Identifying Bioactive Peptides from Poultry By-Products

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Abstract.
Bioactive peptides derived from food proteins are becoming increasingly popular ingredients due to their beneficial effect on the immune system and other functional properties. We aimed to develop a technology for obtaining peptides from poultry by-products and identify their bioactivity.

Pepsin was the main reagent for the in vitro enzymatic hydrolysis. Specialized equipment and methods were used to determine the key indicators. The molecular weight and bioactivity of the resulting peptides were calculated by using the Peptide Mass Calculator and PeptideRanker online resources.

First, we developed a flow chart for obtaining bioactive peptides and produced hydrolysates from poultry by-products. The hydrolysates had identical physicochemical parameters, with no significant differences. The molecular weight distribution revealed that most protein fractions were represented by peptides with a molecular weight below 20 kDa. Then, we evaluated the bioactivity of the peptides. The hydrolysate obtained using pepsin with an activity of 30 units per 100 g of material showed higher bioactivity in the FD peptides (0.922094). The hydrolysate obtained using pepsin with an activity of 45 units per 100 g of material had greater bioactive properties in the CYG peptides (0.947378).

Based on the results, we designed a flow chart for obtaining hydrolysates from poultry by-products and evaluated the bioactive properties of the peptides obtained. For further work, these properties should be confirmed by in vitro experiments to determine the reliability of our data and identify specific bioactive properties of the peptides.

Keywords. Peptides, hydrolysis, hydrolysates, waste-free technologies, in vitro, bioactive properties

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Получение и идентификация биоактивных пептидов из вторичных сырьевых ресурсов переработки птицы

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Аннотация.
Биоактивные пептиды, полученные из пищевых белков, становятся все более популярными на рынке пищевых ингредиентов. Они способствуют укреплению иммунного статуса организма, а также обладают другими функциональными свойствами. Цель исследования состояла в разработке технологии получения пептидов из вторичных сырьевых ресурсов переработки птицы и идентификации их биоактивности.

В качестве основного реагента для проведения исследования использовался фермент пепсин. Ферментативный гидролиз проводили in vitro. Для определения основных показателей применяли специальное оборудование и методики. Молекулярную массу и биоактивность полученных пептидов рассчитывали с помощью онлайн-ресурсов Peptide Mass Calculator и PeptideRanker.

На первом этапе исследования была разработана принципиальная схема производства биоактивных пептидов. Были получены гидролизаты из вторичных сырьевых ресурсов переработки птицы. По физико-химическим показателям сухие гидролизаты были идентичны друг другу, значимых различий не выявлено. Из результатов анализа молекулярно-массового распределения выявлено, что основные фракции представлены пептидами с молекулярной массой ниже 20 кДа. В гидролизате образца № 1, полученного с применением пепсина активностью 30 ед. на 100 г сырья, большей биоактивностью обладают пептиды FD. Их биоактивные свойства равны 0,922094 ед. Три пептидные последовательности гидролизата образца № 2, полученного с применением пепсина активностью 45 ед. на 100 г сырья, обладают биоактивными свойствами. Большей биоактивностью обладают пептиды CYG (0,947378 ед.).

Была разработана принципиальная схема получения гидролизатов из вторичных сырьевых ресурсов переработки птицы. Проведена оценка биоактивных свойств полученных пептидов. Для дальнейшей работы биоактивные свойства следует подтверждать экспериментальными исследованиями in vitro, которые помогут определить достоверность полученных данных и конкретные биоактивные свойства изучаемых пептидов.

Ключевые слова. Пептиды, гидролиз, гидролизаты, безотходные технологии, in vitro, биоактивные свойства

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Introduction
Meat is a source of complete protein in the human diet. Meat proteins have a high nutritional value and therefore contribute to the normal physiological status. They are converted into various forms during cooking, processing, and digestion. For example, bioactive peptides obtained from meat proteins through an enzymatic reaction can help maintain the immune status of the human body. Not only muscle meat is a good source of protein and bioactive peptides, but offal is as well. Therefore, meat by-products have increasingly been studied and used to produce functional ingredients and bioactive peptides [1–6].
Consumers are becoming increasingly aware of the immune-boosting properties of bioactive peptides derived from dietary proteins. Bioactive peptides are small fragments of dietary proteins, mostly consisting of 2–20 amino acid residues. They can be ligands and therefore have many targets in the human body, such as the immune, cardiovascular, digestive, and endocrine systems [7–11].

Hydrolysis is the main process of isolating peptides. Many studies confirm that the proteins of meat by-products are hydrolyzed by proteolytic enzymes under controlled conditions, enabling bioactive peptides to form. The main proteolytic enzymes that can be used to hydrolyze proteins are pepsin, trypsin, chymotrypsin, corolase, papain, as well as enzymes of microbial origin, such as Neutrase from Bacillus amyloliquefaciens and Alcalase from Bacillus licheniformis [12–15].

