Technology for the production of proteolytic enzymes from the digestive organs of freshwater fish in north-west Russia

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Abstract. This article describes the enzymatic activity of proteolytic enzymes, the general chemical composition of the digestive organs of carnivorous and herbivorous fish in the period from October to November 2017 to April 2018. The dependence of the enzymatic activity on the pH of the medium and the mass fractions of protein and fat is shown. Based on the data obtained, enzymes were isolated from the digestive organs of bream (at pH 9.5) and pike-perch (at pH 2.5). To determine the activity of the test substance, we used the corrected absorbance for the test suspension (the difference in absorbance between the test sample and the control sample) obtained using raw materials for the period January to February 2018. We also carried out the purification of enzyme preparations using ion exchangers (Q-XL, SP-XL) and size exclusion chromatography (Sephadex G-75 Superfine), determining the molecular weight of enzyme preparations prepared from the digestive organs of pike-perch and bream.

1. Introduction

Biological catalysts (enzymes, or enzymes) are widely used in various industries. Their use in the food industry is widespread in the production of various food products. The comprehensive biotechnology development programme in the Russian Federation envisages a significant increase in enzyme production. The food industry is interested in the three subclasses of esterase hydrolases, glycosidases and proteases. Proteolytic enzymes are currently in greatest demand in the food industry. They are used for various purposes: for enzymatic hydrolysis of yeast biomass to create functional ingredients [1], for biologically active additives to food and functional food products [2], for enzymatic modification of meat and bone collagen-containing raw materials [3] and secondary fish raw materials [4], for processing waste from commercial crab cutting [5], for enzymatic treatment of unconventional water bodies [6], to separate biologically active peptides and create functional products, including for sports nutrition, from secondary fish raw materials [7], to obtain food and functional products based on collagen-containing fish raw materials [8], to obtain fish protein hydrolysates for food purposes, to process milk, meat and fish raw materials [9–12], to process keratin-containing raw materials, meat and fish waste for the purpose of producing feed products.

Enzymatic preparations are used from plant raw materials (papain, ficin, bromelin) and from the enzyme-containing organs of farm animals (pepsin, trypsin, pancreatin, collagenase, lipase, etc.). Numerous enzymes are also contained in large quantities in commercial water bodies (fish,
crustaceans, molluscs, algae). The main place where enzymes are localised is in the digestive organs - the stomach, intestines, pancreas and liver. Proteolytic enzymes, lipase and collagenase also take first place in the list of enzymes. A wide range of enzymes of various profiles for bioconversion of raw materials of plant and animal origin enables microbiological synthesis. But the need for enzymes from natural sources still exists and is even growing.

An important source for the production of enzyme preparations is waste from the cutting of fish. This secondary raw material includes the main enzyme parts - the digestive organs of the stomach, intestines, pancreas and liver. The raw materials are available and cheap. The predominant research so far has been the use of oceanic raw materials. But in recent years, deep cutting of fish has been developing at coastal fish processing plants. This creates the prospect of using recycled fish from coastal fishing for the production of target products in various directions, including enzyme preparations.

As a result of the use of digestive organs of fish for the production of enzyme preparations with the maximum possible preservation of proteolytic enzymes, elements of complex processing of secondary fish raw materials are being formed. As secondary fish (digestive organs, scales, insides of fish and other objects) are not sufficiently used in the Russian Federation, the task of developing technologies aimed at complex processing of aquatic bioresources is urgent. In this regard, research into the development and justification of technology for the production of enzyme preparations from fish digestive organs in North-West Russia is relevant.

In the Kaliningrad State Technical University, the Department of Food Biotechnology has conducted research on the technology for producing proteolytic enzyme preparations from the digestive organs of two types of commercial fish - pike-perch and bream. These facilities differ in terms of the structure of the digestive system and the nature of nutrition. Pikeperch is an active predator and has a clearly defined stomach. In bream, digestion takes place in the intestines. These fish therefore have enzyme complexes that are active at different levels of active acidity.

The extraction of enzymes is carried out using the extraction method. The conditions of the process must ensure that enzymes are fully excreted and remain active.

In order to ensure that the process is relevant, it was necessary to study the chemical composition of the digestive organs of the pikeperch and bream, the activity of their enzymes and the temperature and time parameters of the enzyme preparation process.

