

The Ames test confirmed the absence of direct and indirect mutagenic activity in the keratin preparations, both before and after SGID (Fig. 5). In the direct assay, revertant counts for TA98 and TA100 remained low, did not differ significantly from C- ($p > 0.05$) and no dose-dependent trends were observed. In the indirect assay, performed in the presence of the metabolic activation system (S9 fraction), revertant counts for all keratin preparations also remained low, with no significant differences relative to C- regardless of strain used ($p > 0.05$). No dose-dependency was observed in either strain. The average counts for C- were 2.75 for TA98 and 4.38 for TA100. C+, i.e. 2-aminoanthracene at 0.55 μg/mL for TA98 and 1.25 μg/mL for TA100, induced strong mutagenic responses, with average revertant counts of 43.4 and 45.5, respectively, confirming the validity of the assay.

4. Discussion

The antioxidant activity assay of KI and its enzymatic hydrolysates revealed significant differences influenced by the degree of hydrolysis (DH) and MW. It is well-established that the strongest antioxidant activity in protein hydrolysates is attributed to low MW peptides, typically those between 3 and 6 amino acids in length (Zou et al., 2016). It has been determined by measuring the activity of peptides fractionated via ultrafiltration or chromatography (Tadesse & Emire, 2020). This study demonstrated that HP-SEC with post-column ABTS derivatization, a method that has not been previously used for protein hydrolysates, offers a fast and labour-saving alternative, enabling the simultaneous determination of peptide size distribution and antiradical activity profiling in a single analysis. Strong correlation between DH of KI and its hydrolysates and protein-normalized total antioxidant activity values (Fig. 3A, B and C) existed, as indicated by R^2 of 0.97, 0.91 and 0.83 for TE, EE and GAE values, respectively. However, HP-SEC analysis showed that the antiradical activity was affected by peptides across various MW fractions, not solely by the smallest peptides (Fig. 2B). It was particularly evident in KI-S, which revealed the highest DH of 36 % and the highest content of peptides below 0.5 kDa at 45 % (Fig. 2A) among all hydrolysates, but their contribution to antiradical activity was only 20 % (Fig. 2B). Thus, even despite extensive hydrolysis, larger peptides still played an important role in the antioxidant properties of the keratin hydrolysates.

To further enhance the antioxidant and sensory properties of the keratin hydrolysates, the Maillard reaction was carried out as an additional modification step. It was observed that the Maillard reaction between KI-S and saccharides occurred only at temperatures above 90 °C (Fig. 6 and Fig. 7). Chung et al. (2012) also reported no browning during heating of equimolar Glc-Gly solution at 90 °C, with significant increases in A_{420} observed only after 9 h. The occurrence of the Maillard reaction between KI-S and saccharides at 105 and 120 °C was supported by notable changes in DG (Fig. 6), browning intensity (Fig. 7), and antioxidant properties (Fig. 2 and Fig. 3). Xyl exhibited greater Maillard-reactivity than Glc, as evidenced by higher DG (Fig. 6) and browning intensity (Fig. 7) under the same temperature and time. Pentoses (Xyl) are generally more readily involved in the Maillard reaction than hexoses (Glc) due to their higher ratio of open-chain structures, which contain more reactive carbonyl groups, as illustrated in the scheme of early and intermediate reaction stages (Fig. 8). This facilitates Schiff base formation with amino acids, accelerating the Amadori rearrangement and promoting the formation of pigmented compounds (Mikami & Murata, 2015; Staroszczyk, 2023).

At alkaline conditions applied in this study (pH 9), the Maillard reaction was likely directed toward the formation of α-dicarbonyl compounds and reductones, which develop readily in this environment due to enhanced deprotonation of amino groups and accelerated Amadori rearrangement (Nooshkam et al., 2019; Staroszczyk, 2023). These pathways favour the generation of strong electron-donating and reducing compounds but also promote Strecker degradation, particularly when nucleophilic amino acids such as Cys, are present in abundance (Mikami & Murata, 2015). All keratin-derived MRPs developed a distinct meat-like aroma, which was progressively more pronounced in more intensely glycated samples. These observations agree with results reported for other Maillard systems involving Cys with Glc or Xyl, where heating generated volatile sulphur compounds such as 2-methyl-3-furanthiol, thiophenes, thiazoles and mercapto-ketones – key contributors to roasted and savoury flavours (Li & Liu, 2022; Theng et al., 2024). Such compounds are typically formed through Strecker degradation of Cys or via cyclization and rearrangement reactions of carbonyl-thiol

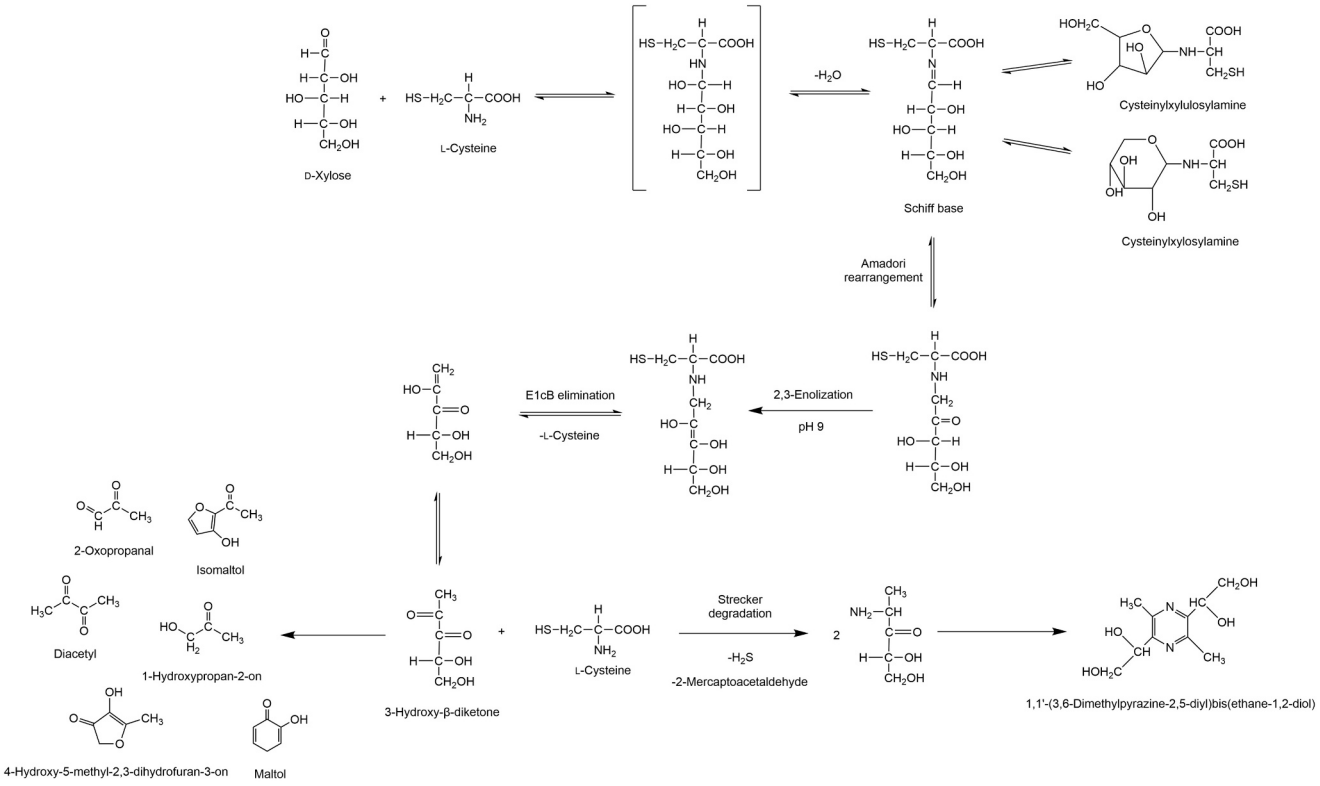

Fig. 8. Scheme of the early and intermediate stages of the Maillard reaction between L-Cys and D-Xyl under alkaline conditions. Chemical structures were drawn using ChemDraw® 20.1.1.

adducts under elevated temperatures and mildly alkaline conditions (Mikami & Murata, 2015). Lasekan (2013) identified potent meat-like aroma compounds: 3-methylbutanal, hexanal, 2,4-decadienal, γ-dodecalactone, 2-methyl-3-furanthiol, 2-ethyl-3,5-dimethylpyrazine, 3-hydroxy-4,5-dimethyl-2(5H)-furanone, bis(2-methyl-3-furyl)disulphide, 2-methylthiophene and 2-thiophenecarboxaldehyde in mixtures of alkaline-enzymatic hydrolysate of feather keratin with Glc heated at 155 °C for 2 h. Although this is the only study to date addressing keratin hydrolysate-derived MRPs, their antioxidant activity, toxicity and behaviour during SGID remain unexplored. Our prior study confirmed that Cys was among the most abundant amino acids in KI, comprising nearly 9 % of the total amino acid content (Taraszkiewicz et al., 2025), thereby serving as a plausible precursor for the formation of volatile sulphur compounds.

No major changes were observed in the HP-SEC chromatograms monitored at 215 nm after the Maillard reaction, likely due to the low MW of the saccharides used (Glc 0.18 kDa; Xyl 0.15 kDa). The minimal presence of high MW MRPs detected by HP-SEC (Fig. 2 C, D, E, F) suggests that melanoidin formation appeared negligible. Consisted with this, although A_{420} increased in all MRPs (Fig. 7B), this browning was not attributed to polymeric compounds. Whereas melanoidins are widely recognized for their contribution to A_{420}, several low MW MRPs, particularly oligomeric precursors or "pre-melanoidins" such as norfuraneol, methylglyoxal-norfuraneol adducts, diaminopyrrolones, 2-acetylpyrroles, furfural derivatives and imidazole-based chromophores also contribute to visible browning (Bork et al., 2023; Gribkova et al., 2023). According to Nooshkam et al. (2019), the MW of MRPs depends on thermal intensity, i.e. milder conditions (55–80 °C, up to a few hours) favour the formation of low MW compounds, while prolonged heating at higher temperatures (>100 °C, >24 h) promotes the development of high MW melanoidins. Numerous studies have shown that melanoidins formed for over 24 h mainly have MW above 10 kDa (Wang et al., 2011). Žilić et al. (2012) reported that both soluble and insoluble melanoidins can form during advanced stages of the Maillard reaction, with the ratio of insoluble to soluble MRPs increasing under severe heating (180 °C, 10 min). All KI-S-derived MRPs remained fully water-soluble, further indicating limited progression of the Maillard reaction under the conditions tested and minimal melanoidin formation.

