Determination of characteristic species-specific protein zones of fish fermentolysates using the method of electrophoretic analysis

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Abstract. The article presents studies on the justification of the most effective technological modes of enzymatic hydrolysis of secondary fish cutting products by the enzyme preparation Collagenase. The optimal duration of enzymatic hydrolysis was established, which is 16 hours for skin fermentolysates, 24 hours for scales fermentolysates and 36 hours for fins fermentolysates. The molecular weights of the protein zones of the obtained fermentolysates are determined, which are mainly represented by low molecular weight digestible proteins with a molecular weight of 13 to 33 kD, which indicates the high availability and degree of protein destruction by human gastrointestinal tract enzymes (from 92.39 to 96.87%).

1. Introduction
The formation of market relations, their further development and improvement require a clear approach to the characteristics of food systems, the problem of multilateral study of which is the most relevant, since the modern technology of their production has undergone significant changes. They relate to the feedstock and auxiliary components used at all stages of production, including new technologies for the production of protein products from secondary raw materials of fish origin.

Protein-containing wastes of the fishing industry are a source of collagen and its hydrolysis products, which are widely used in many sectors of the economy. The main structural unit of collagen is tropocollagen, which consists of three chains, each of which includes 1000 amino acid residues. Collagen is rich in glycine and proline. Collagen contains rare hydroxyproline and hydroxylysine [1–3].

Collagen is the main fibrous structure of the skin, bones, tendons, cartilage and swim bladder of fish. A collagen-like substance is also isolated from fish scales and muscles. Collagen of fish, depending on the type of source of origin, is divided into fibrous collagen of the dermis of skins and tendons, hyaline collagen of bone tissue - ossein, chondrin collagen of cartilage, ichthyaline collagen of a fish bubble - ichthyocol, collagen of fish fins - ichthylepilin and collagen of fish scales - ichthyolipidine [2, 4].

Recently, interest in fish collagen has increased significantly, due to the fact that spongiform encephalopathy (cattle rabies disease) has become such a serious problem that the use of collagen of...
animal origin becomes unsafe. Science does not have information about the transmission of viruses from fish skin to humans. In addition, fish collagen is hypoallergenic (as 96% identical to human protein) [5].

Almost any existing method for processing collagen-rich raw materials involves the breakdown of protein macromolecules to monomers included in it. The resulting protein hydrolysates contain biologically valuable compounds such as polypeptides and free amino acids.

The transformation of the structure of connective tissue into a form easily digestible by the human body includes physical and enzymatic treatment that causes the breakdown of hydrolytic collagens. The most promising methods for the destruction of collagen are the methods using specific enzymes, as it becomes possible to obtain desalted hydrolysates; mild processing conditions preserve the full set of amino acids and the nutritional value of the resulting products, their solubility and digestibility are significantly increased; the accumulation of protein degradation products by molecular weight and functionality can be controlled [6, 7].

It should be noted that the potential use of such products is not limited to consumer products. There are literature data that collagen processing products are successfully used in medical nutrition, as well as in the nutrition of people who lead an active lifestyle, as a protein fortifier in various protein-containing complexes.

Among the existing methods for producing protein hydrolysates, enzymatic hydrolysis is considered the most promising. Its feature, in contrast to chemical methods, is hydrolysis at a temperature of 35-50°C. In this case, the destruction of amino acids and their racemization do not occur [8].

When selecting an enzyme preparation for the processing of fish secondary products of cutting (SPC), splitting connective tissue proteins and producing protein fermentolysates, the following factors were taken into account: the activity of the enzyme preparation and its specific action with respect to collagen.

It is known from published data [7, 8], that native collagen is not hydrolyzed by conventional peptide hydrolases. The main enzyme of its catabolism is collagenase, which cleaves peptide bonds between glycine and leucine residues at once in three \( \alpha \)-chains of tropocollagen at approximately \( \frac{1}{4} \) distance from the C-terminus. The resulting fragments are water-soluble, they spontaneously decompose into separate chains, which are hydrolyzed by various proteases to amino acids. As a result, the molecular weight of the protein changes and its absorption by the body increases.

