Fish supportive, connective and integumentary tissues in food biotechnology

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Abstract. The relevance of the study is due to the insufficient use of the biopotential of the supportive, connective and integumentary tissues of fish (bones, cartilage, fins, heads, scales, skin), which are considered by many scientists as sources of valuable biologically active substances that can be used as functional ingredients in food biotechnology. Collagen and its hydrolysis products, chondroitin sulfate, glycosamine, hyaluronic acid, minerals (calcium, phosphorus, potassium, magnesium, etc.) are the most important components in maintaining human health and, especially, in the prevention and treatment of diseases of the musculoskeletal system. Therefore, the use of hydrolyzed fish tissues as sources of biologically active substances is promising. The biotechnological method of raw material degradation (enzymatic hydrolysis) is the most appropriate, allowing not only to preserve the biopotential of the native tissues, but also to ensure the reliability and sustainability of the production process. The article presents the results of the study of the chemical composition of fish supportive, connective and integumentary tissues, describes the technologies for obtaining new products using these biologically active substances generated by enzymatic hydrolysis.

1. Introduction
Fish products biotechnology currently covers a wide range of scientific knowledge. First of all, they include research in the field of extraction of biologically active substances from raw materials of water origin and their use in the technology of biologically valuable products with specified properties.

According to the Institute of medical and biological problems of the Russian Academy of Sciences, the current health situation in Russia is almost critical, so there are no more than 4% of absolutely healthy people in Russian Federation. At the same time, one of the most common diseases caused by metabolic disorders in the cartilage and bone tissues is osteoarthritis, in which the articular cartilage and adjacent bone tissue are destroyed. It affects 33–48% of the world's population. To prevent this disease, the human diet, along with nutrients for the restoration of degraded tissues, must contain biologically active substances (BAS) involved in their regeneration - vitamins, minerals, antioxidants and minor components.

Secondary natural raw materials, which include the supportive, connective and integumentary tissues of fish (skin, bones, scales, fins, etc.) are considered by scientists as sources of such useful substances for human beings as collagen, glycosaminoglycans, mineral components, lipids [1–5].

There is accurate information about the positive effect of these substances on the metabolism and regeneration of the tissues damaged as a result of metabolic disorders. Collagen, glycosaminoglycans and minerals contained from supportive, connective and integumentary tissues are ready-made "building materials" that can be embedded in the foci of inflammation and reduce its intensity,
participate in the regeneration of damaged tissues. That is why they are part of many medical and preventive medications that support the functions of the human musculoskeletal system.

Minimization of the amount of irrationally recycled by-products of the processing plants by introducing new technologies is an effective way to improve the depth of fish processing and production efficiency. The Department of food biotechnology of the Kaliningrad state technical university is actively working on the research of the potential of fish by-products and developing biotechnologies for using them in production of functional food products.

2. Research objects and methods
The research was carried out using the laboratory facilities of the Department of food biotechnology of the Kaliningrad state technical university (KSTU, Kaliningrad), laboratories of the Atlantic branch of the FGBNU "VNIRO" ("AtlantNIRO") and OOO "Kaliningrad testing center".

As raw materials for the experiments were used fresh, frozen, cooked and dried crushed supportive, connective and integumentary tissues of fish (Baltic cod, pike perch). The studies were performed with dried raw materials to assess the quality and biological value of cod skin. The fish by-products were collected, washed, cleaned of scales and cuts and then dried by convective drying at temperature of 20±2°C and air velocity of 2 m/s until the mass fraction of moisture in the product was no more than 20%, after which it was stored in a hermetically sealed container at temperature of 5±2°C.

For enzymatic hydrolysis, a broad-specificity enzyme (endopeptidase) "Alcalase 2.5 L" (Novozymes, Denmark) with following characteristics was selected: activity -2.5 AU/g (Anson units), optimum pH and temperature: 6.5 –9.0 and 45 –55 °C respectively. For acid-enzymatic hydrolysis of fish skin was used a subsurface serum containing rennet.