The composition of MRPs generated under the experimental conditions was mirrored in their performance in the total antioxidant activity assays. ABTS scavenging activity either increased (in KI-S-X4, KI-S-X5, KI-S-X7), remained unchanged (in KI-S-X6, KI-S-X8, KI-S-G4, KI-S-G5, KI-S-G6), or decreased (in all other MRPs) compared to KI-S (Fig. 3A). Fe^{2+} chelating capacity was drastically diminished in the MRPs (Fig. 3B). The FCR reducing activity was strongly correlated with the severity of the thermal treatment, with the highest values observed in KI-S-G9 and KI-S-X9 (Fig. 3C). To the best of our knowledge, no biological activities of keratin-derived MRPs have been investigated so far. The results obtained suggest that the Maillard reaction predominantly generated low MW, electron-donating compounds (such as reductones and α-dicarbonyl intermediates), which enhanced radical scavenging and reducing properties, without significantly improving metal chelation. Kanzler et al. (2016) established that Maillard intermediates, including kojic acid, maltol, furaneol, norfuraneol and ethylfuraneol, have potent ABTS scavenging and FCR reducing properties, similarly to MRPs derived from KI-S. According to Nooshkam et al. (2019), the radical chain-breaking activity via hydrogen atom donation has also been identified as a potential antioxidant mechanism of intermediate Maillard reaction reductones. Although the specific effects of the Maillard reaction on the Fe^{2+} chelating properties of protein hydrolysates remain poorly understood, the pronounced activity loss observed in the keratin-derived MRPs (67–95 % vs KI-S) is consistent with broader findings on melanoidins. It has been established that efficient Fe^{2+} binding generally requires high MW melanoidins (above 50 kDa), rich in

metal-coordinating groups, specifically, hydroxyl and carbonyl in pyridone and pyranone rings (Gu et al., 2009, 2010; Nooshkam et al., 2019). Although low MW Maillard intermediates also contribute to chelation through π-conjugated system (Bork et al., 2023), their activity is weaker compared to high MW compounds (Gu et al., 2009, 2010). Additionally, amino acid residues responsible for metal-binding properties of peptides, particularly His, Cys, Asp and Glu (Du et al., 2022; Jiang et al., 2022), could have undergone Maillard-driven structural alterations that reduced their affinity for Fe^{2+} chelation, thus diminishing the binding effect observed. Overall, the results of antioxidant assays suggest that the activity of KI-S-derived MRPs resulted mainly from soluble, early-stage low MW intermediates rather than high MW polymers, highlighting their suitability as versatile ingredients in health-promoting food and supplement products.

Three keratin preparations, representive for the subsequent processing stages, i.e., KI, KI-S and KI-S-X4, were subjected to the INFOGEST 2.0 protocol (Brodkorb et al., 2019) to assess their digestibility, functional stability, and safety in conditions simulating human digestion. SGID led to significant keratin proteolysis, yielding completely soluble digests, with resulting peptides below 1 kDa constituting 68–84 % (Fig. 2G). HP-SEC chromatograms registered at 215 nm indicated that KI-SGID had higher content of compounds below 0.5 kDa than KI-S-SGID (Fig. 2G). However, the total content of free amino acids was higher in KI-S-SGID than KI-SGID (Table 2). This discrepancy could have resulted from much lower UV absorptivity of free amino acids than peptides (Johns et al., 2011) as well as the limitations of quantification below the fractionation range of the HP-SEC column. The chromatograms registered at 734 nm (Fig. 2H) and the results of total antioxidant activity (Fig. 3) illustrated the complementary impact of the three processing stages of KI. Ultimately, the strongest antioxidant effect post-SGID was achieved through the combined application of hydrolysis with subtilisin and mild Maillard reaction with Xyl (KI-S-X4), resulting in a preparation abundant in highly active low MW compounds. The potential of processing strategies to improve both bioaccessibility and bioactivity of keratin was thus confirmed.

The most abundant free amino acids released from the keratin digests were Arg, Gly, Val, Phe and Tyr (Table 2). They are well represented in the KI amino acid profile, with respective content of 6.4, 7.0, 6.5, 4.7 and 1.6 g/100 g of lyophilizate (Taraszkiewicz et al., 2025). These amino acids are known for their contribution to antioxidant defences and high nutritional value. Their high concentration confirms high digestibility of the keratin preparations in vitro. No free Pro was detected in all SGID samples, despite the fact that Pro was present at 10.1 g/100 g of lyophilized KI (Taraszkiewicz et al., 2025). Indeed, hydrolysis with gastric (KI-P), intestinal (KI-T and KI-C) or microbial (KI-S) proteases individually also could not release free Pro from KI (Taraszkiewicz et al., 2025). This implies that regardless of prior KI processing, bioaccessible Pro-containing peptides were produced at the digestion endpoint. Structure-activity relationship studies highlight the importance of Pro residues in enhancing the antioxidant capacity of peptides, as well as their ability to inhibit key regulatory enzymes, i.e. angiotensin-converting enzyme, dipeptidyl peptidase IV and prolyl oligopeptidase, which are implicated in the pathophysiology of hypertension, diabetes and neurodegenerative disorders, respectively (Iwaniak et al., 2018; Sinkiewicz et al., 2018; Taraszkiewicz et al., 2024). These findings indicate that the keratin preparations may provide valuable peptides with both nutritional and potential health-promoting activities after digestion, aligning with the aim of developing biofunctional food ingredients.

All keratin preparations obtained, both before and after SGID, were well tolerated by the Caco-2 cells and exhibited neither direct nor indirect mutagenicity in the Ames assay. According to the producer of the MPF Ames test, the use of both TA98 and TA100 strains provides a comprehensive safety assessment, enabling the detection of distinct mutagenic mechanisms, i.e. frameshift mutations and base-pair substitutions, respectively. These preliminary results suggest that the keratin processing methods used yielded protein preparations safe for human consumption. Based on our prior analysis performed with the ToxinPred tool, the peptides in silico released from chicken feather keratin by pepsin, trypsin, chymotrypsin and subtilisin were also predicted to be non-toxic (Taraszkiewicz et al., 2022). The conditions of the Maillard reaction we applied were specifically chosen to avoid production of the most potent Maillard mutagens, i.e. acrylamide, furfural and hydroxymethylfurfural (Staroszczyk, 2023). The former is produced in temperatures >120 °C from free Asn (Staroszczyk, 2023), which was absent in KI-S (Taraszkiewicz et al., 2025), while the latter two are formed at pH ≤ 7 (Staroszczyk, 2023). According to Augustine and Bent (2022), thiol compounds, such as Cys, compete with Asn for α-hydroxycarbonyl compounds, thus impeding the major pathway for acrylamide formation. Altogether, these findings support the overall safety of keratin-derived preparations and support their potential suitability as novel ingredients for food and nutraceutical applications.

5. Conclusions

The feather keratin derived from L-Cys reduction can be effectively processed through enzymatic hydrolysis with pepsin, trypsin, chymotrypsin and subtilisin, and the mild Maillard reaction with Glc and Xyl to produce digestible, antioxidant-rich protein preparations. This three-step feather processing enhanced the bioactivity and maintained the safety of keratin-derived peptides, as the in vitro cytotoxicity and mutagenicity tests indicated. Carefully controlled Maillard reaction conditions avoided the formation of high MW polymers, preserving solubility. SGID of keratin preparations confirmed their high bioaccessibility and capacity to release valuable free amino acids and bioactive Pro-containing peptides. These findings support the use of feather keratin as a sustainable resource of functional food ingredients or nutraceutical compounds. Future investigations should focus on identifying individual peptides and MRPs, exploring the effects on gut health and assessing bioavailability.

CRediT authorship contribution statement

Antoni Taraszkiewicz: Writing – original draft, Visualization, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Agata Sommer:** Writing – review & editing, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation. **Izabela Sinkiewicz:** Writing – review & editing, Resources, Methodology, Formal analysis, Data curation. **Izabela Koss-Mikołajczyk:** Visualization, Methodology. **Barbara Kusznierewicz:** Methodology. **Linda Giblin:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Formal analysis. **Hanna Staroszczyk:** Writing – review & editing, Supervision, Methodology, Formal analysis.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

The data that support the findings of this study are openly available in Bridge of Knowledge at https://doi.org/10.34808/hd66-cp40. Dataset Part III 21.05.2025 (Original data) (mostwiedzy)

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REVIEW

The biological role of prolyl oligopeptidase and the procognitive potential of its peptidic inhibitors from food proteins

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ABSTRACT

Prolyl oligopeptidase (POP) is a conserved serine protease belonging to proline-specific peptidases. It has both enzymatic and non-enzymatic activity and is involved in numerous biological processes in the human body, playing a role in e.g., cellular growth and differentiation, inflammation, as well as the development of some neurodegenerative and neuropsychiatric disorders. This article describes the physiological and pathological aspects of POP activity and the state-of-art of its peptidic inhibitors originating from food proteins, with a particular focus on their potential as cognition-enhancing agents. Although some milk, meat, fish, and plant protein-derived peptides have the potential to be applied as natural, procognitive nutraceuticals, their effectiveness requires further evaluation, especially in clinical trials. We demonstrated that the important features of the most promising POP-inhibiting peptides are very short sequence, high content of hydrophobic amino acids, and usually the presence of proline residue.

KEYWORDS

bioactive peptides; food proteins; procognitive peptides; prolyl oligopeptidase; prolyl oligopeptidase inhibitors; structure-activity relationship

Introduction

The progress in medicine has significantly improved life span and quality, but at the same time, it has resulted in the aging of the global society. It is estimated that by 2050 the number of people older than 60 will surpass the population of younger generations, whereas the number of people over 80 will triple – reaching 380 million (Harper 2014). Consequently, the number of individuals suffering from age-related cognitive disorders increases. While over 46.8 million people were affected by dementia in 2015, this number is projected to increase to 131.5 million by 2050 (Prince et al. 2015). No therapy is known to cure the disease. The only clinically available medications for Alzheimer's disease (AD), the most common cause of dementia, are three acetylcholinesterase (EC 3.1.1.7) inhibitors, namely donepezil, rivastigmine, and galantamine, and the N-methyl-D-aspartic acid (NMDA) receptor antagonist memantine. These drugs improve neurotransmission, but they bring only a slight alleviation from the symptoms of AD, and only in some patients (Benek, Korabecny, and Soukup 2020). Therefore, the discovery of appropriate strategies of the cognitive disorders' prevention and treatment is becoming of crucial importance.

Diet has a direct impact on one's cognitive performance (McEvoy et al. 2019; Klimova, Dziuba, and Cierniak-Emerych 2020). Vitamin deficiencies, primarily A, E, C, B, and folates, high consumption of saccharides, and saturated fatty acids are associated with intellectual decline and increased susceptibility to neurological disorders. On the other hand, a diet rich in polyunsaturated fatty acids, especially of n-3 family, polyphenols, and other antioxidants improves cognition and lowers the risk on mental impairments (Beilharz, Maniam, and Morris 2015; Phillips 2017). A change in nutritional habits is often considered to be easier and safer than pharmacotherapy.

Dietary proteins have traditionally been viewed as a source of amino acids. However, reports from a few recent decades indicate that a complementary criterion, allowing a more complete view of the proteins' biological value should also take into account their usefulness as a source of bioactive peptides (Barati et al. 2020). These protein fragments, usually 2-30 amino acid residues in length, are released from protein precursors e.g. during food digestion in the gastrointestinal (GI) tract, fermentation by proteolytic microorganisms, and enzymatic or chemical hydrolyzis. They can also be obtained by chemical synthesis or through the expression of corresponding genes (Zambrowicz et al. 2013; Iwaniak, Darewicz, and Minkiewicz 2018). Biopeptides not only serve as nutrients but can also exert drug-like properties. The most extensively studied food-derived bioactive peptides are angiotensin-converting enzyme (ACE; EC 3.4.15.1) inhibitory, dipeptidyl peptidase IV (DPP IV; EC 3.4.14.5) inhibitory, and antioxidative peptides. Some of the biopeptides, which efficiency and safety were confirmed in human studies, are applied as active ingredients in functional foods. Examples of commercial products containing bioactive peptides include: Calpis® (Calpis Co. Ltd., Japan), Evolus® (Valio Ltd., Finland), BioZate® (Davisco Foods, USA), and

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Lapis Support (Tokiwa Yakuhin Co. Ltd., Japan) (Iwaniak, Darewicz, and Minkiewicz 2018).

