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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

SCYDLCRPCGPTPLANSCNEPCVRQCQDSRVVIQPSPVVVTLPGPILSSFPQNTAVGSSTSAAVGSI
LSEEGVPISCGGFGISGLGSRFSGRRCLPC

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

SCY - D - L - CRPCG - **PT** - **PL** - A - N - SCN - E - PC - **VRQ** - CQ - D - SR - V - V - **IQ** - **PSP** - V - V - **VT** - L - **PG** - P - **IL** - **SSF** - **PQ** - N - T - A - **VG** - **SST** - SA - A - **VG** - S - **IL** - **SE** - E - G - **VP** - ISCG - G - F - G - **ISG** - L - G - **SRF** - 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

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), VPSSCQ(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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