Thus, the task of developing effective technologies for fish hydrolysates is urgent, since it is inextricably linked with the problem of waste-free rational use of aquatic biological resources, which is extremely important, both from a scientific and economic point of view.

2. Materials and methods
To study the effect on collagen-containing fish raw materials, an enzyme preparation from hepatopancreas of the king crab Collagenase was selected, produced by Bioprogress LLC according to TU 9154-032-11734126-10.

To assess the efficiency of the effect of collagenase on collagen proteins of scales, skins, fins of pink salmon (lat. Oncorhynchus gorbuscha) and silver carp (lat. Hypophthalmichthys), enzymatic hydrolysis was carried out in an aqueous solution with a pH of 6.5. Dosages of the enzyme preparation, justified taking into account the experimentally established proteolytic activity of collagenase, were 0.005; 0.01; 0.015; 0.02; 0.025; 0.03; 0.035; 0.04; 0.045 and 0.05% by weight of the raw material. The hydromodule (the ratio of raw materials : water) was 1: 8, taking into account the complete immersion of the investigated raw materials in the solution, the temperature of the hydrolyzable mixture was 37°C [17].

To separate the mixture of proteins of fish SPC into fractions based on the movement of charged protein macromolecules of different molecular weights in a stationary electric field, electrophoresis (EF) was performed in vertical glass plates in 7.5% polyacrylamide gel (PAAG) at a current of 50 mA / gel at the Bioklon plant [11, 12].
The following proteins were used as taps: Bovine serum albumin (BSA) with a molecular weight of 67,000 Da; Trypsin — molecular weight 24,000 Da; Lysozyme - molecular weight 14,600 Da [13–15].

Samples were prepared as follows: 20 mg of the protein preparation was dissolved in 0.1 ml of distilled water and brought to a final volume of 10 ml with water, after dissolution, an equal volume of solubilizing solution was added and the samples were boiled in a water bath for 3 minutes. 25 mcL of the sample was applied to the pocket of the concentration gel. Next, electrophoresis was performed. After EF, the PAAG gel was removed from the glass plates and placed for 30 min in a staining solution containing 0.05% Coomassie R-250, 8% CH₃COOH, and 15% C₂H₅OH. The gel was washed with hot 10% acetic acid for several days with a periodic change of acid. The resulting gels were placed in a solution of alcohol: glycerol (1: 1, v/v) with a double change of solution, then dried on glass plates in cellophane (Balakovo), and then scanned with a resolution of 300 dpi on an HP Scanjet 3770 scanner in a window for transparent materials (Osterman, 1981).

3. Results and discussion

Since the graphical dependences characterizing biochemical processes involving enzymes specific for the hydrolysis of substrates of different nature, as a rule, have the form of smooth curves that reach a plateau after reaching the so-called “saturation point”, the goal of this stage of the work was to determine the optimal dosages of collagenase to the mass of raw materials providing the effect of maximum hydrolysis of collagen-containing raw materials at the lowest dosage of the enzyme [16].

The criterion for the efficiency of hydrolysis of protein fractions of collagen-containing raw materials of fish origin was the accumulation of hydrolysis products containing peptide bonds in the liquid fraction of the hydrolyzate.

Experimental data on the influence of the mass fraction of collagenase on the accumulation of products of hydrolytic decomposition of the components of fish SPC are presented in Figure 1.