Conventionally, the effects of dietary proteins and peptides on the functions of the central nervous system (CNS) have mainly been attributed to their role as a source of amino acids, acting as precursors of important neurotransmitters, including serotonin, dopamine, epinephrine, and norepinephrine (Van De Rest, Van Der Zwaluw, and De Groot 2013). In recent years though, an increasing number of the whole peptide sequences with cognition-affecting activity have been discovered. Examples include opioid peptides that exert diverse regulatory functions associated with emotion and memory (Z. Liu and Udenigwe 2019), and various neuroprotective peptides, such as acetylcholinesterase inhibitory, antioxidative, anti-inflammatory and immunomodulatory (Lemieszewska et al. 2016; S. Y. Lee and Hur 2019). The cognition-enhancing effects can also be exhibited by peptides showing prolyl oligopeptidase (POP) inhibitory properties, last reviewed over 11 years ago (Wilson, Hayes, and Carney 2011). The purpose of this study is to describe the physiological and pathological aspects of POP activity in the human body and the potential of its peptidic inhibitors derived from food proteins with a focus on their procognitive properties.

Prolyl oligopeptidase and its role in human pathophysiology

Classification, occurrence and structure

Prolyl oligopeptidase (EC 3.4.21.26) also known as prolyl endopeptidase, proline endopeptidase, proline specific endopeptidase, post-proline endopeptidase, endoprolylpeptidase, and post-proline cleaving enzyme is a conserved serine protease belonging to proline-specific peptidases (PSP). PSPs are the only proteolytic enzymes capable of catalyzing the hydrolyzis of peptide bonds formed by Pro residues, except for a few nonspecific metallopeptidases that can hydrolyze such bonds, providing that they are present at peptides' N-terminus (Dunaevsky et al. 2020). Most of the known POPs have demonstrated the ability to hydrolyze only short peptides, up to approx. 30 amino acid residues in length, hence the name prolyl oligopeptidase (Svarcbahs et al. 2019).

POP was first detected in the human uterus as a protease responsible for oxytocin degradation via cleavage of Pro-Leu peptide bond (Walter et al. 1971). It is a soluble, cytoplasmic enzyme, but under inflammatory conditions it can also be secreted into the extracellular matrix (Natunen et al. 2019). The human POP is distributed broadly in numerous body parts including kidneys, thymus, testis, muscles, heart, and the CNS (Dunaevsky et al. 2020). In the brain, the majority of POP expression and activity has been detected in neurons of the brain cortex and nigrostriatal system, particularly in substantia nigra, caudate nucleus, and pallidum (Irazusta et al. 2002; Myöhänen et al. 2007). The immunoreactive POP protein, POP mRNA, and POP activity levels are not always correlated, most likely due to the presence of endogenous POP inhibitors, and other PSPs in the examined tissues, exhibiting similar specificity while being resistant to POP specific inhibitors (Bracke et al. 2019; Svarcbahs et al. 2019). The difficulty in confirming the data on POP distribution is additionally influenced by the unknowingness of its specific antibodies (Svarcbahs et al. 2019) and the fact that studies on this protease were performed using various methods, under different conditions (Dunaevsky et al. 2020).

The human POP is composed of 710 amino acid residues, with a molecular weight of approximately 80 kDa, almost three times higher than classical serine proteases (trypsin and chymotrypsin). This enzyme is a cylindrically shaped, monomeric protein, consisting of two domains. The first is catalytic, with an α/β hydrolase fold, and the second, unique β-propeller, is composed of a seven-fold repeat of four-stranded β sheets. The active center resides at the interface of the domains, in the cavity gorge. The order of amino acid residues forming the catalytic triad is unusual. In classical serine proteases it is Asp-His-Ser or His-Asp-Ser, while in POP it is Ser-Asp-His (Dunaevsky et al. 2020). Another unique feature of POP is a system of flexible loops that is vital for its enzymatic activity. The system is comprised of a loop of the noncatalytic domain (called loop A), a loop of the catalytic domain (called loop B), and other catalytically important loops. The system controls the proteolysis via motion and rearrangement of the loop structure. It regulates ligand entry and binding to the active site, thus influencing the enzyme's specificity, and is stabilized by inhibitor binding. It has also been suggested to be a promising target for inhibitor design (Szeltner et al. 2013; Tsirigotaki et al. 2017). The 3 D structure of human POP, accessed from the UniProt database (The UniProt Consortium 2019), is presented in Figure 1.

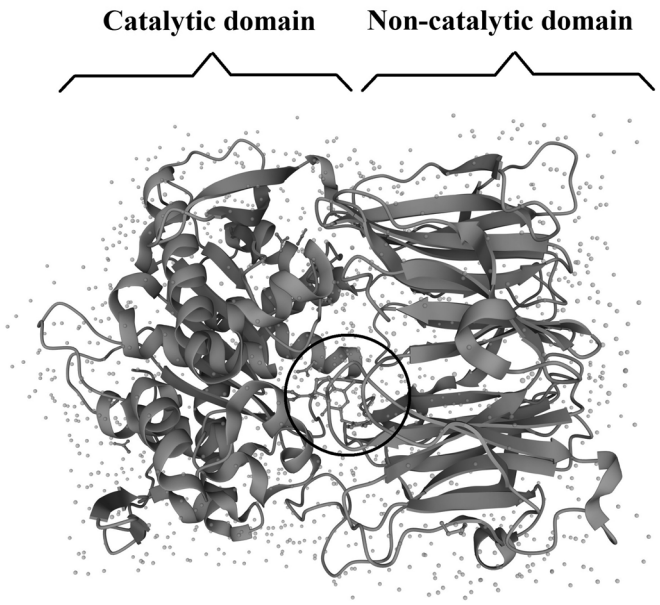

Figure 1. The 3 D structure of human POP with the inhibitor (6 s)-1-chloro-3-[(4-fluorobenzyl)oxy]-6-(pyrrolidin-1-ylcarbonyl)pyrrolo[1,2-a]pyrazin-4(6h)-one bound at the active stie (circled) [the UniProt database (accession date: 22.12.2022)].

Enzymatic activity

Most PSPs are classified as exopeptidases, with POP and fibroblast activation protein being the only exceptions, exhibiting endopeptidase activity (Dunaevsky et al. 2020). POP breaks the peptide bonds at the carboxyl side of Pro, with a recognition sequence of X-Pro-Y, where X is a peptide or protected amino acid, and Y is a peptide, an amino acid, an amide, an alcohol, or an aromatic amine (Walter, Simmons, and Yoshimoto 1980). Early studies on the specificity of this enzyme have suggested that the noncatalytic domain acts as a gate to the active site that limits the access of substrates larger than approx. 30 amino acids. However, a few POPs capable of hydrolyzing longer peptides, as well as intact proteins, have been reported (Kang, Yu, and Xu 2014). Therefore, the major factor influencing the POP specificity is substrate's accessibility to the enzyme's active site, rather than its chain length.

POP is responsible for the maturation and degradation of short, Pro-containing neuropeptides and hormones, including substance P, thymosin β4, thyrotropin-releasing hormone, and arginine-vasopressin that are known to be important modulators of cognitive processes. POP activity is also associated with several neurological and mental disorders. This is why, despite the ubiquitous localization of POP in the body, the enzyme found in the CNS is the most important therapeutic target (García-Horsman, Männistö, and Venäläinen 2007). POP also participates in angiotensin processing, therefore it is a member of the renin-angiotensin-aldosterone system – one of the major physiological mechanisms for the maintenance of water-electrolyte homeostasis and regulation of blood pressure (Serfozo et al. 2020). The hydrolytic activity of POP is measured using various chromogenic and fluorogenic substrates such as Z-Gly-Pro-Leu-Ala (Yoshimoto et al. 1988), Z-Gly-Pro-Leu-Gly-Pro (Sattar et al. 1990), or Z-Gly-Pro-4-nitroanilide (Kang, Yu, and Xu 2014).

Non-enzymatic activity

Although customarily the main biological function of POP had been considered to be related to its hydrolytic properties, investigations have revealed that peptide hydrolyzis alone cannot fully explain the enzyme's role in the processes in which it was relevant. Therefore, recent studies have paid more attention to the enzyme's non-catalytic activity resulting from POP's involvement in direct, physiologically important protein-protein interactions (PPIs) (Männistö and García-Horsman 2017). The most important of POP's binding partners include α-tubulin, neurospecific growth-associated protein-43 (GAP-43), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and α-synuclein (αS) (Dunaevsky et al. 2020). The strength of the PPIs depends on the protease's conformation, which in turn is influenced by the presence of its ligand. It was also speculated that POP enzyme-substrate complex might require the PPIs to form an enzymatically active tertiary complex (Männistö and García-Horsman 2017). However, the protease's conformational dynamics and its relevance in the enzyme's role are not yet fully elucidated, as even catalytically inactive POP mutants were reported to maintain some of its' regulatory functions (Svarcbahs et al. 2019). More details on the significance of these PPIs with particular proteins is described in the following sections.

Activity in cellular growth

POP is engaged in age-dependent processes. Studies on rodents demonstrated that POP mRNA levels are the highest during perinatal growth and at birth, then progressively decline as the animals age (Agirregoitia et al. 2010), to greatly increase again in old age (C. H. Jiang et al. 2001). The rise of POP levels in early life stages is likely due to its involvement in proliferation and differentiation of various types of cells, including liver (Matsubara et al. 1998), ovary (Kimura et al. 1998), testis (Kimura, Matsui, and Takahashi 2002) and neurons (Moreno-Baylach et al. 2008; Hannula, Männistö, and Myöhänen 2011). According to Höfling et al. (2016), the mice with the POP gene knocked out manifested alterations in brain development including reduced volume of the brain cortex and disruption in synaptic plasticity. They also demonstrated increased expression of some neuropeptides receptors and polysialylated-neural cell adhesion molecule, an important mediator of neuroplasticity, while exhibiting reduced anxiety but increased activity similar to attention deficit hyperactivity disorder (ADHD). D'Agostino et al. (2013) reported that the POP- gene-trapped mice exhibited a decrease in hippocampal synaptic spine density and long-term potentiation, impairment in hippocampal-mediated learning, and reduced levels of GAP-43 that is involved in axon guidance and growth cone formation. Di Daniel et al. (2009) described the ability of POP to form PPI with GAP-43, independently of the enzyme's catalytic activity, and the possibility of fixing the alternations in growth cone dynamics of POP knock-out cells via transfection of either wild type or catalytically inactive POP mutant.

Role in inflammation

POP is linked to inflammatory processes. Along with other proteases capable of hydrolyzis of bigger proteins it generates immunoactive peptides: N-acetyl-Pro-Gly-Pro from endogenous collagen and N-acetyl-Ser-Asp-Lys-Pro from thymosin β4, both exerting anti-inflammatory properties and acting as markers of inflammation. The level of the former peptide was correlated with POP levels in murine model of inflammatory pulmonary diseases. Additionally, POP levels were increased in the brain of mice after inflammatory insult (Penttinen et al. 2011). The POP-deficient mice have shown no inflammatory response to lipopolysaccharide treatment, unlike wild-type animals (Höfling et al. 2016), and the POP gene-disrupted mice exhibited decreased intensity of inflammatory responses to a high-fat diet (D.-X. Jiang et al. 2020). Furthermore, Roda et al. (2014) suggested that the model of valproic acid (VPA) interaction with POP indicates a promising possibility of using POP inhibitors as therapeutics in inflammatory disorders.

Significance in neuropsychiatric disorders

The POP plasma activity level is altered in people affected by neuropsychiatric disorders. It is reduced in individuals suffering from anorexia, bulimia nervosa (Maes et al. 2001), and depression, but increased in those with mania, schizophrenia (Maes et al. 1995), and post-traumatic stress disorder (Maes et al. 1999). Significant alterations of POP plasma activity, that is higher mean and standard deviation, were observed in children with autistic spectrum disorders as compared to the non-autistic group (Momeni et al. 2005).