The efficacy and safety of bioactive peptides must be proven by clinical studies on living organisms before they can be approved for use by regulatory and supervisory authorities [16–18]. Some food-derived peptides have antioxidant, immunomodulatory, antihypertensive, anticancerous, anti-inflammatory, antimicrobial, hypocholesterolemic, intestine-modulatory, antidiabetic, opioid, and metal-chelating properties [19–23]. Their biological activity mainly depends on their amino acid composition, sequence, length, and charge [24, 25].

Currently, hundreds of peptides with different biological action have been identified and isolated from various food sources, including milk, whey, eggs, fish, rice, soybeans, peanuts, chickpeas, corn, and algae [24]. However, only a few of them are marketed as functional nutraceutical products. For example, bioactive peptides derived from milk and fish are more commonly used as food ingredients than peptides from other food sources. Table 1 presents some bioactive peptides obtained from various protein sources and lists their properties [26].

Antioxidant peptides usually contain hydrophobic amino acids and residues of histidine, phenylalanine, tryptophan, or tyrosine.

Thus, the food industry needs to develop technologies for isolating bioactive peptides from by-products, including meat by-products.

We aimed to develop a technology for obtaining peptides from poultry by-products and study the bioactivity of some peptide sequences.

To achieve this aim, we set to:
– select enzymes and develop a scheme for hydrolyzing a homogeneous mass of chicken skin;
– develop two alternative schemes for producing bioactive peptides through the hydrolysis of poultry by-products;
– conduct an electron microscopy of the obtained hydrolysates;
– determine the main physicochemical parameters of the hydrolysates; and
– perform a comparative analysis of the peptides.

**Study objects and methods**

We studied broiler chicken skin in a homogeneous state. First, we selected proteolytic enzymes and hydrolysis conditions that could increase the bioactivity and yield of hydrolysates. In particular, we chose a protein source and peptide sequence that would be most effective for isolation and isolation of bioactive peptides.

### Table 1. Some bioactive peptides and their properties

| Protein source         | Peptide sequence                                                                 | Bioactive properties          |
|------------------------|----------------------------------------------------------------------------------|-------------------------------|
| Cow’s milk whey lactoferrin | Glu-Asn-Leu-Pro-Glu-Lys-Ala-Asp-Arg-Asp-Glu-Tyr-Glu-Leu | Osteoblast-proliferating     |
| Beans (Phaseolus vulgaris) | Gly-Leu-Thr-Ser-Lys, Leu-Ser-Gly-Asn-Lys, Gly-Glu-Gly-Asp-Glu-Ala, Met-Pro-Ala-Cys-Gly-Ser-Ser, and Met-Thr-Glu-Glu-Tyr | Anticancerous                  |
| Soy                    | Met-Ile-Thr-Leu-Ala-Ile-Pro-Val-Asn-Lys-Pro-Gly-Arg                               | Immunomodulatory              |
| Chicken egg lysozyme   | Val-Ala-Trp-Arg-Asn-Arg-Cys-Lys-Gly-Thr-Asp, Trp-Arg-Asn-Arg-Cys-Lys-Gly-Thr-Asp, Ala-Trp-Ile-Arg-Gly-Cys-Arg-Leu, Trp-Ile-Alg-Gly-Cys-Arg-Leu, and Ile-Arg-Gly-Cys-Arg-Leu | Antioxidant                    |
| Egg white              | Ala-Glu-Glu-Glu-Tyr-Pro, Asp-Glu-Asp-Thr-Gln-Ala-Met-Pro, Pro-Val-Asp-Glu-Asn-Asp-Glu-Gly, Gln-Pro-Ser-Ser-Val-Asp-Ser-Gln-Thr-Ala-Met, and Gln-Glu-Arg-Tyr-Pro | Antioxidant                    |
| Casein                 | Tyr-Gln-Lys-Phe-Pro-Gln-Thr-Leu-Gln-Tyr                                          | Antihypertensive               |
| Egg yolk               | Ile-Arg-Trp and Ile-Gln-Trp                                                       | Antidiabetic                  |
| Tuna                   | Gly-Asp-Leu-Gly-Lys-Thr-Thr-Thr-Val-Ser-Asn-Asp-Thr-Ser-Pro-Lys-Thr-Lys-Thr-Pro    | Antihypertensive               |
| Freshwater clam (Corbicula fluminea) | Val-Lys-Pro and Val-Lys-Lys                                                   | Hypocholesterolemic           |
| Soy                    | Tyr-Val-Val-Asn-Pro-Asp-Asn-Asp-Glu-Asn and Tyr-Val-Val-Asn-Pro-Asp-Asn-Asp-Glu-Asn | Hypocholesterolemic           |
pepsin produced by Meito Sangyo Co. (Japan) for our experiments as a potentially more suitable enzyme for obtaining peptides from poultry by-products (Table 2). As can be seen in Table 2, the enzyme has high proteolytic properties and is activated at temperatures that are favorable for protein structures.
Enzymatic hydrolysis was carried out in vitro. MM-5 laboratory magnetic stirrers were used to ensure a uniform treatment of the materials with an enzyme solution throughout the experiment (100 rpm, 28 ± 2°C). The acidity was maintained with 1M hydrochloric acid. The hydrolysates were dried in a B-290 Mini Spray Dryer (Buchi, Sweden) at 100 ± 2°C and at a solution supply rate of 3.5–4.0 mL/min. The dried hydrolysates were microphotographed with a JEOL JED-2300 electronic microscope (Japan). The mass fraction of protein was determined on a RapidN Elementar nitrogen (protein) analyzer. This analyzer uses the Dumas method that involves combusting the samples and registering total nitrogen on a thermal conductivity detector. Molecular weight distribution was performed by polyacrylamide gel electrophoresis in the presence of an anionic detergent (sodium dodecyl sulfate). The amino acid sequence of the peptides was determined by matrix-activated laser desorption/ionization on a MALDI Biotyper (Bruker). The molecular weight was calculated by using the Peptide Mass Calculator. The bioactivity of the peptides was assessed in silico using the PeptideRanker online server that ranks peptides by the predicted probability of their bioactivity. The structure of the peptides was modeled by using the PepDraw online tool.