An important indicator for the characterisation of enzymes is their molecular weight, the values of which can be used to determine whether the enzymes belong to a particular class.

The aim of this study is to study the chemical composition of the digestive organs of freshwater fish in pikeperch and bream, the activity of their enzymes and the temperature and time parameters at the stage of selecting enzymes to maintain maximum activity, and the creation of technology for the production of proteolytic enzyme preparations.

2. Research methods

Within the framework of a scholarship from the German Ecological Foundation (www.dbu.de), a series of experimental studies on the processing of secondary fish raw materials were carried out at the ANiMOX research laboratory in Berlin. This development work made it possible to optimise the technological production stages, determine the proteolytic activity of enzyme preparations from the digestive organs of pike-perch and bream and determine the molecular weight of the enzymes.

In the experiments, the digestive organs of pike-perch and bream provided by the fish processing company "Za Rodinu" were used as raw materials. The raw materials obtained were prepared fresh, frozen and stored at a temperature not exceeding minus 20°C until the experiments.

The enzymatic activity of proteolytic enzymes was determined by a modified method of protease analysis using a previously ground digestive tract of frozen fish to obtain a solution of the enzyme preparation [13, 14]. The protease assay method is based on hydrolysis of the substrate at 35°C. Incubation of the sodium caseinate solution with the enzyme preparation under investigation was carried out for 30 min until peptides and amino acids were formed at different pH values (2.5; 5.5; 7.2;
The enzymatic activity was evaluated based on the amount of tyrosine contained in the hydrolysis products that were not precipitated by trichloroacetic acid. The activity of the test substance was determined using the corrected absorbance for the test suspension (the difference in absorbance between the test sample and the control sample) taking into account its dilution U/g.

The effectiveness of the extraction of enzymes from crushed feedstock was determined by the results of relevant experiments at the ratios of feedstock and buffer solutions of 1:1 and 1:2, the buffer solutions had pH of 2.5; 7.2 and 9.5, the duration of extraction varied from 1 to 4 hours at temperatures of 35–40°C. The results were assessed by the dry substance content of the extracts and the remaining dense residue and activity of the extracted proteases.

To improve the extraction efficiency and increase the yield of the finished product, additional fine crushing of the raw materials with a mixer was performed, as well as re-extracting enzymes from the dense residues obtained.

In order to purify the complex enzyme preparation, enzymes were separated and bound by liquid chromatography (Pharmacia chromatograph). Cation and anionic ion exchangers were used [15–17]. Protein absorption in fractions eluated from ion exchangers was determined with the UV-3100 spectrophotometer, mark "VWR".

The aim of the experiments was to separate enzymes by binding them to the ion-exchange material of the chromatographic column, selective elution with increasing concentration of counterions, collection and study of individual enzyme properties.

Determination of molecular weight was carried out using exclusive chromatography on a Merck-Hitachi LaChrom (L-7000 Serie) unit with software that allows calculation of the molecular weight of polymers (enzymes) based on the values of absorption in the eluted fractions and their retention time. The enzyme molecular masses were calculated using Bio-Rad Gel Filtration Standard (cat #151 1901) with components with known molecular masses: albumin (beef, 690.000 kDa), ovalbumin (chicken, 44.000 kDa), myoglobin (horse, 17.000 kDa), vitamin B12 (1.350 kDa).

Calculations were made using software in the Excel system. The results reports (PDF) are reflected and saved on an HPLC computer. The evaluation is performed by the Excel system for all fractions of each sample, from which tables with results and chromatogram charts are generated and stored.

### 3. Results and discussion

The general chemical composition of the digestive organs of fish of different species is shown in Table 1. The composition of the digestive system influences the chemical composition, especially the fat content. From October to November 2017, the digestive organs were tested for fats, which often have a high level of activity.