García-Horsman, Männistö, and Venäläinen (2007) described two theories relating the POP activity to neuropsychiatric disorders. The first is connected to several behavior-modulating neuropeptides that are involved in the pathophysiology of depression, i.e. thyrotropin-releasing hormone and arginine-vasopressin, which levels are modified by altered POP activity. The second is related to POP's association with the metabolism of inositol 1,4,5-triphosphate (IP3), a vital cellular second messenger in the cascade of neuropeptide signaling. Schulz et al. (2002) reported that the level of this protease's expression was inversely correlated to IP3 concentration and POP inhibition enhanced substance P-mediated stimulation of IP3 production. Interactions of POP with other enzymes controlling IP3 level were also described by other authors (Williams et al. 1999; Harwood 2011).

POP inhibition was shown to reverse the effect of three traditional mood-stabilizing drugs: VPA, lithium, and carbamazepine, all exerting IP3 depleting activity (Williams et al. 2002). Concurrently, VPA was demonstrated to be a POP inhibitor itself. It might explain VPA's ability to treat both mania and depression, assuming that steady mood is affected by stable IP3 signaling – which can be both reduced by VPA treatment due to its IP3 diminishing activity and elevated via the drug's POP inhibiting properties (Cheng et al. 2005). This theory is consistent with the previous study, which revealed that antidepressants and VPA restore plasma POP activity to near control levels in depressed and manic patients, respectively (Maes et al. 1995).

Relevance in neurodegeneration

POP is involved in several neurodegenerative disorders, including dementia with Lewy bodies, AD, Parkinson's disease, Huntington disease, and multiple sclerosis (García-Horsman, Männistö, and Venäläinen 2007; Svarcbahs et al. 2019; Trallero et al. 2019). Although the precise etiology of these pathologies is unknown, common to them is chronic oxidative stress (Singh et al. 2019) and accumulation of insoluble aggregates of misfolded proteins in neurons, ultimately resulting in their death (Gandhi et al. 2019).

In *post mortem* brains of patients with AD and Parkinson's disease, POP strongly colocalized with three proteins susceptible to the pathological misfolding and directly involved in the neurodegenerative diseases, i.e. αS, β-amyloid (βA), and τ-protein (Hannula et al. 2013). These proteins often co-occur, accelerate each other's aggregation and share several protein interactors (Irwin and Hurtig 2018; Yan, Uronen, and Huttunen 2020).

POP was suggested to be one of γ-secretases, i.e. enzymes responsible for the generation of neurotoxic peptides from βA, as POP inhibition reduced the generation of βA in neuroblastoma cells and hindered βA-like deposition in the brain of mice used as a model of accelerated senescence (Barelli et al. 1999).

Moreover, POP lack or inhibition resulted in a decrease in reactive oxygen species (ROS) production. Puttonen et al. (2006) demonstrated that POP inhibitors are able to prevent some neurotoxin-induced cell stress-related factors, such as ROS production and nuclear translocation of GAPDH which was suggested to be a proapoptotic mediator and intracellular sensor of oxidative stress. According to Dokleja, Hannula, and Myöhänen (2014), POP inhibition improve the viability of SH-SY5Y human neuroblastoma cells by decreasing ROS production via the reduction of αS aggregate formation. Svarcbahs et al. (2018) used POP knock-out cells and mice to show that POP enhances αS-mediated toxicity both in vitro and in vivo, as well as impairs the ability of proteasomal systems to degrade it.

Role in autophagy and cancer

POP intensifies aggregation of αS via PPI and negatively regulates its autophagy, one of the major catabolic mechanisms of dysfunctional component removal. These processes are dampened by POP inhibitors, although this dampening effect's intensity for particular inhibitors is not correlated with their inhibitory potency, i.e. IC_{50} values (half-maximal POP inhibitory concentrations) (Kilpeläinen et al. 2020). POP regulates autophagy via negative regulation of protein phosphatase 2 A, a regulator of cell cycle and growth, which reduced levels lead to aggregation of αS and τ-protein hyperphosphorylation resulting in AD, and disturbances in cell proliferation, inducing carcinogenesis (Svarcbahs et al. 2020).

POP protein and activity levels are elevated in tumors. In several cancer cell lines, i.e. neuroblastoma, gastric cancer, and human breast cancer, in which protein phosphatase 2 A activity alterations are seen, POP inhibition resulted in a reduction of the cell proliferation rate. The mechanism of anticancer activity is not precisely examined, although it is known that G_0/G_1 arrest is involved (Sakaguchi et al. 2011; Suzuki et al. 2014; S. Tanaka, Kanayo, and Sakaguchi 2017).

POP inhibitors from food proteins

Several synthetic POP inhibitors have been developed, potent and selective, with IC_{50} values reaching the nanomolar range. Some of them have been experimentally proven to be efficacious cognition-enhancing agents, even in clinical trials. However, none has entered the pharmaceutical market so far, due to insufficient information on their pharmacokinetics, pharmacodynamics, bioavailability, toxicity, and incomplete understanding of the POP's physiological role and its association with pathological conditions (Babkova et al. 2017; García-Horsman 2020). The majority of strong POP

inhibitors are low molecular weight, substrate-like peptidic molecules based on N-acyl-L-prolyl-pyrrolidine scaffold. Usually, the P1 site is a pyrrolidine ring with an electrophile in its 2S-position, the P2 site is an α-aminoacyl group, and the P3 site is a hydrophobic acyl group (Kilpeläinen et al. 2020). The first discovered POP inhibitor was N-benzyloxycarbonyl-L-prolyl-L-prolinal (i.e. Z-pro-prolinal) (Wilk and Orlowski 1983). Some peptides derived from food proteins also exhibit POP inhibitory activity (Wilson, Hayes, and Carney 2011). Sequences of 63 such peptides are deposited in the BIOPEP-UWM database (accession date: 11.01.2023), each containing at least one Pro residue. The full list of these peptides is available in the database when searching for compounds with "antiamnestic" activity (Minkiewicz, Iwaniak, and Darewicz 2019).

Milk protein-derived POP inhibitors

Among the food proteins, the proteins of milk have been the most widely studied as precursors of bioactive peptides. POP-inhibiting peptides have been detected in milk or milk products obtained from various mammalian species, including cows, sheep, and humans. Examples of milk protein-derived peptides with POP inhibitory properties, and other bioactivities of peptides with the same sequence (accessed from the BIOPEP-UWM database on 10.01.2023) are shown in Table 1.

In silico studies

A theoretical study based on BIOPEP-UWM revealed that bromelain and papain may release peptides containing POP inhibiting motifs from bovine α_{S1}- and β-casein (Iwaniak and Taraszkiewicz 2022).

In vitro studies

The first evidence of milk protein-derived POP inhibitors was provided by Asano, Nio, and Ariyoshi (1991) and Asano, Nio, and Ariyoshi (1992) who synthesized various Pro-containing peptides and peptide derivatives sharing sequence homology with human, bovine, buffalo, and ovine β-caseins. Their POP repressive properties were sequence-dependent. The majority of the most effective peptides contained hydrophobic amino acid residues, giving them easy access to the enzyme's active site. Pripp (2006) used the data about the sequences and the IC_{50} values of these peptides to create a quantitative structure-activity relationship model. It demonstrated that hydrophobicity and molecular bulkiness of amino acids in positions P3, P2, and P1' of inhibitory sites of the peptides were positively correlated with their POP inhibitory potency. Several of these peptides were released during simulated GI digestion of both raw and pasteurized human milk (Wada and Lönnerdal 2015).

Sørensen et al. (2004) found potent POP inhibitory peptides in water-soluble extracts from Cheddar, Norvegia, Jarlsberg, and Blue cheeses. These peptides were characterized by molecular weight in the range of 1 to 3.5 kDa and diverse hydrophobicity. Similarly, Srinivas and Prakash (2010) reported that bovine α-casein hydrolyzate by chymotrypsin expressed POP inhibitory activity with IC_{50} at 1.3 mg/mL. According to Öztürk and Akın (2021), several POP inhibiting peptides were released during 180 days of Tulum cheese ripening.

Hsieh et al. (2016) used thermolysin, bromelain, and the GI enzymes to hydrolyze sodium caseinate. All of the obtained hydrolyzates exhibited POP repressing properties. The most potent activity was shown by hydrolyzate prepared by bromelain. Nine oligopeptidic α-, β-, and κ-casein-derived competitive POP inhibitors were identified (see Table 1).

It used to be believed that Pro residue is necessary for POP inhibition, therefore the majority of studies aiming to discover them were focused on Pro-rich proteins or peptides. Sistla (2013) demonstrated that Pro-free peptides Gln-Lys-Ala-Leu-Asn-Glu-Ile-Asn-Gln-Phe and Thr-Lys-Lys-Thr-Lys-Leu-Thr-Glu-Glu-Glu-Lys-Asn-Arg-Leu from α_s-casein also exhibit POP inhibitory activity (see Table 1).

The first study to reveal the ability of casein-derived peptides to inhibit POP from human cells was reported by Juillerat-Jeanneret, Robert, and Juillerat (2011). The authors established that bovine milk caseins fermented by lactic acid

Table 1. Milk protein-derived peptides exhibiting POP inhibitory activity in vitro.

Source	Peptide sequence	IC_{50} [μM]	Other bioactivities	References
human β-casein	Ile-Tyr-Pro-Phe-Val-Glu-Pro-Ile	8	n. a.	(Asano, Nio, and Ariyoshi 1991)
bovine α_{S1}-casein	Phe-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe-Gly	60	n. a.	(Juillerat-Jeanneret, Robert, and Juillerat 2011)
bovine α_{S2}-casein	Gln-Lys-Ala-Leu-Asn-Glu-Ile-Asn-Gln-Phe	0.0058	n. a.	(Sistla 2013)
bovine α_{S2}-casein	Thr-Lys-Lys-Thr-Lys-Leu-Thr-Glu-Glu-Glu-Lys-Asn-Arg-Leu	0.036	n. a.	(Sistla 2013)
bovine β-casein	Asn-Leu-His-Leu-Pro-Leu-Pro-Leu-Leu	150	anticancer	(Juillerat-Jeanneret, Robert, and Juillerat 2011)
bovine β-casein	Leu-Pro-Pro	875	ACE inhibitor	(Siltari et al. 2012; Martin and Deussen 2019)
bovine β-casein	Val-Pro-Pro	761	ACE inhibitor, anti-inflammatory	(Siltari et al. 2012; Chakrabarti and Wu 2015; Martin and Deussen 2019)
bovine κ-casein	Ile-Pro-Pro	486	ACE inhibitor, anti-inflammatory, α-amylase inhibitor, α-glucosidase inhibitor	(Siltari et al. 2012; Chakrabarti and Wu 2015; Martin and Deussen 2019; Martini et al. 2021)
bovine sodium caseinate	Pro-Ile-His-Asn-Ser-Leu-Pro-Gln-Asn-Ile-Pro-Pro-Leu-Thr-Gln-Thr-Pro-Val	29.8	n. a.	(Hsieh et al. 2016)
bovine lactoferrin	Ser-Val-Asp-Gly-Lys-Glu-Asp-Leu-Ile-Trp	1808.4	n. a.	(Manzanares et al. 2018)
bovine lactoferrin	Ac-Arg-Lys-Trp-His-Phe-Leu-Trp-NH_2	296.4	n. a.	(Manzanares et al. 2018)

n. a. – no activity according to the BIOPEP-UWM database.

bacteria contain peptides capable of inhibiting several enzymes present in extracts of HT-29 and SW480 human colon carcinoma cells, including POP. However, the peptides exhibited no such activity in the intact cells, as they were probably not absorbed. These peptides were characterized by a lack of cytotoxicity and IC_{50} values in the range of 30 to 250 μM. The concentration of these peptides in fermented milk can exceed these values (Juillerat-Jeanneret, Robert, and Juillerat 2011).