The experiments were performed at the Department of Animal Origin Food Technology and the Scientific and Educational Center of the Research and Innovation Department, Kemerovo State University.

Results and discussion

The flow chart for producing bioactive peptides from poultry by-products is presented in Fig. 1. It includes two sets of parameters for enzymatic hydrolysis of a homogeneous mass of chicken skin.

Chicken skin was homogenized with a laboratory homogenizer. The homogeneous mass was hydrolyzed with pepsin in two variations during 12 h at 28 ± 2°C. The enzyme was then thermally inactivated at 45 ± 2°C and neutralized with a weak alkali to pH 7.0 ± 2.0. Next, the hydrolysate was fractionated into protein and fat parts using a CM 6 M Multi centrifuge at 3000 rpm. The protein part was then subjected to thermal water extraction in order to dissolve the protein fractions at 70 ± 5°C. The resulting solution was filtered using a MFU-R-45-300 laboratory ultrafiltration unit (Russia) at a controlled pressure of 3.0 bar and a difference of 0.2–0.5 kgf/cm² in the discharge and return headers. The protein part of the solution went into the retentate, while the water and minerals went into the permeate. Further, protein hydrolysate samples were obtained by spray drying at 90 ± 2°C and a solution supply rate of 3.0 ± 0.2 mL/min. The samples were then microphotographed with an electronic microscope (magnified 500 times), as can be seen in Fig. 2.

First, we evaluated the color and particle size of the hydrolysates. As we can see in Fig. 2, the samples differed in color, with sample 1 having a darker creamy color and sample 2 having a whitish color. Also, the hydrolysates differed in particle size, although they were dried under the same conditions. In particular, sample 2 had a more finely dispersed structure, which can be explained by a deeper hydrolysis of this sample.

Next, we determined the main physicochemical parameters of the hydrolysates for further studies (Table 3).

As can be seen in Table 3, the samples had no significant differences in physicochemical parameters. Since the hydrolysates had a high protein content (over 90%), they can be classified as a high-protein product.

Next, we analyzed the distribution of protein fractions by polyacrylamide gel electrophoresis in the

| Parameters, % | Sample 1 | Sample 2 |
|---------------|----------|----------|
| Protein       | 90.7 ± 0.1 | 91.4 ± 0.2 |
| Fat           | 0.60 ± 0.03 | 0.40 ± 0.06 |
| Moisture      | 8.1 ± 0.1  | 7.8 ± 0.2  |
| Ash           | 0.60 ± 0.04 | 0.40 ± 0.03 |
presence of an anionic detergent, sodium dodecyl sulfate. Table 4 shows the molecular weight distribution of the hydrolysates.

As can be seen in Table 4, most peptide fractions had a molecular weight below 20 kDa, especially those in sample 2. Thus, we can assume that the hydrolysates contained peptides with bioactive properties, especially sample 2.

Then, we determined the amino acid sequence of the hydrolysates under study (Table 5).

According to Table 5, sample 1 had a chain of 41 peptides, while sample 2 was represented by 27 peptides. We can also see that the first and the second variants of hydrolysis, which used pepsin with lower and higher activity, respectively (Fig. 1), split the protein into 14 and 8 fragments of amino acids (peptide sequences), respectively.