**Table 1. General chemical composition of the digestive organs of fish in different months of the year in %.

| Fish species and time of catch | Humidity (g) | Fat (g) | Protein (g) | Minerals (g) |
|-------------------------------|-------------|--------|-------------|-------------|
| Pike perch February           | 71.95       | 10.8*  | 15.87       | 1.08        |
| April                         | 73.61       | 10.1** | 15.28       | 0.86        |
| June                          | 67.69       | 14.56**| 16.86       | 0.89        |
| August-September (year 2018) | 69.71       | 11.48**| 17.76       | 1.05        |
| October-November (year 2018)  | 64.42       | 5.28*  | 10.44       | 0.86        |
| December (year 2018)          | 72.87       | 8.26** | 16.19       | 0.62        |
| Bream February                 | 70.45       | 10.66* | 16.82       | 1.09        |
| April                         | 68.59       | 13.2** | 15.89       | 0.96        |
| June                          | 73.33       | 9.9**  | 16.68       | 0.95        |
| August-September (year 2018)  | 70.22       | 10.22**| 18.68       | 0.98        |
| October-November (year 2018)  | 68.50       | 25.55* | 14.6        | 1.09        |
| December (year 2018)          | 71.01       | 9.02** | 16.95       | 0.87        |

* - Digestive organs with fat content; ** - Digestive organs with fat removed during sample preparation
Due to the accumulation of reserve surface fat, its content in fish digestion bodies is high. The lowest fat content for pike-perch was recorded in October-November (5.28%). Starting from December, the fat content increases, reaching its maximum value in June. The breamer's fat content is practically high throughout the year (from 10.66% in winter in February to 25.55% in October-November). This has necessitated the manual removal of fat from the digestive organs. In experiments (starting in December), fat was removed from the digestive surface during sample preparation. This had an impact on the results (Table 1), but the fat content remained high throughout the year even with manual fat removal (10.1–14.56% for pike-perch and 9.02–13.2% for bream) [13].

The digestive organs had a high protein content (16.5–18.58%), including enzymatically active compounds.

Changes in the activity of proteolytic enzymes in pike-perch's digestive organs during the year at different pH levels are shown in Figure 1, and in bream's digestive organs in Figure 2.

**Figure 1.** Change in proteolytic enzyme activity in the digestive organs of pike-perch during the year at different pH levels.

**Figure 2.** Changes in proteolytic enzyme activity in the breasts' digestive organs during the year at different pH levels.
Acidic, slightly acidic, neutral and alkaline proteases are present in the digestive organs of the fish species studied. The highest enzymatic activity was measured in the pH zones alkaline (for bream) and acidic (for pike-perch). During the year, the maximum activity in the digestive organs of pike-perch is measured in acidic and slightly acidic zones at pH 2.5 and 5.5. Only in November was a significantly higher enzyme activity observed in pike-perch in the alkaline pH zone.

In November - December (year 2018) low proteolytic activity of enzymes in the acidic zone was observed in the digestive organs of pike-perch in the acidic zone (pH 2.5 to 5.5). During these months, however, a significant increase in activity in the alkaline range was observed (pH 7.2–9.5). In herbivorous bream, the maximum activity in the digestive organs is determined in the alkaline range, i.e. at a pH of 9.5.

This development work within the framework of the DBU grant at ANiMOX served to optimise the technological regime of the production process of enzyme preparations from the digestive organs of pike-perch and bream.

When modelling and determining the optimal factors for obtaining an enzyme preparation, the orthogonal central second-order composition plan was used for two factors. The duration and temperature conditions of thermoregulation were chosen as planning factors. The responses are the activity of the isolated enzymes and the mass of the non-reactive residue. Modelling was used for two pH values at which the process is carried out - 2.5 for the production of enzymes from the digestive organs of pike-perch and 9.5 for the digestive organs of bream.

The range of changes in the factors for the production of enzyme from the digestive organs of pike-perch and bream subject to optimisation (duration of extraction and temperature of extraction) and the limits of their variation are given in Table 2.