In vivo studies

Although not confirmed to have POP-inhibiting activity, Colostrinin™ – a complex of low molecular weight (0.5-3 kDa), Pro-rich (about 20%) peptides isolated from ovine, bovine, caprine, and human colostra was shown to have procognitive properties (Sokołowska et al. 2008). According to Stańczykiewicz et al. (2017), consumption of Colostrinin™ at the dose of 4 μg had beneficial effects on the cognitive functions of young rats. Lemieszewska et al. (2018) isolated a peptide with sequence Arg-Pro-Lys-His-Pro-Ile-Lys-His-Gln, sharing sequence homology with sheep and beef αS1-caseins, from Colostrinin™ and demonstrated its anti-apoptotic and neuroprotective activity in adrenal pheochromocytoma PC12 cells. Colostrinin™ was also shown to improve the cognitive performance of patients with moderate AD (Bilikiewicz and Gaus 2004).

Manzanares et al. (2018) were the first to reveal that peptides obtained via hydrolyzis of lactoferrin, glycoprotein from milk whey, and a few sequence-related synthetic peptides, exhibited POP repressive properties in vitro. They also protected transgenic nematode *Caenorhabditis elegans*, a convenient in vivo model used in screening for potential AD drugs, from the toxicity caused by βA-derived peptides. According to the authors, the protective effect was not related to antioxidative activity, as these peptides did not show such properties, instead the importance of tryptophan residue for the bioactivity was highlighted.

The most widely studied ACE inhibitory peptides from milk proteins are so-called lactotripeptides: Leu-Pro-Pro and Val-Pro-Pro from β-, and Ile-Pro-Pro from κ-casein. Their bioavailability and antihypertensive properties were confirmed in numerous animal and human studies, and they are the active ingredients of nutraceutical drinks, e.g. Calpis® (Calpis Co. Ltd., Japan), Evolus® and Valio® (Valio Ltd., Finland) (Iwaniak, Darewicz, and Minkiewicz 2018). These peptides were also identified in cheeses, although their concentration varies significantly between different cheese types (Iwaniak and Mogut 2020). Siltari et al. (2012) studied whether any other vascular function regulating enzymes are also inhibited by the lactotripeptides and found that these molecules are POP inhibitors, unlike the free amino acids of which they are composed. Human studies revealed that the consumption of lactotripeptides promotes cognitive performance. According to Hamasaki et al. (2019), it improved brain neural activation related to enhanced cognitive function and decreased arterial stiffness in middle-aged and older adults, regardless of their involvement in physical activity. Akazawa et al. (2018) reported that it increased middle cerebral blood flow velocity.

Although an inverse correlation between milk consumption and the frequency of cognitive disorders was shown in meta-analyses, which are placed at the top of pyramid in the evidence-based medicine (J. Lee et al. 2018; Wu and Sun 2016), the general evidence is not strong enough to draw a solid conclusion on that subject. This is due to several significant study limitations, including methodological variability and a limited number of high-quality research papers.

Meat and fish protein-derived POP inhibitors

Several POP-inhibiting peptides have been discovered in meat and fish proteins, mostly in collagen and a few in other proteins. The examples of such peptides, along with other bioactivities of peptides with the same sequence (accessed from the BIOPEP-UWM database on 10.01.2023) are shown in Table 2.

In silico studies

Minkiewicz, Dziuba, and Michalska (2011) reported that proteinase K and proteinase P1 can potentially release various biopeptides from bovine muscle proteins, including POP inhibitors. According to these authors, among the bovine proteins analyzed, collagen and elastin were found

Table 2. Meat protein-derived peptides exhibiting POP inhibitory activity in vitro.

Source	Peptide sequence	IC_{50} [μM]	Other bioactivities	References
collagen	Pro-Gly	n. d.	ACE inhibitor, antithrombotic, DPP IV inhibitor, regulating the stomach mucosal membrane activity	(Cheung et al. 1980; Ashmarin et al. 1998; Lan et al. 2015)
collagen	Gly-Pro	n. d.	ACE inhibitor, antithrombotic, DPP IV inhibitor, regulating the stomach mucosal membrane activity	(Yoshimoto et al. 1978; Ashmarin et al. 1998; Byun and Kim 2002)
bovine collagen	Pro-Pro-Gly	2700	DPP IV inhibitor	(Lafarga, O'Connor, and Hayes 2014; Lafarga, O'Connor, and Hayes 2015)
collagen	Pro-Gly-Pro	n. d.	antithrombotic, chemotactic, inhibitor of insulin secretion, regulating the stomach mucosal membrane activity	(Ashmarin et al. 1998; O'Reilly et al. 2009)
bovine serum albumin	Pro-Pro-Leu	2860	ACE inhibitor, DPP IV inhibitor	(Wang et al. 2011; Lafarga, O'Connor, and Hayes 2014; Lafarga, O'Connor, and Hayes 2015)
porcine and bovine myosin	Ala-Pro-Pro-His	3950	ACE inhibitor	(Lafarga, O'Connor, and Hayes 2014; Lafarga, O'Connor, and Hayes 2015)

n. d. – not determined.

to be the best sources of such peptides, due to the high content of Pro and Gly residues. Lafarga, O'Connor, and Hayes (2015) synthesized a few POP inhibitory peptides which they had predicted *in silico* to be released from meat proteins by various proteases. These peptides were characterized by diverse inhibitory activity (see Table 2), resistance to further degradation by the GI enzymes and lack of toxicity, according to *in silico* analyses. B.-B. Huang, Lin, and Chang (2015) identified 7 proteins in extracts of tilapia frame and skin, and established that their theoretical hydrolyzates by various proteases contain POP inhibiting peptides. Kęska and Stadnik (2016) found that several POP inhibitory peptides can potentially be released from porcine myofibrillar proteins hydrolyzed by GI enzymes, i.e. pepsin, trypsin, and chymotrypsin. Collagens from cow, pig, sheep, chicken, duck, horse, salmon, rainbow trout, goat, rabbit, and turkey hydrolyzed *in silico* with various proteases were found out to be theoretically promising sources of POP inhibiting peptides, whose frequency of occurrence in the collagens' sequences was only inferior to ACE and DPP IV inhibitory peptides (Iwaniak et al. 2020). Adopting a similar approach, based on the BIOPEP-UWM database, we predicted that some POP inhibitory peptides can be released during enzymatic hydrolyzis of chicken feather and pig hair keratins (Taraszkiewicz et al. 2022).

In vitro studies

Sørensen et al. (2004) reported that hydrolyzates of cod, salmon, and trout muscle proteins produced by endogenous and/or exogenous proteases contain diverse peptides exhibiting strong POP inhibitory activity. According to Sila et al. (2015), barbel skin gelatin hydrolyzates produced by various proteases contained POP and DPP IV inhibiting peptides, the potency of which was sequence-dependent and length-independent.

Similarly, hydrolyzates of proteins from sardine and tuna head, muscle, and viscera prepared by Alcalase contained <3 kDa peptides with moderate POP and ACE inhibitory properties (Martínez-Alvarez et al. 2016). The bioactivity of these peptides was enhanced considerably after simulated gastric digestion, due to the release of smaller peptides, while remaining unchanged following continued intestinal digestion (Martínez-Alvarez et al. 2016).

Lajmi et al. (2019) studied the effect of hydrolyzis conditions by trypsin on the release of ACE and POP inhibitory peptides from proteins found in smooth hound collagenous by-products, i.e. skin, tail, and fins. The authors reported that the optimal pH and temperature of the process affect the desired bioactivity of peptides to be produced because the most potent POP inhibitory activity was obtained in the hydrolyzate produced at 35 °C at pH 7, whereas the greatest ACE inhibition was exhibited by the hydrolyzate obtained at 45.9 °C at pH 9.

POP inhibitory peptides were also detected in proteins derived from meat offal. Ohmori et al. (1994) isolated such peptide with sequence Met-Pro-Pro-Pro-Leu-Pro-Ala-Arg-Val-Asp-Ala-Leu-Asn from bovine brain. Papain, collagenase, and Alcalase generated POP repressing peptides from heat- and pressure-treated proteins found in bovine lungs, which inhibitory potency was dependent on the enzyme used and method of pretreatment applied (Lafarga and Hayes 2017).

In vivo studies

Porcine brain hydrolyzate exhibited POP inhibiting properties and improved cognitive performance of βA-peptide-infused rats (Y.-T. Liu et al. 2019).

Collagen is a rich source of bioactive peptides, POP inhibitors in particular, owing to a unique structure of this protein, comprising of a repetitive sequential motif of Gly-Pro-Hyp (Iwaniak et al. 2020). A set of so-called glyprolines, i.e. short peptides derived from this motif, including Pro-Gly, Gly-Pro, Pro-Gly-Pro, Gly-Pro-Gly-Gly, and cyclic Pro-Gly, inhibit POP and potentiate memory consolidation processes in the CNS (Ashmarin et al. 1998). Besides, the glyprolines show a variety of other bioactive properties, both positive and negative, depending on their structure and organ or system they affect, including antithrombotic, anti-inflammatory, chemoattractive, hemostasis regulatory, and neuroprotective (Misiura and Miltyk 2019). Human studies revealed that following collagen hydrolyzate ingestion, several short Pro-containing peptides were quickly absorbed into the blood where they remained for a long time, which confirms their bioavailability (Sato, Jimi, and Kusubata 2019; Koizumi et al. 2019).

Collagen hydrolyzates have shown some promising results in improving cognitive capabilities in in vivo studies. They were reported to improve the recovery rate of rats suffering from surgical brain injury by angiogenesis promotion (K.-F. Huang et al. 2014) and exhibit neuroinflammation lowering properties in these animals (Chen et al. 2019). They also enhanced spatial memory and learning, reduced the oxidative stress, and upregulated the expression of brain-derived neurotrophic factor in aged mice (Pei et al. 2010), as well as induced changes in the human brain, along with improvements in language cognitive functions (Koizumi et al. 2019). However, most likely due to a limited number of studies, no systematic reviews have assessed the collagen's effectiveness in preventing mental impairments.

Findings from the latest systematic review regarding meat consumption's effects on cognitive function were inconsistent (Zhuang et al. 2020). Meta-analysis of several studies indicated some protective effect of meat consumption toward cognitive disorder's frequency but no strong conclusions can be currently drawn due to limited representativeness and possible publication bias (Zhuang et al. 2020).

Plant protein-derived POP inhibitors

Plant-based bioactive peptides were investigated less extensively than those from proteins of animal origin. However, some examples of POP inhibiting peptides have been found in plant-derived proteins. Sequences of several POP inhibitory peptides originating from plant proteins are presented in Table 3.

Table 3. Plant protein-derived peptides exhibiting POP inhibitory activity in vitro.

Source	Peptide sequence	IC_{50} [μM]	References
corn γ-zein	His-Leu-Pro-Pro-Pro-Val	80	(Maruyama et al. 1992)
corn γ-zein	Leu-Pro-Pro-Val	240	(Maruyama et al. 1992)
soybean cell wall protein	Lys-Pro-Pro-Ile	380	(Maruyama et al. 1992)
soybean cell wall protein	Lys-Pro-Pro-Val	270	(Maruyama et al. 1992)
red wine	Val-Glu-Ile-Pro-Glu	17	(Yanai, Suzuki, and Sato 2003)
rice glutelin	pGlu-Leu-Phe-Asn-Pro-Ser-Thr-Asn-Pro-Trp-His-Ser-Pro	24.3	(Saito et al. 1997)
rice glutelin	Ser-Pro-Phe-Trp-Asn-Ile-Asn-Ala	42.8	(Saito et al. 1997)
cocoa seed protein	Asn-Tyr-Asp-Asn-Ser-Ala-Gly-Lys-Trp	483.8	(Martorell et al. 2013)
cocoa seed protein	Asp-Asn-Tyr-Asp-Asn-Ser-Ala-Gly-Lys-Trp-Trp-Val-Thr	122.1	(Martorell et al. 2013)

In silico studies

In silico analysis of cereal storage proteins by Cavazos and Gonzalez de Mejia (2013) revealed that some POP inhibitory peptides occur within sequences of wheat gliadin and glutenin, barley hordein, oat globulin, and rice glutelin. According to Amin et al. (2022), POP inhibiting peptides can theoretically be released from large subunit of RuBisCO from green seaweed *Ulva lactuca* by the action of pancreatic elastase, chymotrypsin C, papain, ficin, bromelain, calpain 2, and pepsin.