Tissue enzymatic hydrolysis was monitored by the accumulation formol titratable nitrogen and dry substances. Sampling and preparation of samples, determination of the mass fraction of water, protein, fat, ash, minerals, formol titratable nitrogen was determined according to the Russian State Standard GOST 7636-85. Water activity was determined using device "Lab Master – Aw" (NOVASINA, Switzerland), according to the Russian State Standard GOST R ISO 21807. The amino acid composition of the protein in the raw material was analyzed by capillary electrophoresis using device "Kapel", followed by computer based data processing using Multichrom program for Windows. The fatty acid composition of lipids in raw materials was determined by gas capillary chromatography with preliminary isolation of the lipid fraction from the material under study by petroleum ether and their subsequent determination. The mass fraction of oxyproline is determined by the Newman and Logan colorimetric method, the essence of which is to isolate oxyproline during acid hydrolysis of a product sample, conduct a color reaction with its oxidation products, and measure the intensity of the developing color using a photoelectrocolorimeter [6].

3. Results and discussion
In this work the supportive, connective and integumentary tissues of the Baltic fish arts pikeperch (Sander lucioperca) and cod (Gadus morhua) were researched. The data on the study of the mass composition of these species is presented in Table 1. It can be seen that the share of supportive, connective and integumentary tissues accounts for 14.5% in cod and 16.5 % in pikeperch.

Table 1. Mass composition of pikeperch and cod.

| Sample  | Head | Skin | Bone and cartilage tissue | Fins | Entrails | Muscle tissue |
|---------|------|------|---------------------------|------|----------|---------------|
| Pikeperch | 18   | 4    | 7.5                       | 5    | 10       | 55.5          |
| Cod     | 19   | 3.5  | 8.0                       | 3    | 15       | 51.5          |

The chemical composition of the supportive, connective and integumentary tissues under consideration is shown in Table 2. It can be seen that the reduction of the main BAS content occurs
during the heat treatment of raw materials. However, pre-heat treatment of tissues is appropriate for their preparation for subsequent enzymatic hydrolysis.

Table 2. Chemical composition of the supportive, connective and integumentary tissues of pikeperch and cod, %.

| Indicator | Pikeperch | Pikeperch after heat treatment | Cod | Cod after heat treatment |
|-----------|-----------|-------------------------------|-----|-------------------------|
| Water     | 35.7      | 31.1                          | 64.0| 57.4                    |
| Protein   | 25.6      | 24.9                          | 14.4| 12.1                    |
| Fat       | 9.6       | 8.4                           | 3.0 | 1.8                     |

Table 3 shows the results of studying the mineral composition of the supportive, connective and integumentary tissues of pikeperch and cod after heat pretreatment for enzymatic hydrolysis. The tissues are rich in calcium and phosphorus, and the former is more contained in the supportive, connective and integumentary tissues of pikeperch, and phosphorus is more contained in similar tissues of cod. These mineral components are essential for the prevention and treatment of diseases of the musculoskeletal system and should be supplied in sufficient quantities with food or as part of dietary supplements.

Table 3. Mineral composition of raw materials of the supportive, connective and integumentary tissues.

| Indicator | Pikeperch | Cod |
|-----------|-----------|-----|
| K, %      | 0.05      | 0.07|
| P, %      | 4.2       | 5.6 |
| Ca, %     | 5.5       | 4.1 |
| Na, %     | 0.25      | 0.1 |
| Mn, mg/kg | 8.3       | 22.0|
| Fe, mg/kg | 10.3      | 3.0 |

Organoleptic assessment of the quality of dried cod skin was performed for the compliance with the requirements of the technical specification "Dry fish skin" (project), which showed that they correspond to standard quality (Table 4).