A comparative analysis of the obtained graphical dependence showed (Fig. 1) that the maximum accumulation in the liquid fraction of fish protein fermentolysates of collagen hydrolytic decomposition products is achieved at different dosages of collagenase depending on the type of raw material. The greatest accumulation of hydrolyzed protein was observed during enzymatic hydrolysis of silver carp skins (8.1 mg/cm³) and pink salmon (7.5 mg/cm³), with the lowest dosage of the enzyme 0.02-0.025% by weight of the raw material. At the same time, the accumulation of hydrolyzed protein in the liquid fraction of the hydrolyzate during the processing of the pink salmon fins and silver carp fins with collagenase was less intense than during the treatment of skins and scales with the largest mass fraction of the introduced enzyme.

From the data in Figure 1, we can additionally conclude that the optimal dosages of collagenase to the mass of raw materials for the complete hydrolytic decomposition of the components of the fish SPC were: for pink salmon skins - 0.02%; for silver carp skin - 0.025%; for pink salmon scales - 0.03%; for silver carp scales - 0.035%; for pink salmon fins - 0.035%; for silver carp fins - 0.04%.

Thus, for a more complete hydrolytic decomposition of the components of SPC of silver carp, a higher concentration of the enzyme is required than for similar raw materials obtained during cutting of pink salmon.

To justify the duration of the enzymatic treatment of fish SPC, the established optimal dosages of the enzyme preparation Collagenase for each type of secondary collagen-containing raw material were introduced into the reaction mixture. The duration of enzymatic hydrolysis ranged from 4 to 40 hours. The effectiveness of the enzyme was judged by the accumulation of hydrolyzed protein (Fig. 2).
Figure 1. The influence of the mass fraction of collagenase on the accumulation of products of hydrolytic decomposition of the components of fish SPC.

Figure 2. The effect of the duration of enzyme treatment on the accumulation of products of hydrolytic decomposition of the components fish SPC.
It was experimentally established (Fig. 2) that it is advisable to carry out enzymatic processing of various types of fish SPC at the selected optimal dosages of collagenase for 16-36 hours, depending on the type of collagen-containing raw materials, since further incubation of the reaction mixtures does not lead to an increase in target indicators. The optimal duration of enzymatic hydrolysis is 16 hours for skin enzymes, 24 hours for scales enzymes and 36 hours for fin enzymes.

When the processing time was shorter than these indicators, there was insufficient hydrolysis of protein fractions by the collagenase enzyme, and therefore, their complete extraction from collagen-containing raw materials was not achieved.

The results of studies to substantiate the most effective technological modes of enzyme hydrolysis of fish SPC allowed us to offer a comprehensive technology for processing scales, fins, and skins of pink salmon and silver carp as the main raw material for obtaining dry food fermentolyzates.

An increase in the concentration of hydrolyzed protein in fermentalyzates and, accordingly, the degree of enzyme hydrolysis correlates well with changes in other parameters of fermentalyzates, such as, for example, its molecular weight composition [9, 10].

In order to determine the molecular weights of the protein zones, Lammley electrophoresis was performed in the presence of sodium dodecyl sulfate (SDS) with protein taps of known molecular weights. (Laemmli, 1970).

Protein spectra of fermentolyzates are presented in Figure 3.

During the experiment, it was proved that all protein zones that appeared in the gel during electrophoresis can be divided into 3 zones: the upper, cathode, with $R_f$ from 0.013 to 0.465 (the most "heavy" proteins with a molecular weight of 36,000 to 90,000 Da); the middle part of the gel with $R_f$ from 0.507 to 0.653 (proteins with a molecular weight of from 20,000 to 33,000 Da); the lower part, the anode, with $R_f$ from 0.792 to 0.889 (low molecular weight proteins from 13,000 to 16,000 Da) (Table 1).

Samples of fermentolyzates from pink salmon fins and silver carp fins have absolutely similar protein spectra with zones located in the middle and lower parts of the polyacrylamide gel.