In vitro studies

Maruyama et al. (1992) synthesized several peptides with sequences homologous to fragments of several Pro-rich proteins, i.e. corn γ-zein, soybean cell wall protein, and carrot 33-kDa protein. They also hydrolyzed the γ-zein using 10% (E/S) subtilisin in an attempt to generate POP inhibitors. The obtained peptides were characterized by diverse, sequence-dependent inhibitory potency (see Table 3).

The POP inhibiting peptides were also discovered in some alcoholic drinks. Saito et al. (1997) isolated some POP inhibitory peptides, originating from rice glutelin, from sake, a traditional Japanese alcoholic beverage, and sake by-product (sake cake) hydrolyzate by pepsin (see Table 3). Yanai, Suzuki, and Sato (2003) obtained two POP inhibitory oligopeptides from proteins present in grape juice and wine (see Table 3). These peptides were also able to reduce the degradation rate of memory-related neuropeptides: arginine-vasopressin, substance P, and neurotensin in vitro. The authors found that the concentration of these peptides in the analyzed materials depends on the grape kind and the winemaking conditions.

Hsieh et al. (2016) used thermolysin, bromelain, and the GI enzymes to obtain hydrolyzates of wheat gluten and soy protein isolate, and established that their POP inhibitory potency at the concentration of 5 mg/mL was in the range of 58–72% and 56–79%, respectively, depending on the protease applied.

A hydrolyzate of corn gluten meal with 1% pepsin and its in vitro digesta exhibited POP inhibitory and antioxidant properties i.e. radical scavenging and ferric-reducing power, upregulation of catalase expression, and cellular antioxidant activity in human neuroblastoma SH-SY5Y cells (Chanajon, Noisa, and Yongsawatdigul 2022).

According to Suwanangul et al. (2022), hydrolyzates of proteins from oilseed meal (sacha inchi) exhibited POP inhibitory activity with IC_{50} values ranging from 0.67 to 20 mg/mL depending on peptide size, with the fraction below 1 kDa exhibiting the highest inhibition.

In vivo studies

Martorell et al. (2013) investigated neuroprotective potential of "Barquillo" – a protein-rich cocoa by-product, its hydrolyzates and peptides. The authors reported that treatment of "Barquillo" with Termamyl 120 L and Alcalase 2.4 L increased its POP inhibitory activity from approx. 23 to 43% and improved its protective ability against neurotoxicity caused by βA-peptides in *Caenorhabditis elegans*. Several peptides originating from cocoa's 21 kDa seed protein were identified (see Table 3). Notably, the most promising peptide Asp-Asn-Tyr-Asp-Asn-Ser-Ala-Gly-Lys-Trp-Trp-Val-Thr was also identified in different commercial chocolate samples (Martorell et al. 2013).

According to Katayama et al. (2014), a diet supplemented with 7% (w/w) soy peptide mixture composed of 64% di- and tripeptides prevented cognitive impairment during a 26-week mice trial. Similarly, according to Maebuchi et al. (2013), a supplementation of di/tripeptide-rich soybean protein hydrolyzate at a dose of 8 g/day over 8 weeks improved the delayed and immediate memory scores substantially in patients with mild cognitive dysfunction. Mazza et al. (2017) assayed the effects of dietary choices on cognitive decline progression over 12 months in Italian elderly individuals. According to the authors, diets including plant-based proteins and legumes improved the participants' cognitive performance. However, no systematic reviews were published on the impact of dietary plant protein-derived peptides on cognitive functions. The examples of food protein-derived peptides/hydrolyzates exhibiting procognitive properties in vivo are given in Table 4.

Bioavailability of POP inhibitory peptides

One of the major concerns influencing the physiological effects of bioactive peptides is their limited bioavailability. It is reflected in differences between in vitro and in vivo studies regarding their biological effects and can be caused by interactions with food matrix and GI tract components such as mucin or proteases in particular. Orally administrated peptides are vulnerable to proteolytic degradation in the stomach, intestine, brush border, and plasma. Generally, short peptides with Pro, Arg, or Trp residues at C-terminus exhibit high protease resistance (Udenigwe et al. 2021).

Table 4. Food protein-derived peptides exhibiting procognitive activity in vivo.

Peptide/hydrolyzate	Organism	Procognitive effect	Reference
Colostrinin™ (complex of 0.5-3 kDa peptides containing 20% Pro residues)	Rat	Increase of ability to concentrate	(Stańczykiewicz et al. 2017)
Colostrinin™ (complex of 0.5-3 kDa peptides containing 20% Pro residues)	Human	Improvement of cognitive sympoms and daily function during moderate AD	(Bilikiewicz and Gaus 2004)
Ser-Val-Asp-Gly-Lys-Glu-Asp-Leu-Ile-Trp and Ac-Arg-Lys-Trp-His-Phe-Leu-Trp-NH_2	*Caenorhabditis elegans*	Inhibition of βA oligomerization and protection against βA peptide-induced toxicty	(Manzanares et al. 2018)
Casein hydrolyzate containing 1.4 mg Val-Pro-Pro and 2 mg Ile-Pro-Pro	Human	Increase of cerebral blood flow velocity	(Akazawa et al. 2018)
Casein hydrolyzate containing 1.4 mg Val-Pro-Pro and 2 mg Ile-Pro-Pro	Human	Improvement of brain neural activation related to enhanced cognitive function and decrease of arterial stiffness	(Hamasaki et al. 2019)
Porcine brain hydrolyzate	Rat	Protection against βA peptide-induced toxicity, improvement of performance on reference, spatial and working memory tests	(Y.-T. Liu et al. 2019)
Chum salmon skin collagen hydrolyzate containing 86% oligopeptides	Mouse	Improvement of spatial memory and learning, reduction of oxidative stress in brain and number of apoptotic neurons	(Pei et al. 2010)
Porcine collagen hydrolyzate with average molecular weight of 1.2 kDa	Human	Induction of changes in the brain and improvement of language cognitive functions	(Koizumi et al. 2019)
Asp-Asn-Tyr-Asp-Asn-Ser-Ala-Gl y-Lys-Trp-Trp-Val-Thr	*Caenorhabditis elegans*	Protection against oxidative damage and βA peptide-induced toxicty	(Martorell et al. 2013)
Soy protein hydrolyzate containing 64% di- and tripeptides	Mouse	Prevention of cognitive impairment	(Katayama et al. 2014)
Soybean protein hydrolyzate rich in di/ tripeptides	Human	Improvement of delayed and immediate memory scores, increased dopamine serum level	Maebuchi et al. (2013)

Peptides that survive the GI transit and reach the gut can either exhibit their bioactivity there or can be absorbed into the bloodstream from which they reach their molecular targets (Xu et al. 2019). The transport of peptides across the intestinal epithelial cell monolayer occurs through either single or multiple mechanisms, depending on the peptides' properties e.g. molecular weight, amino acid composition, hydrophobicity, charge, and side chain flexibility (Xu et al. 2019). Di- and tripeptides are absorbed mostly via the PepT1 transporter present on the apical side of the gut epithelium. Larger peptides pass the intestinal barrier through paracellular transport via tight junctions, transcytosis via vesicles, and/or passive transcellular diffusion (Sato 2021). Although the absorbability of most peptides does not exceed 1%, some of them can reach the blood intact at concentrations within the micromolar range and remain bioactive in the plasma for several minutes to hours (Xu et al. 2019).

An additional difficulty in the search for effective POP inhibitors is that they must have the ability to penetrate the blood-brain barrier (BBB) – selective, semipermeable tissue formed by endothelial cells, serving as a regulatory interface between the blood and the CNS. It is one of the most challenging obstacles for molecules designed to be therapeutics for the CNS-related disorders, where numerous potential drug candidates fail (Banks 2015). The first food-derived POP inhibitory peptide whose ability to cross the BBB was confirmed was Pro-Pro-Leu (Hayes et al. 2016), previously generated from meat proteins using *in silico* analysis (Lafarga, O'Connor, and Hayes 2015). Several other small peptides capable of penetrating the brain were also discovered, including Pro-Gly, Pro-Hyp, Tyr-Pro, Trp-Tyr, Met-Lys-Pro, and Gly-N-methylated-Gly (Gly-Sar), although only the first of these is known to exhibit POP inhibitory properties (Ashmarin et al. 1998; Matsui, Yoshino, and Tanaka 2020). The transportability of peptides across the BBB is largely understudied, however, the known examples share a few common features such as small size up to several amino acid residues and high protease resistance (Matsui, Yoshino, and Tanaka 2020). The peptide sequence is also important, since Pro-Tyr, a sequence-reversed analogue of the brain transportable Tyr-Pro was not uptaken (M. Tanaka et al. 2019; Matsui, Yoshino, and Tanaka 2020).

Summary and future perspectives

The use of bioactive peptides from food proteins appears to be a very promising strategy in the prevention of various diseases. The biopeptides often have a similar mechanism of action to commonly prescribed synthetic pharmaceuticals, but unlike the latter, they are usually completely side-effect-free, although the possibility of adverse allergic reactions should not be ignored.

Numerous examples of biopeptides have been discovered, varying in origin, structure, and type of bioactivity, although the number of known POP inhibitory peptides is, comparatively, low and their potential health effects are largely unexplored. To the best of our knowledge, the majority of studies on POP repressing peptides have been done in vitro and none of the human studies, nor systematic reviews in which they were mentioned, was specifically designed to investigate the relationship between POP inhibitory properties of the peptides and their ability to influence the cognitive functions.

The difficulty in carrying out and interpreting the results of studies aimed to assess the neuroprotective potential of POP inhibitory peptides from foods is influenced by several factors. First of all, numerous peptides present nonspecific, multifunctional pharmacological properties. The IC_{50} values for the same inhibitor may differ depending on whether they were determined for the POP of bacterial, animal, or human origin. Moreover, some inhibitor-sensitive

biochemical processes that are controlled by POP are not dependent on its proteolytic activity. Lastly, most of the research concerning peptidic POP inhibitors has been performed on relatively pure proteins/hydrolyzates/peptides. The actual dietary sources of these peptides are often consumed while being a part of complex food matrices, comprising a myriad of CNS-affecting compounds capable of interacting and changing each other's properties in a manner that is often difficult to predict.

Several suggestions on the direction of future research concerning the POP inhibitory peptides can be made. Firstly, the efforts should be focused on optimizing the process of protein hydrolyzis in terms of obtaining very short peptides, as only these have been shown to be transportable beyond the BBB and potentially capable of exerting physiologically relevant effects. At the same time, longer peptides should not be excluded from consideration, since they can act as pro-drugs, from which shorter, more bioavailable peptides can be released following continued hydrolyzis by endogenous enzymes. On the other hand, hydrolyzates characterized by very high hydrolyzis degrees may contain mostly free amino acids instead of peptides, which do not exhibit POP inhibitory properties. Thus, the simulated GI digestion of the peptides in question should be applied whenever possible, to assess whether their inhibitory potency and potential bioavailability would be affected. Secondly, the search for POP inhibitory peptides should not be narrowed to Pro-rich proteins only, as Pro-free peptides also exhibited notable repressive properties in vitro. Instead, proteins rich in hydrophobic amino acid residues should be investigated more extensively.