Table 4. Organoleptic indicators of cod skin quality.

| Indicator name | Characteristic and norm |
|----------------|-------------------------|
| Appearance     | Skin of whole fish, even, the surface is clean, without scales, grayish |
| Consistency    | Dense                   |
| Smell          | Fishy, characteristic for fresh skin, without foreign odors. Mild iodine odor is allowed. |

The chemical composition of the skin of the Atlantic cod (*Gadus morhua*) caught in March-April 2019 in the Baltic Sea is presented in Table 5.
Table 5. General chemical composition of cod skin

| Sample name         | Water | Protein* | Fat * | Minerals * | Collagen, from total protein | Water activity a_w |
|---------------------|-------|----------|-------|------------|------------------------------|-------------------|
| Fresh cod skin      | 71.1  | -        | -     | -          | -                            | 0.979             |
| Dried cod skin      | 16.8  | 90.7     | 0.3   | 9.0        | 57.3                         | 0.615             |

* in terms of dry matter

From the data presented in Table 5 it can be seen that cod skin is 90.7% protein, while connective tissue share is 57.3% of the total protein content; mineral substances content is 9%. Low fat content (0.3%) will have a good effect on the storage capacity of both the skin itself and on the organoleptic properties of the product in which the hydrolyzed skin can be introduced as a functional component.

The value of water activity $a_w$ at the level of 0.979 indicates that the following microorganisms can develop in fresh skin: Pseudomonas, Escherichia, Proteus, Shigella, Klebsiella, Bacillus, Cl. perfringens, Cl. botulinum E, G, as well as some types of yeast. Analysis of the indicators of microbiological safety of cod skin showed the absence of pathogenic and opportunistic microflora, the value of Quantity of Mesophilic Aerobic and Facultative Anaerobic Microorganisms is no more than 1.9×10⁴ CFU / g.

Skin drying leads to decrease in the value of water activity $a_w$ to the level of 0.615. In this regard, it can be testified that the microbiological contamination decreases, hydrolytic processes slow down, which will have a positive effect on the storage capacity of the skin as a raw material for the smooth operation of production.

Spectrophotometric determination of hydroxyproline in the samples showed its presence at the level of 6.1%.

Table 6 shows the results of chromatographic analysis of the amino acid composition of cod skin, from which it can be seen that cod collagen contains a significant amount of nonessential and partially nonessential amino acids: glycine (5.77%), proline (2.58%), arginine (2.4%), alanine (2.35%) and essential - valine (2.52%) and lysine (1.3%).

Table 6. Content of amino acids in cod skin, %

| Amino acid name       | Cod skin |
|-----------------------|----------|
|                       | Essential| Nonessential amino acids |
| Valine                | 2.52     |                        |
| Leucine               | 0.93     |                        |
| Isoleucine            | 0.46     |                        |
| Lysine                | 1.30     |                        |
| Methionine            | 0.41     |                        |
| Cystine               | less than 0.1 |                        |
| Phenylalanine         | 0.61     |                        |
| Tyrosine              | 0.3      |                        |
| Threonine             | 0.67     |                        |
| Tryptophan            | less than 0.1 |                        |
| Arginine              | 2.4      |                        |
| Histidine             | less than 0.5 |                        |
| Proline               | 2.58     |                        |
| Serine                | 1.54     |                        |
| Alanin                | 2.35     |                        |
| Glycine               | 5.77     |                        |
| Aspartic acid + asparagine | 1.83  |                        |
| Glutamic acid + glutamine | 2.26  |                        |
The research results confirm the literature data on the amino acid composition of fish collagen, which is characterized by the obligatory presence of hydroxyproline, which is a feature of the connective tissue of fish, and almost complete absence of tryptophan, which is an integral part of the muscle tissue of animals and fish [7]. Glycine, proline and hydroxyproline are the most important amino acids in collagen, accounting for 50% of the total amino acids in protein. The content of proline and hydroxyproline is especially important for the gelling effect, which can potentially have a positive effect on the formation of the structure of brine cheese. Therefore, cod skin hydrolysate can be recommended as a functional additive for the prevention of musculoskeletal system diseases and a technological additive for structure formation of brine cheese. Analysis of the fatty acid composition of cod skin is presented in Table 7. It shows that fatty acids which are typical for fish skin predominate: palmitic, oleic, linoleic and stearic acids.