Next, we used online resources to determine the molecular weight and bioactivity of individual peptide sequences, as well as modelled their structure (Tables 6 and 7).

The PeptideRanker service has a threshold value of 0.5 for peptides’ bioactive properties, i.e. any peptide with an estimated value above 0.5 is ranked as bioactive. However, literature shows that using a higher threshold, particularly 0.8, reduces the number of false positive results. Therefore, we assessed the bioactive properties of peptides based on a threshold bioactivity value of 0.8 and the maximum value of 1.

According to Table 6, the FD peptides (38–39 in the sequence) and the NW peptides (40–41 in the sequence) had greater bioactivity values of 0.922094 and 0.934148, respectively. Their structure shows aromatic rings which are mainly represented by the aromatic α-amino acid of tyrosine.

As can be seen in Table 7, sample 2 had three peptide sequences with high bioactive properties. They were the CYG peptides (25–27), the GHG peptides (13–15), and the AYG peptides (22–24), with bioactivity values of 0.947378, 0.839383, and 0.815664, respectively. Structurally, these bioactive peptides had aromatic rings represented by the aromatic α-amino acid of tyrosine.

We found no correlation between the bioactivity values of the peptides and their molecular weight.
Table 6. Individual peptide sequences for hydrolysate Sample 1

| The number of peptide in the chain | Peptide sequence in a one-letter code* | Molecular weight, g/mol | Bioactivity | Peptide structure |
|-----------------------------------|---------------------------------------|-------------------------|-------------|------------------|
| 1–4                               | PILG                                  | 398                     | 0.492674    |                  |
| 5–8                               | PILV                                  | 440                     | 0.291507    |                  |
| 9–11                              | ILA                                   | 315                     | 0.237443    |                  |
| 12–14                             | ILG                                   | 301                     | 0.480965    |                  |
| 15–17                             | ILV                                   | 343                     | 0.123381    |                  |
| 18–20                             | ILT                                   | 345                     | 0.154222    |                  |
| 21–23                             | PSV                                   | 301                     | 0.166459    |                  |
| 24–26                             | GVS                                   | 261                     | 0.109533    |                  |
| 27–29                             | HGL                                   | 325                     | 0.446783    |                  |
| 30–32                             | HVI                                   | 367                     | 0.0887486   |                  |
| 33–35                             | SVP                                   | 301                     | 0.194373    |                  |
| 36–37                             | SV                                    | 204                     | 0.0523218   |                  |
| 38–39                             | FD                                    | 280                     | 0.922094    |                  |
| 40–41                             | NW                                    | 318                     | 0.934148    |                  |

* A – alanine; C – cysteine; D – aspartic acid; E – glutamic acid; F – phenylalanine; G – glycine; H – histidine; I – isoleucine; K – lysine; L – leucine; M – methionine; N – asparagine; P – proline; Q – glutamine; R – arginine; S – serine; T – threonine; V – valine; W – tryptophan; Y – tyrosine.

* A – аланин; C – цистеин; D – аспартат; E – глутамат; F – фенилаланин; G – глицин; H – историдин; I – изолейцин; K – лизин; L – лейцин; M – метионин; N – аспарагин; P – пролин; Q – глутамин; R – аргинин; S – серин; T – треонин; V – валин; W – триптофан; Y – тирозин.
products. Then, we evaluated the bioactive properties of the peptides obtained. The hydrolysate sample obtained with a lower pepsin activity (30 units per 100 g of material) had greater bioactivity values in the FD and NW peptides (0.922094 and 0.934148, respectively). The sample obtained with a higher pepsin activity (45 units per 100 g of material) had higher bioactivity in the CYG, GHG, and AYG peptides (0.947378 units, 0.839383, and 0.815664, respectively). However, these data obtained in silico need to be confirmed by in vitro experiments for further work. Such experiments will determine their reliability and identify specific bioactive properties of the studied peptides.

**Conclusion**

Based on the research results, we designed a flow chart for obtaining hydrolysates from poultry by-products. Then, we evaluated the bioactive properties of the peptides obtained. The hydrolysate sample obtained with a lower pepsin activity (30 units per 100 g of material) had greater bioactivity values in the FD and NW peptides (0.922094 and 0.934148, respectively). The sample obtained with a higher pepsin activity (45 units per 100 g of material) had higher bioactivity in the CYG, GHG, and AYG peptides (0.947378 units, 0.839383, and 0.815664, respectively). However, these data obtained in silico need to be confirmed by in vitro experiments for further work. Such experiments will determine their reliability and identify specific bioactive properties of the studied peptides.
Contribution
The authors were equally involved in writing the manuscript and are equally responsible for plagiarism.

Conflict of interest
The authors declare that there is no conflict of interest regarding the publication of this article.

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