### Table 2. Overview of experiments to optimise the factors extraction time and temperature for the production of the protease enzyme from the digestive organs of pike-perch and bream.

| Enzyme sample | Material | Duration of extraction and temperature of extraction | Remarks | Protease activity (liquid) U/g |
|---------------|----------|-----------------------------------------------------|---------|-------------------------------|
|               | Duration, hour | Temper | Weight Material, g | Mass of the buffer solution, g and its pH |
|               |           | atur, °C |                             |                                 |
| 1             | 1.0       | 35       | 18.0347                   | 18.0, pH 2.5                     | 1.83 |
| 2             | 3.0       | 35       | 15.0225                   | 15.0, pH 2.5                     | 1.87 |
| 3             | 5.0       | 35       | 15.1632                   | 15.0, pH 2.5                     | 1.87 |
| 4             | 5.0       | 35       | 16.6809                   | 17.0, pH 2.5, Enzyme precipitation 75% isopropanol | 0.715 (trophic enzyme) |
| 5             | 3.0       | 35       | 25.0                      | 25.0, pH 2.5, 1.75 |
| 5 (1)         | 3.0       | 35       | 25.0                      | 25.0, pH 2.5                     | 1.74 |
The activity of proteolytic and lipolytic enzymes in the digestive organs of pike-perch and bream, varies according to their physiological state, which in turn is linked to the fish's habitat and its biological properties. It has been found that the highest proteolytic activity in the digestive organs occurs during the period of intensive feeding of fish, which is from May to September.

It is therefore advisable to use fish to isolate enzymes during this period. During the same period, however, the greatest amount of fat is stored in the digestive organs, which makes the process more difficult. Table 2 shows the experimental results. After this experiment we can already say that a high activity is observed at a temperature of 35 °C degrees and a duration of 3 hours. A double extraction of samples 5 and 6 was also carried out. As the results of the proteolytic activity show, enzymes still remain in the digestive organs after the second extraction. It is therefore advisable to double the extraction when preparing enzyme preparations. On the other hand, it is not recommended to carry out the precipitation of enzymes with isopropanol, as shown in experiment 4, as the activity of the protease enzyme is greatly reduced. No significant lipase activity was detected in the samples.

In the following, the amounts of dry substance in the enzyme solution and in the sediment were determined (Table 3).

| Rehearsal | Enzymatic solutions | Remaining sediments | Proportion of enzyme (%) |
|-----------|---------------------|---------------------|--------------------------|
|           | Dry substances enzyme solution (g) | Weight of the enzyme solution (g) | Dry matter enzyme solution (g) | Dry matter (sediment) weight | Sediment (g) | Dry matter (g) |
| 1         | 5.40% | 13.8324 | 0.747 | 14.65% | 18.7213 | 2.742 | 21.4 |
| 2         | 6.07% | 12.2886 | 0.746 | 16.13% | 12.4751 | 2.012 | 27.0 |
| 3         | 6.88% | 13.5348 | 0.932 | 16.80% | 13.4148 | 2.254 | 29.2 |
| 4         | 6.37% | 19.7334 | 1.257 | 15.54% | 14.3742 | 2.234 | 36.0 |
| 5         | 7.29% | 17.7986 | 1.298 | 16.89% | 16.4072 | 2.771 | 31.9 |
| 6         | 7.06% | 15.1207 | 1.068 | 15.39% | 13.4056 | 2.069 | 34.0 |
| 5 (1)     | 4.50% | 1.8774 | 0.084 | 17.89% | 1.6339 | 0.292 | 22.4 |
| 6 (1)     | 4.48% | 1.9538 | 0.088 | 23.24% | 0.9855 | 0.229 | 27.7 |
| 7         | 6.68% | 22.5947 | 1.509 | 14.96% | 18.0652 | 2.703 | 35.8 |
| 8         | 8.86% | 19.7208 | 1.747 | 19.79% | 16.7825 | 3.321 | 34.5 |

Table 3. Results of the determination of dry substances in the enzyme solution and in the remaining sediment.
The highest proportion of enzymes was found in Samples 4 and 7. It follows that the extraction parameters of 4–5 hours at 35 degrees Celsius are suitable for the production of enzyme preparations from the digestive organs of pike-perch and bream.

Cleaning and separation of enzymes from experimental specimens of the preparations was performed by liquid chromatography using ion exchangers (Q-XL anion exchanger and SP-XL cation exchanger).

The cationic ion exchanger is a Sepharose SP-XL, a highly acidic cationic exchanger with a sulphopropyl group using Na⁺ or K⁺ as positive charge ions. The anion exchanger is a Sepharose Q-XL, a highly acidic cation exchanger with a sulfoethyl group, using the PO₄³⁻ ion as an opposite charge ion [15–17].