Table 7. Content of fatty acids in cod skin, %.

| Indicator name | Cod skin |
|----------------|----------|
| Butyric acid (c 4: 0) | less than 0.1 |
| Caproic acid (c 6: 0) | less than 0.1 |
| Caprylic acid (c 8: 0) | less than 0.1 |
| Capric acid (c 10: 0) | 0.4 |
| Lauric acid (c 12: 0) | 11.2 |
| Myristic acid (c 14: 0) | 6.4 |
| Myristoleic acid (c 14: 1) | less than 0.1 |
| Palmitic acid (c 16: 0) | 38.6 |
| Palmitooleic acid (c 16: 1) | 1.3 |
| Stearic acid (c 18: 0) | 4.3 |
| Oleic acid (c 18: 1) | 28.4 |
| Linoleic acid (c 18: 2) | 5.8 |
| Linolenic acid (c 18: 3) | 0.2 |
| Arachidic acid (from 20: 0) | less than 0.1 |
| Behenic acid (from 22: 0) | less than 0.1 |
| Others | 3.4 |

Using biodegraded fish supportive, connective and integumentary tissues some innovative technologies of biologically active compositions, enriched and functional food products were developed (see Table 8). Detailed research results on the justification of the parameters of each technology were published earlier [8–11].

Table 8. Characteristics of the technology and quality of new biologically active substances and food products enriched with hydrolyzed fish supportive, connective and integumentary tissues.

| Product/origin | Features of the technological process | Product feature |
|----------------|---------------------------------------|-----------------|
| Biological product “Chondroeffectin” / pikeperch supportive, connective and integumentary tissues (bones, fins, skin, scales, bones and cartilage of the head and ridge) | The key technological operations are the preparation of raw materials, its fermentation and drying of semi-finished products. The preparation of raw materials includes washing, removing excess moisture, boiling, removing the remnants of muscle tissue and grinding to a particle size of no more than 5 mm. The resulting minced material goes to fermentation, it is carried out in an environment of water-alcohol plant extract with a hydromodulus “raw materials:extract 1:9”. The process is carried out in a bioreactor. The enzyme "Chondroeffectin" is an amorphous powder that has a specific taste and smell with hints of dried fish products and rosehip. Main chemical composition: sulfate ions (1.2%), total nitrogen (11.38%), total phosphorus (0.65 %), minerals (5.3 %), lipids (4.56 %), vitamin C (5.63 |
preparation "Food grade Collagenase" is introduced in amount of 0.8% by weight of the suspension. The fermentation process is carried out with periodic stirring for 12–16 hours at temperature of 42 °C. At the end, the enzyme is inactivated, bringing the temperature in the reactor to 75–80 °C and kept for 15 minutes. After filtration, the hydrolysate is cooled and dried. Drying is carried out in mild conditions at a temperature of 50–55 °C. Spray drying results in the mass fraction of water in the product of no more than 10%. The filtered dense residue is also dried in a vacuum drying unit at temperature below 60 °C until the mass fraction of water in the product is no more than 10%. Finished dry products are grounded to a powdery state in mills and fine grinding machines, sifted, weighed, packaged in 10 g, packed in consumer packaging (up to 100 units).