![Figure 3. Protein spectrum of fermentolyzates: 1 - from pink salmon fins; 2 - from silver carp fins; 3 - from pink salmon scales; 4 - from silver carp scales; 5 - from pink salmon skin; 6 - from silver carp skin; M – protein taps: BSA - bovine serum albumin. The arrow shows the current direction.](image-url)
The spectrum of the fermentolizate from pink salmon scales is similar to that of the fins, except that protein zones with Rf 0.653 (molecular weight 20400 Da) are present in samples 3 (fermentolyzate from silver salmon scales) and 4 (fermentolyzate from silver carp scales) in minor amounts. The sample of fermentolysate from silver carp scales is distinguished by the presence of pronounced zones with Rf 0.097 and 0.111 (molecular weight 76000 and 74000 Da, respectively), which are absent in the first three samples, but present in sample No. 6 (fermentolyzate from silver carp skin).

Table 1. Molecular weights of fermentolyzate proteins

| Protein zone | Rf, relative unit | Lg Mr | Molecular weight, Da | Sample No., zone availability |
|--------------|------------------|-------|----------------------|------------------------------|
| BSA          | 0.236            | 4.83  | 67000                |                              |
| Trypsin      | 0.569            | 4.38  | 24000                |                              |
| Lysozyme     | 0.888            | 4.16  | 14600                |                              |
| №1           | 0.013            |       | At the beginning     | 6                            |
| №2           | 0.028            | 4.95  | 89000                | 6                            |
| №3           | 0.097            | 4.88  | 76000                | 4,6                          |
| №4           | 0.111            | 4.87  | 74000                | 4,6                          |
| №5           | 0.139            | 4.82  | 66000                | 6                            |
| №6           | 0.167            | 4.81  | 64600                | 6                            |
| №7           | 0.208            | 4.79  | 61700                | 6                            |
| №8           | 0.236            | 4.76  | 57500                | 6                            |
| №9           | 0.333            | 4.68  | 47900                | 6                            |
| №10          | 0.465            | 4.56  | 36000                | 6                            |
| №11          | 0.507            | 4.52  | 33000                | 1,2,3,4,5,6                  |
| №12          | 0.542            | 4.42  | 26300                | 1,2,3,4,5,6                  |
| №13          | 0.604            | 4.37  | 23400                | 1,2,3,4,5,6                  |
| №14          | 0.625            | 4.34  | 21900                | 1,2,3,4,5,6                  |
| №15          | 0.653            | 4.31  | 20400                | 1,2,3,4,5,6                  |
| №16          | 0.792            | 4.21  | 16200                | 1,2                          |
| №17          | 0.806            | 4.18  | 15100                | 1,2,3                        |
| №18          | 0.854            | 4.16  | 14500                | 1,2,3                        |
| №19          | 0.875            | 4.13  | 13500                | 1,2,3                        |
| №20          | 0.889            | 4.11  | 13000                | 1,2,3                        |

The largest number of protein zones is possessed by sample No. 6 — fermentolyzate from silver carp skin. Although the division of the bands is not clear enough, nevertheless, 15 protein zones are visualized in the gel in the cathode and middle parts of the gel; however, in the anode part of the gel, as well as the sample from the pink salmon skin, there are no bands.

Thus, a study of the molecular mass distribution of the molecules of the protein fractions of fermentolizes showed that protein zones are represented mainly by low molecular weight digestible proteins with a molecular weight of 13 to 33 kDa, which indicates a high availability and degree of protein destruction by human gastrointestinal tract enzymes (from 92.39 to 96.87%).

4. Conclusion
The obtained fermentolysates are amino acid-peptide mixtures obtained in accordance with processes carried out in a living organism. Therefore, they are physiological, easily absorbed by different methods of introduction. Moreover, they are non-toxic, allergen-free, do not give anaphylactic reactions and other side effects. Such fermentolizes can be recommended to increase the general status of the body, with preventive purposes as the enrichment of food products with easily digestible protein and in the nutrition of people involved in power sports.

Based on the studies, a method for producing fish protein fermentolysates (RF patent No. 2711915 dated 01/23/2020) by introducing the enzyme preparation Collagenase into the reaction system is proposed.
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