Pharmacia chromatograph and Ecoview UF-3200 spectrophotometer were used in the research.

The purpose of these experiments was to bind the enzymes to the ion exchange material, then selectively elute them with increasing concentrations of counterions, and collect and study the fractions as individual pure enzymes.

In studies using ion-exchange materials, the binding and elution of enzymes from pikeperch organs was performed by ion-exchange chromatography under acidic conditions and SP XL Sepharose was used as the column material. A phosphate buffer with a concentration of 0.01 mol/L was used in the pH 4-6 range. Table 4 shows the results of these ion-exchange chromatography work. Albumin with a known molecular weight was used as a standard test. Chromatograms were created for each experiment to capture protein peaks, inactive proteases and peptides.

| Enzyme sample       | Material samples | Dilution | Chromatogram number | Column material     | pH  | Number of peaks |
|---------------------|------------------|----------|---------------------|---------------------|-----|-----------------|
| Standard-Test       | Albumin          | 1:100    | CH-01-03            | SP-XL-Sepharose     | 6.0 | 1               |
| Standard-Test       | Albumin          | 1:100    | CH-04               | SP-XL-Sepharose     | 6.0 | 1               |
| Standard-Test       | Albumin          | 1:100    | CH-05               | SP-XL-Sepharose     | 5.0 | 1               |
| 7                   | Pike-perch       | 1:10     | CH-06               | SP-XL-Sepharose     | 5.0 | 1               |
| 7                   | Pike-perch       | 1:10     | CH-07               | SP-XL-Sepharose     | 4.0 | 3               |
| 7                   | Pike-perch       | 1:10     | CH-08               | SP-XL-Sepharose     | 6.0 | 2               |
| 8                   | Bream (Bowel)    | 1:10     | CH-09               | SP-XL-Sepharose     | 6.0 | 2               |
| 8                   | Bream (Bowel)    | 1:10     | CH-10               | QXL-Sepharose       | 8.0 | 3               |
| 8                   | Bream (Bowel)    | 1:10     | CH-11               | QXL-Sepharose       | 8.0 | 3               |

This table shows the conditions under which the peaks from the study were obtained. As you can see, the number of peaks depends on the pH values and the activity of the enzyme preparation.

A chromatogram with analysis of proteins and proteases was prepared for each experiment. Experiment 7 is shown as an example in Figure 3. In experiment 7 a buffer with a pH of 4.0 was used, as the enzymes are active in the acidic pH range. Before starting the protein fractionation analysis, 10 cm anionic column material (SP-XL-Sepharose) with a diameter of 1 cm and a length of 30 cm was added to the column and sample 7 was diluted with phosphate buffer solution at pH = 4.0 in a ratio of 1:10. A programme of elution with increasing salt gradient was then carried out, which is shown in the figure by increasing the conductivity.
Figure 3. Elution diagram of separation of pike-perch enzyme extract on SP-XL-Sepharose. Starting buffer: 0.01 mol/l phosphate buffer with pH = 4.0 with conductivity sodium chloride gradient up to 1.0 M. Flow rate: 1 ml/min. The fraction collector was set to a time of 5 min/fraction, whereby 5 mL per fraction was collected. The elution process took 2.5 hours.

In experiment 7 in Figure 3 we see 3 peaks. The first protease peak contains the unbound enzyme eluted directly from the column, the other two peaks show the enzyme in a bound state. With continuous NaCl ionic strength gradients the 2 bound enzymes are eluted.

In a similar way, we carried out the binding and elution of the liquid enzyme preparation from the digestive organs of sea bream into different protein fractions using ion chromatography. For this purpose, the same phosphate buffer with a concentration of 0.01 mol/L at a pH value of 8.0 was used, as the enzymes are active in the alkaline pH zone. Before starting the protein fractionation analysis, 10 cm cationic column material (Q-XL-Sepharose) with a diameter of 1 cm and a length of 30 cm was added to the column and sample 8 was diluted 1:10 with phosphate buffer solution, pH 8.0.
Figure 4. Elution scheme of the separation of the enzyme extract from the digestive organs of sea bream on QXL-Sepharose. Starting buffer: 0.01 mol / l phosphate buffer with pH = 8.0 with sodium chloride conductivity gradient up to 1.0 M. Flow rate: 1 ml / min. The fraction collector was set to a time of 5 min/fraction, whereby 5 mL per fraction was collected. The elution process took 2.5 hours.