In summary, although some milk, meat, fish, and plant protein-derived peptides have the potential to be applied as natural, procognitive nutraceuticals, their effectiveness requires further evaluation, especially in clinical trials. We demonstrated that the important features of the most promising POP-inhibiting peptides are very short sequence, high content of hydrophobic amino acids, and usually the presence of Pro residue (Table 5).

Table 5. List of abbreviations.

Abbreviation	Explanation
αS	α-synuclein
βA	β-amyloid
ACE	angiotensin-converting enzyme
AD	Alzheimer's disease
ADHD	attention deficit hyperactivity disorder
BBB	blood-brain barrier
CNS	central nervous system
DPP IV	dipeptidyl peptidase IV
GAP-43	growth-associated protein 43
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GI	gastrointestinal
IC_{50}	half-maximal inhibitory concentration
IP3	inositol 1,4,5-triphosphate
NMDA	N-methyl-D-aspartic acid
POP	prolyl oligopeptidase
PP2A	protein phosphatase 2 A
PPI	protein-protein interaction
PSP	proline-specific peptidase
ROS	reactive oxygen species
VPA	valproic acid

Disclosure statement

No potential conflict of interest was reported by the authors.

Author contributions

Conceptualization, A.T.; investigation, A.T.; writing-original draft, A.T.; writing-review and editing, I.S., A.S. and H.S.; project administration, A.T. and H.S.; supervision, I.S. and H.S. All authors have read and agreed to the final version of the manuscript.

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Academic achievements

Published scientific papers

1. **Taraszkiewicz, A.**, Sommer, A., Sinkiewicz, I., Koss-Mikołajczyk, I., Kusznierewicz, B., Giblin, L., & Staroszczyk, H. (2025). Valorising feather keratin: Antioxidant activity, cytotoxicity and mutagenicity during processing and gastrointestinal digestion. *Food Chemistry*, *493P2*, 145820. https://doi.org/10.1016/j.foodchem.2025.145820 (Q1, IF_{2024}=9.8, $MNiSW_{2025}$*=200)
2. **Taraszkiewicz, A**., Sinkiewicz, I., Sommer, A., Kusznierewicz, B., Giblin, L., & Staroszczyk, H. (2025). Chemical composition and techno-functional properties of high-purity water-soluble keratein and its enzymatic hydrolysates. *Food Chemistry*, 472, 142641. https://doi.org/10.1016/j.foodchem.2024.142641 (Q1, IF_{2024}=9.8, $MNiSW_{2025}$*=200)
3. **Taraszkiewicz, A**., Sinkiewicz, I., Sommer, A., & Staroszczyk, H. (2024). The biological role of prolyl oligopeptidase and the procognitive potential of its peptidic inhibitors from food proteins. *Critical Reviews in Food Science and Nutrition*, 64(19), 6567–6580. https://doi.org/10.1080/10408398.2023.2170973 (Q1, IF_{2024}=8.8, $MNiSW_{2024}$*=200)
4. Iwaniak, A., & **Taraszkiewicz, A.** (2022). Fragmentomic analysis of biopeptides *in silico* released from milk proteins. *Polish Journal of Natural Sciences*, 37(1), 125–147. https://doi.org/10.31648/pjns.7726 (Q4, $MNiSW_{2022}$*=40)
5. **Taraszkiewicz, A**., Sinkiewicz, I., Sommer, A., Dąbrowska, M., & Staroszczyk, H. (2022). Prediction of bioactive peptides from chicken feather and pig hair keratins using *in silico* analysis based on fragmentomic approach. *Current Pharmaceutical Design*, 28(10), 841–851. https://doi.org/10.2174/1381612828999220114150201 (Q2, IF_{2022}=3.1, $MNiSW_{2022}$*=70)
6. Dąbrowska, M., Sommer, A., Sinkiewicz, I., **Taraszkiewicz, A.**, & Staroszczyk, H. (2022). An optimal designed experiment for the alkaline hydrolysis of feather keratin. *Environmental Science and Pollution Research*, 29(16), 24145–24154. https://doi.org/10.1007/s11356-021-17649-2 (Q1, IF_{2022}=5.8, $MNiSW_{2022}$*=100)

Σ IF 37.3; Σ MNiSW* 810; H-index (Scopus) 3; total citation number (Scopus): 52

** MNiSW – Ministerstwo Nauki i Szkolnictwa Wyższego (Ministry of Science and Higher Education, Poland). The number refers to the official ministerial score of the journal, used in national academic evaluation in Poland.*

Unpublished scientific papers

1. Staroszczyk, H., Sommer, A., **Taraszkiewicz, A.**, & Michalec M. Changes in the structure and thermal properties of feather keratin induced by L-Cys extraction followed by enzymatic hydrolysis. https://doi.org/10.2139/ssrn.5382858 *(under review)*

Participation in grants

1. “Characterization of bioactive peptides from chicken feather keratin and products of their transformations occurring during the Maillard reaction”, project no: DEC-2021/41/N/NZ9/04466, funder: National Science Centre, Poland, granting programme: Preludium 20, total funding: 209,920 PLN, realization period: 03.01.2022-02.01.2026, role: PI
2. “WasteValue | Anaerobic biorefinery for resource recovery from waste feedstock”, project no: NOR/POLNOR/WasteValue/0002/2019-00, funder: The National Centre for Research and Development, Poland, granting programme: EEA and Norway grants - Applied Research Programme, total funding: 6,871,859.90 PLN, realization period: 12.01.2020-30.04.2024, role: co-investigator

Scientific communications

1. Sommer, A., Staroszczyk, H., & **Taraszkiewicz, A.** Thermal and structural properties of reduced keratin and its enzymatic hydrolysates, 15th Conference on Calorimetry and Thermal Analysis, 08-12.09.2024, Zakopane, Poland *(poster)*

2. Niedźwiedzka, A., Lichocki, P., **Taraszkiewicz, A.**, & Sommer, A. Bacterial cellulose as a matrix in vegan food, XIth International Session of Young Scientific Staff “Food in the face of the challenges of the modern world”, 16-17.05.2024, Gdańsk, Poland *(oral presentation)*
3. **Taraszkiewicz, A.,** Sinkiewicz, I., Bietto, F., Dold, C., Oliver, C., Sommer, A., Staroszczyk, H., & Giblin, L. Effects of enzymatic hydrolysis, the Maillard reaction and *in vitro* gastro-intestinal digestion on antioxidant properties of feather keratin, 8th International Conference on Food Digestion, 09-11.04.2024, Porto, Portugal *(oral presentation)*
4. **Taraszkiewicz, A.** Charakterystyka bioaktywnych peptydów z keratyny z piór drobiowych oraz produktów ich przemian zachodzących w trakcie reakcji Maillarda [Characterization of bioactive peptides from chicken feather keratin and products of their transformations occurring during the Maillard reaction], Seminar of the Polish Society of Food Technologists, Gdańsk Branch, 15.02.2024, Gdańsk, Poland *(invited lecture)*
5. **Taraszkiewicz, A.**, Sinkiewicz, I. Sommer, A., Kusznierewicz, B., & Staroszczyk, H. Właściwości przeciwutleniające, emulgujące i pianotwórcze hydrolizatów keratynowych oraz ich modyfikacja glukozą na drodze reakcji Maillarda [Antioxidant, emulsifying and foaming properties of keratin hydrolysates and their modification with glucose via the Maillard reaction], II Ogólnopolska Konferencja Naukowa “Żywność i żywienie w pigułce”, 22.04.2023, Gdańsk, Poland *(oral presentation)*
6. Świderska, P., Sommer, A., **Taraszkiewicz, A.**, & Staroszczyk, H. Aktywność przeciwutleniająca hydrolizatów keratynowych [Antioxidant activity of keratin hydrolysates], II Ogólnopolska Konferencja Naukowa “Żywność i żywienie w pigułce”, 22.04.2023, Gdańsk, Poland *(poster)*
7. **Taraszkiewicz, A.**, Sommer, A., Mańko, J., Sinkiewicz, I., & Staroszczyk, H. Charakterystyka izolatów keratyny z piór drobiowych w aspekcie produkcji biopeptydów i biodegradowalnych opakowań [Characterisation of chicken feather keratin isolates in terms of the production of biopeptides and biodegradable packaging], III Wielkopolska Konferencja Nauka Gospodarce “Partnerstwo nauki i przemysłu źródłem rozwoju”, 09-10.06.2022, Poznań, Poland *(poster)*
8. **Taraszkiewicz, A.**, Sinkiewicz, I., & Staroszczyk, H. Chicken feather keratin as a source of bioactive peptides useful in prevention of metabolic disorders – *in silico* and *in vitro* hydrolysis, NutRedOx COST Action 16112 meeting “ROS roundabout – many exit options”, 19-21.09.2021, Gdańsk, Poland *(oral presentation)*
9. **Taraszkiewicz, A.**, Sinkiewicz, I., & Staroszczyk, H. Inhibitory endopeptydazy prolinowej pochodzące z białek żywności [Prolyl endopeptidase inhibitors derived from food proteins], XLV Konferencja Komitetu Nauk o Żywności i Żywieniu PAN “Żywność w strategii Zielonego Ładu”, 01-02.07.2021, Gdańsk, Poland *(oral presentation)*
10. **Taraszkiewicz, A.**, Sinkiewicz, I., Serafin, A., & Staroszczyk, H. Właściwości przeciwutleniające i pianotwórcze enzymatycznych hydrolizatów keratynowych [Antioxidative and foaming properties of enzymatic keratin hydrolysates], VI Pomorskie Spotkania z Mikrobiologią “Drobnoustroje - wrogowie i sprzymierzeńcy”, 24-25.06.2021, Gdańsk, Poland *(poster)*
11. **Taraszkiewicz, A.,** Sinkiewicz, I., & Staroszczyk, H. Analiza *in silico* bioaktywnych peptydów pochodzących z keratyny z piór drobiowych [*In silico* analysis of bioactive peptides derived from chicken feather keratin], XXVIII Ogólnopolskie Sympozjum Bromatologiczne “Innowacyjne podejście do bezpiecznej żywności i racjonalnego żywienia”, 28-29.09.2020, Gdańsk, Poland *(poster)*

Scientific internships

1. Internship at the Food Bioavailability Laboratory, Department of Food Biosciences, Teagasc Food Research Centre, Moorepark, Ireland, supported by “STER - Internationalisation of Doctoral Schools” programme, supervisor: Dr. Linda Giblin, 31.07.2023-31.10.2023

2. Internship at the Food Bioavailability Laboratory, Department of Food Biosciences, Teagasc Food Research Centre, Moorepark, Ireland, supported by Erasmus+ programme, supervisor: Dr. Linda Giblin, 05.09.2022-19.12.2022

Mentorship over diploma theses

Bachelor theses

1. Agnieszka Bławat, Production of Maillard reaction products from keratin hydrolysates, 2025
2. Jakub Dziadosz, Determination of mutagenicity of keratin hydrolysates and products of their transformations occurring during the Maillard reaction against *Salmonella enterica* var. Typhimurium TA98, 2025
3. Martyna Dędza, Determination of mutagenicity of keratin hydrolysates and products of their transformations occurring during the Maillard reaction against *Salmonella enterica* var. Typhimurium TA100, 2025
4. Agnieszka Bielawska, Production of keratin hydrolysates by digestive enzymes, 2025
5. Magdalena Kopka, Functional properties of enzymatic keratin hydrolysates, 2023
6. Julia Fularczyk, Production of keratin hydrolysates with selected enzymes, 2023
7. Agata Serafin, Antioxidant activity of enzymatic keratin hydrolysates, 2021