| Biological product "Proteomineral"/pikeperch supportive, connective and integumentary tissues (bones, fins, skin, scales, bones and cartilage of the head and ridge) | See above | "Proteomineral" is a finely dispersed easily crumbling powder. It has smell and taste characteristic for such products, with hints of dried fish smell, enriched with aromas of rosehip fruits. Main chemical composition: total nitrogen (4.13 %), total phosphorus (6.98%), calcium (12.84 %), lipids (2.8 %). "Proteomineral" is recommended for use as a functional product that helps maintain the functions of the human musculoskeletal system, as a source of substances of anti-arthrotic and anti-osteoporotic action. |
|---|---|---|
| Soft drink enriched with hydrolyzed collagen/ pikeperch skin | Fish skin enters the processing line in a chilled state. It is manually cleaned and washed in cold running water at a temperature of 18°C for 30 minutes in order to remove mucus, dirt from the surface of the raw material, and extract ballast globulin and albumin proteins. Then the skin is crushed to a particle size of 2-3 mm. Water is added to the crushed skin, heated up to 40°C and kept for 50 minutes. | The drink is an opaque liquid without sediment and foreign inclusions. Opalescence is allowed due to the characteristics of the raw materials used. The taste is sweet and sour, slightly astringent, balanced, with a |
After the specified time, the liquid fraction is filtered out and used to isolate hyaluronic acid. The solid fraction is bleached with a peroxide-salt solution, the process is carried out for 12 hours. The peroxide-salt solution is drained, then the bleached solid fraction is subjected to swelling in a 1.0–1.2% solution of sodium hydroxide, taken in a ratio of 1:1 to the mass of the solid fraction, 24 hours at a temperature of 20–25°C. The resulting mixture is neutralized with a 3% solution of boric acid. The treatment of swollen solid fraction is carried out with a solution of enzyme preparation "Pancreatin", taken in an amount of 0.5–0.6% by weight of solids, for 1.5–2.0 hours at temperature of 37–40 °C, and enzyme preparation is prepared by mixing with water in the ratio 1:3. Fractions washed with cold running water to remove residual "Pancreatin". The resulting collagen hydrolysate is sent for drying in drying chambers with forced air circulation at a temperature of 18–20°C for 12 hours.

The technological scheme for the production of a drink enriched with the obtained collagen hydrolysate includes the following main operations: receipt and storage of raw materials, preparation of raw materials, water filtration, water disinfection, water quality improvement, water softening, preparation of sugar syrup, preparation of blended syrup, mixing syrup with water, adding collagen hydrolysate, filling and capping, pasteurization, labeling, storage and transportation.

| Brine cheese “Marine” / cod skin | The essence of the proposed technological solution for the preparation of brine cheese lies in the acid-enzymatic hydrolysis of dry cod skin and subsequent introduction of the produced hydrolysate into the cheese mass. |
|----------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Evaluation of the quality of the finished product showed that produced cheese has a characteristic appearance, the surface is even, with traces of cheese cloth. Color is white with a grayish tint interspersed with fucus and fish skin, the consistency is uniform, moderately dense, the taste and smell are cheesy, smoked, moderately salty taste, with a characteristic taste of algae and fish. |

noticeable pleasant hint of orange. The smell is pleasant, with a slight aroma of orange. The color is orange. The drink is recommended for people suffering from joint diseases and athletes.
Physicochemical and biochemical indicators of the quality of brine cheese “Marine” demonstrate its high quality: mass fraction of water – 56.3%; protein – 17.6%, incl. collagen 23% of total protein; fat – 17.8%; mineral substances – 4.7%, incl. salt – 4.0%. Spectrophotometric determination of hydroxyproline in the composition of brine cheese (control sample) was 2.42 mg%, brine cheese “Marine” (experimental sample) – 6.77 mg%, which is 2.8 times more. Determination of the biological value of the brine cheese “Marine” (per 100 grams of the finished product) showed that when using 70 g of the fortified product, the daily human need for calcium is satisfied by 36.8%.

4. Concluding remarks
1. Biological potential of the fish arts pikeperch (Sander luciopercai) and cod (Gadus morhua) is characterized by high content of collagen, calcium, phosphorus, a glycosaminoglycan.
2. The possibility of the production of enzymatic hydrolysates from fish supportive, connective and integumentary tissues in form of functional food products was studied.
3. Biologically active compositions "Chondroeffectin" and "Proteomineral" are recommended for use as functional products that help maintain the functions of the human musculoskeletal system, as sources of substances of chondroprotective and anti-osteoporotic action respectively.
4. Soft drink enriched with collagen hydrolysate is recommended for use by people suffering from joint diseases as well as athletes.
5. Brine cheese “Marine” is developed for the prevention of musculoskeletal system diseases. When using 70 g of an enriched product, the content of oxyproline is 2.8 times higher than the standard product, and the daily human need for calcium is satisfied by 36.8%.

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