In experiment 8 in Figure 4 we see 3 peaks. The first peak of the protease contains an unbound enzyme that elutes directly from the column, the other two peaks show the enzyme in a bound state. As the ionic strength gradient of NaCl continuously increases, two peaks of the bound enzyme elute one after the other.

Samples with active enzymes were then collected and freeze dried. It is planned to use them for further studies.

Separation of the liquid enzyme preparation from the digestive organs of pike-perch and bream was performed by size exclusion chromatography on different protein fractions. A phosphate buffer with a concentration of 0.1 mol / L was used to obtain different pH values. In this case a solution with pH 5.0 and 8.0 was used. Before starting the analysis of the protein molecular weight separation, app. 50 mL column material (Sephadex G-75 Superfine) with a diameter of 1 cm and a length of 65 cm was added to the column and an undiluted sample was added in a quantity of 0.5 ml. This Sephadex G-75 Superfine is used to separate protein compounds including enzymes by molecular weight.

To determine the molecular weight on the column, a molecular weight standard with different defined molecules was first used.

The results of the molecular weight standard are shown in Figure 5.
Figure 5. Molecular weight standard (69 kDa albumin; 44 kDa ovalbumin; 17 kDa myoglobin; 1.35 kDa vitamin B12).

From here you can use this Figure 5 in Excel to calculate the molecular weight of enzymes from the digestive organs of pike-perch and bream.

Figure 6. Separation of enzymes (bream) on Sephadex G-75 Superfine. Column filling: 1.0×70 cm. Buffer 0.1M Na₂HPO₄ with pH 8. flow rate: 0.2 ml/min. The fraction collector was set to a time of 10 min/fraction, which collected 2 mL per fraction. The elution process took 6.8 hours.

As shown in Figures 6, the peak area of the active enzymes of the bream is 18 to 22 cm. The molecular weight of the enzyme is therefore about 22.5 kDa. This means that there are enzymes in the bream's digestive system which have a molecular weight similar to that of elastase and chymotrypsin.
and which hydrolyse proteins in the alkaline range. In this case, these samples yield highly purified enzyme with significantly less impurities. The original sample also contains a peptide with a high molecular weight.

![Graph showing enzymatic activity and protein absorption](image-url)

**Figure 7.** Separation of the enzyme (pike-perch) on Sephadex G-75 Superfine. Column filling: 1.0×70 cm. Buffer 0.1M KH$_2$PO$_4$ with pH 5. flow rate: 0.2 ml/min. The fraction collector was set to a time of 10 min/fraction, which allowed 2 mL per fraction to be collected. The elution process took 6.8 hours.

As shown in Figure 7, the peak area of the active pike-perch enzyme is 16 to 20 cm. The molecular weight of the pike-perch enzyme is therefore around 29.5 kDa. This means that there are enzymes in the digestive system of pike-perch that are similar in molecular weight to pepsin enzymes and hydrolyse proteins in the acidic pH range. In this case, a highly purified enzyme with significantly less impurities was obtained in these samples. The original sample also contains a high molecular weight peptide.

4. **Summary of the results**

1) For the production of protease-containing enzyme raw materials from the digestive organs of pike-perch and bream, it is best to use raw materials from April to September, as these fish species show high proteolytic activity during this period.

2) The highest enzymatic protease activity of pike-perch is observed in the acidic pH range. Bream has enzymatic activity in the alkaline pH range.

3) As further research has shown, it is better to use double extraction when producing an enzyme preparation, as enzyme active substances remain in the raw materials after the first extraction.

4) Anions and cation exchangers could not completely bind the pike-perch and bream enzyme. However, a separation of fractions was possible.

5) Another method of separation and purification of the protease from bream and pike-perch was preparative size exclusion chromatography, which involved the detection, purification and separation of the protease and also the molecular weight of the protease enzymes obtained by extraction from the
digestive organs of pike-perch and bream. From this, a transfer from chromatography to ultrafiltration with separation at 50 and 5 kDa can be deduced.

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