Master theses

1. Magdalena Kopka, Inhibitory activity of keratin hydrolysates against dipeptidyl peptidase IV, 2024
2. Ziółkowska Zuzanna, Physical and biological properties of reduced keratin hydrolysates and products of their transformations occurring during the Maillard reaction, 2022

Participation in R&D works

1. “Development of innovative technology of lyophilizates production using wave emitters for process optimization and improvement of product quality”, funder: Agency for Restructuring and Modernisation of Agriculture, granting programme: Rural Development Programme 2014-2020, total funding: 6,831,757.97 PLN, realization period: 01.12.2023-31.12.2024, role: co-investigator
2. “Optimization of conditions for the extraction of proteins, peptides and lipids from poultry industry by-products” for Cedrob S.A., Warsaw, Poland, realization period: 01.04.2022-01.10.2022, role: co-investigator

Scientific peer reviewing

1. *Critical Reviews in Food Science and Nutrition* (Q1) – 1 article
2. *Biomass Conversion and Biorefinery* (Q2) – 1 article
3. *Food & Function* (Q1) – 1 article

Membership in scientific society

1. Member of the Polish Society of Food Technologists [Polskie Towarzystwo Technologów Żywności] 1.10.2021-present

Co-organisation of scientific conferences

1. XIth International Session of Young Scientific Staff “Food in the face of the challenges of the modern world”, 16-17.05.2024, Gdańsk University of Technology, Gdańsk, Poland
2. XLV Konferencja Komitetu Nauk o Żywności i Żywieniu PAN „Żywność w strategii Zielonego Ładu”, 01-02.07.2021, Gdańsk University of Technology, Gdańsk, Poland
3. VI Akademia Sokowa „Produkty sokownicze – od roślin po korzyści zdrowotne", 21.11.2019, Gdańsk University of Technology, Gdańsk, Poland

Participation in popular science event

1. Participation in Gdynia Design Days with a poster “Nowe życie piór kurzych” [New life of chicken feathers] summarizing the results obtained during the realization of the Preludium 20 project, 22-30.06.2024, “Experyment” Science Centre, Gdynia, Poland

Rewards and distinctions

1. Recipient of three financial bonuses in the Nitrogenium Supporting Excellence in Publishing programme (Gdańsk University of Technology) for publication of articles:
    a. “Valorising feather keratin: Antioxidant activity, cytotoxicity and mutagenicity during processing and gastrointestinal digestion”, 2025
    b. “Chemical composition and techno-functional properties of high-purity water-soluble keratein and its enzymatic hydrolysates”, 2025
    c. “The biological role of prolyl oligopeptidase and the procognitive potential of its peptidic inhibitors from food proteins”, 2024
2. Recipient of pro-quality scholarship in the Francium Supporting Outstanding Doctoral Candidates programme (Gdańsk University of Technology), 2023
3. Recipient of outgoing mobility grant for the best PhD students supporting 3-month research internship funded by the project "STE(E)R-ING towards International Doctoral School” financed by the Polish National Agency for Academic Exchange under the programme “STER - Internationalisation of Doctoral Schools” (Gdańsk University of Technology), 2023
4. Distinction for the oral presentation entitled “Właściwości przeciwutleniające, emulgujące i pianotwórcze hydrolizatów keratynowych oraz ich modyfikacja glukozą na drodze reakcji Maillarda” [Antioxidant, emulsifying and foaming properties of keratin hydrolysates and their modification with glucose via the Maillard reaction] at II Ogólnopolska Konferencja Naukowa “Żywność i żywienie w pigułce” (Medical University of Gdańsk), 2023
5. Distinction for activity for the academic community (University of Warmia and Mazury in Olsztyn), 2018

Gdańsk, 26.08.2025

Co-authors' declaration regarding the description of contribution and percentage share in the preparation of the scientific manuscript

Taraszkiewicz, A., Sinkiewicz, I., Sommer, A., Dąbrowska, M., & Staroszczyk, H. (2022). Prediction of bioactive peptides from chicken feather and pig hair keratins using *in silico* analysis based on fragmentomic approach. *Current Pharmaceutical Design*, 28(10), 841–851. https://doi.org/10.2174/1381612828999220114150201

Author	Description of contribution	Percentage of contribution	Signature
Antoni Taraszkiewicz	I conceptualized the study, developed the methods, performed all computations and data interpretation, prepared figures and tables, wrote the original draft, and participated in preparation of responses to reviewers.	50	Antoni Taraszkiewicz
Izabela Sinkiewicz	I supervised the research, co-developed the methods, critically revised and edited the manuscript, and participated in preparation of responses to reviewers.	30	
Agata Sommer	I assisted with journal formatting, article revision, and preparation of responses to reviewers.	5	Agata Sommer
Małgorzata Dąbrowska	I assisted with computations and literature search.	5	
Hanna Staroszczyk	I supervised the research, provided administrative and strategic guidance, critically reviewed and edited the manuscript, coordinated responses to reviewers, and served as the corresponding author.	10	

Gdańsk, 08.12.2022

Statement on the percentage of participation in the creation of scientific article

Authors of the following article:

Taraszkiewicz Antoni, Sinkiewicz Izabela, Sommer Agata, Dąbrowska Małgorzata, Staroszczyk Hanna. Prediction of Bioactive Peptides from Chicken Feather and Pig Hair Keratins using *In Silico* Analysis Based on Fragmentomic Approach. *Current Pharmaceutical Design*, 2022, 28(10), 841-851. DOI: 10.2174/1381612828999220114150201

state that the percentage of their participation in its creation is as follows:

- Antoni Taraszkiewicz – 50%
- Izabela Sinkiewicz – 30%
- Agata Sommer – 5%
- Małgorzata Dąbrowska – 5%
- Hanna Staroszczyk – 10%

Hanna Staroszczyk
Agata Sommer
Izabela Sinkiewicz
Małgorzata Dąbrowska
Antoni Taraszkiewicz

Gdańsk, 26.08.2025

Co-authors' declaration regarding the description of contribution and percentage share in the preparation of the scientific manuscript

Taraszkiewicz, A., Sinkiewicz, I., Sommer, A., Kusznierewicz, B., Giblin, L., & Staroszczyk, H. (2025). Chemical composition and techno-functional properties of high-purity water-soluble keratein and its enzymatic hydrolysates. *Food Chemistry*, 472, 142641. https://doi.org/10.1016/j.foodchem.2024.142641

Author	Description of contribution	Percentage of contribution	Signature
Antoni Taraszkiewicz	I conceptualized and designed the study, co-developed the methods, performed all experiments, data analysis and interpretation, curated the dataset, prepared figures and tables, provided resources, secured funding, managed project administration, wrote the original draft, and participated in the preparation of responses to reviewers.	44	Antoni Taraszkiewicz
Izabela Sinkiewicz	I supervised the research, co-developed the methods, provided resources, verified the data, revised the manuscript, and participated in the preparation of responses to reviewers.	14	
Agata Sommer	I assisted with experiments, preparation of figures and data analysis, co-developed the methods, revised the manuscript, and participated in the preparation of responses to reviewers.	14	Agata Sommer
Barbara Kuźnierewicz	I led the development of the HP-SEC analytical method and provided guidance on data interpretation.	4	B.
Linda Giblin	I supervised the research, provided resources, contributed to funding acquisition, critically reviewed and edited the manuscript, and performed a native-speaker English language check.	10	
Hanna Staroszczyk	I supervised the research, provided administrative and strategic guidance, co-developed the methods, critically reviewed and edited the manuscript, coordinated responses to reviewers, and served as the corresponding author.	14	

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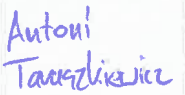
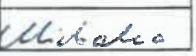
Co-authors' declaration regarding the description of contribution and percentage share in the preparation of the scientific manuscript

Staroszczyk, H., Sommer, A., Taraszkiewicz, A., & Michalec M. Changes in the structure and thermal properties of feather keratin induced by L-Cys extraction followed by enzymatic hydrolysis *(under review)*

Author	Description of contribution	Percentage of contribution	Signature
Hanna Staroszczyk	I conceptualized the study, developed the methods, supervised the research, prepared figures and tables, analysed and interpreted the data, wrote the original draft, reviewed and edited the manuscript, managed project administration, contributed to funding acquisition and served as the corresponding author.	50	
Agata Sommer	I prepared samples for analysis, performed FT-IR, TGA and DSC measurements, data analysis and interpretation and assisted with literature search and manuscript revision.	30	
Antoni Taraszkiewicz	I curated the dataset, secured funding, assisted with sample preparation, FT-IR, TGA and DSC measurements, project administration, literature search and manuscript revision.	15	
Marek Michalec	I performed XRD measurements.	5	

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Co-authors' declaration regarding the description of contribution and percentage share in the preparation of the scientific manuscript

Taraszkiewicz, A., Sommer, A., Sinkiewicz, I., Koss-Mikołajczyk, I., Kusznierewicz, B., Giblin, L., & Staroszczyk, H. (2025). Valorising feather keratin: Antioxidant activity, cytotoxicity and mutagenicity during processing and gastrointestinal digestion. *Food Chemistry*, *493P2*, 145820. https://doi.org/10.1016/j.foodchem.2025.145820

Author	Description of contribution	Percentage of contribution	Signature
Antoni Taraszkiewicz	I conceptualized and designed the study, co-developed methods, performed all experiments, data analysis and interpretation, curated the dataset, prepared tables and assisted with figures, secured funding, managed project administration, wrote the original draft, and participated in the preparation of responses to reviewers.	50	Antoni Taraszkiewicz
Agata Sommer	I performed data analysis, verification and interpretation, prepared figures, co-developed methods, assisted with experiments, critically revised the manuscript, and participated in the preparation of responses to reviewers.	16	A. Sommer
Izabela Sinkiewicz	I co-developed methods, provided resources, verified the data, and assisted with manuscript revision.	6	
Izabela Koss-Mikołajczyk	I assisted with the preparation of figures and provided advice regarding the MTT and Ames tests.	4	Koss-Mikołajczyk
Barbara Kusznierewicz	I led the development of the HP-SEC with post-column ABTS derivatization analytical method and provided guidance on data interpretation.	4	
Linda Giblin	I supervised the research, provided resources, co-developed methods, contributed to funding acquisition, verified the data, critically reviewed and edited the manuscript, and performed a native-speaker English language check.	10	
Hanna Staroszczyk	I supervised the research, co-developed the methods, critically reviewed and edited the manuscript, coordinated responses to reviewers, and served as the corresponding author.	10	

Gdańsk, 26.08.2025

Co-authors' declaration regarding the description of contribution and percentage share in the preparation of the scientific manuscript

Taraszkiewicz, A., Sinkiewicz, I., Sommer, A., & Staroszczyk, H. (2024). The biological role of prolyl oligopeptidase and the procognitive potential of its peptidic inhibitors from food proteins. *Critical Reviews in Food Science and Nutrition*, 64(19), 6567–6580. https://doi.org/10.1080/10408398.2023.2170973

Author	Description of contribution	Percentage of contribution	Signature
Antoni Taraszkiewicz	I conceptualized the review, conducted the literature search using the Scopus, Web of Science, PubMed, Google Scholar, UniProt and BIOPEP-UWM databases, handled project administration, wrote the original draft, and participated in the preparation of responses to reviewers.	70	Antoni Taraszkiewicz
Izabela Sinkiewicz	I supervised the work, reviewed and edited the manuscript, and participated in the preparation of responses to reviewers.	10	
Agata Sommer	I reviewed and edited the manuscript and participated in the preparation of responses to reviewers.	10	Agata Sommer
Hanna Staroszczyk	I supervised the work, provided administrative assistance, reviewed and edited the manuscript, coordinated responses to reviewers, and served as the corresponding author.	10	