

Article

Method of Enzyme Production from the Hepatopancreas of the Red King Crab (*Paralithodes camtschaticus*): The Effect of Temperature and Defatting Conditions on Enzymatic Activity

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Abstract

Processing crabs for meat generates large quantities of waste (secondary raw materials). Internal organs are typically discarded, which causes environmental problems and highlights the need for the integrated use of marine bioresources. The red king crab hepatopancreas is an excellent source of various proteins, including enzymes with different substrate specificities. In this study, an improved method was developed for producing a complex enzyme preparation from the hepatopancreas of red king crab (*Paralithodes camtschaticus*) harvested by a large industrial enterprise in the Barents Sea. Physicochemical analysis revealed high protein (16%) and lipid (10%) contents in the hepatopancreas. The developed method involves centrifugation to rapidly separate the fat fraction from the aqueous protein fraction and acetone washing to remove residual lipids. The use of low temperatures (0 to 5 °C) for autolysis is justified to prevent a decrease in enzymatic activity of the final preparation. Obtained enzyme preparations were analyzed for protease, collagenase, and endo- and exochitinase activity. They exhibited high proteolytic ($A_{\text{prot}} = 252 \mu\text{mol Tyr} \cdot \text{g}^{-1} \cdot \text{min}^{-1}$), exochitinolytic ($A_{\text{exo}} = 0.94 \mu\text{mol GlcNAc} \cdot \text{g}^{-1} \cdot \text{min}^{-1}$), and collagenolytic ($A_{\text{col}} = 210 \text{ mIU} \cdot \text{mg}^{-1}$) activities. It has been shown that enzymatic activities depend on seasonality (winter and summer) of the crab catch, which is due to differences in the physiological processes of crabs during seasonal cyclical temperature changes in their natural habitat. The developed process flowsheet for producing an enzyme preparation from the red king crab hepatopancreas can be used as the basis for technology by enterprises engaged in the comprehensive processing of marine bioresources.

Keywords: enzyme preparation; hepatopancreas; red king crab; enzymatic activity; autolysis; proteins; lipids

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1. Introduction

Enzyme-based biochemical catalysis is used in various industries to produce fine organics, biodegradable substances, biofuels, pharmaceuticals, and food products [1]. In recent years, biocatalysis has become a well-established industrial technology. A key

driver in its development is the production of new enzymes with the required substrate specificity, catalytic activity, and stability [2].

One promising approach for producing effective enzymes is the use of marine invertebrate proteinases [3,4], particularly from commercially available species such as crustaceans and mollusks. Developing technologies to extract proteolytic enzymes from crustacean internal organs enables the rational use of harvested biological resources and the production of enzyme preparations for medicine, microbiology, and the food industry [5].

The red king crab (*Paralithodes camtschaticus*), also known as the Kamchatka crab, holds significant economic importance in various regions worldwide, particularly in the North Atlantic, Barents Sea, and Far Eastern seas. Its value arises from the high demand for its meat as a delicacy, as well as its impact on local economies and ecosystems. Over the past two decades, the red king crab has been commercially harvested in the North Atlantic and the Barents Sea [6,7]. Processing crabs for meat generates large quantities of solid and liquid waste (secondary raw materials), including shells and internal organs. To reduce environmental impact and ensure comprehensive bioresource utilization, this underutilized but economically promising waste, which contains numerous valuable biologically active compounds, must be processed [8]. While chitin and chitosan are traditionally produced from shells [9], the internal organs remain largely unused.

The hepatopancreas, which combines liver and pancreatic functions, is the primary source of enzyme preparations among king crab internal organs. The hepatopancreas contains various biologically active proteins, including enzymes with different substrate specificities [10]. The hepatopancreas accounts for approximately 8% of the animal's mass in secondary resources generated during crab-meat processing [11,12].

The Kamchatka crab hepatopancreas contains chitinolytic [13,14] and lipolytic [15] enzymes, hyaluronidase [16], elastase [17], collagenase [18], and other hydrolytic enzymes. Complex enzyme preparations with collagenolytic activity are commonly obtained from the hepatopancreas [19,20], and studies show these preparations comprise multiple enzymes with distinct substrate specificities [10].

Extracting enzyme preparations from the Kamchatka crab hepatopancreas presents several challenges, including lipid removal (extraction), extraction of water-soluble proteins (enzymes), and purification of enzymes from inactive ballast proteins [21–23]. All extraction and fractionation steps must be performed at low temperatures (−20 to −10 °C in acetone and approximately 0 °C in aqueous solutions) to prevent enzyme inactivation [24].

Based on these considerations, enzyme preparation methods can be divided into two groups [18]. The first involves lipid extraction with organic solvents (acetone and butanol) at subzero temperatures (down to −20 °C). The second group includes hepatopancreas homogenization in cold water or buffer, followed by separation of the aqueous enzyme solution via centrifugation or fractionation into lipid, aqueous, and solid phases (insoluble precipitate). This process may involve raw material autolysis to enhance lipid and water-soluble proteins yields, as well as phase separation using organic solvents.

Autolysis is frequently employed in enzyme isolation to increase lipid yield through enzymatic hydrolysis of lipoproteins and other lipid–protein complexes. Autolysis also boosts water-soluble protein yields by degrading high-molecular-weight proteins and releasing low-molecular-weight enzymes from the insoluble precipitate [25]. Temperature is a critical factor for autolysis: at low temperatures, enzyme activity decreases, slowing or halting the process. Autolysis of crab hepatopancreas is described in several patents, with protocols involving incubation for 6 h at 5–20 °C [26], 14–15 h at 21 °C [27], and 6–15 h at room temperature [28]. Unfortunately, the literature lacks clear data on how autolysis affects the activity of hepatopancreatic enzymes.

The study aimed to develop an improved method for producing a complex enzyme preparation from red king crab hepatopancreas. We focused on two main aspects: (1) significantly altering the sequence of operations related to lipid removal (defatting method) and (2) examining the influence of autolysis temperature on enzyme preparation activity.

Additionally, we aimed to evaluate the impact of the red king crab harvest season on its enzymatic activity. This aspect is important because the biological cycles of the king crab—such as molting, spawning, and migrations—as well as its metabolic activity, including enzyme activity, are directly influenced by seasonal changes in its habitat.

2. Materials and Methods

2.1. Hepatopancreas of the Red King Crab

Red king crabs (*Paralithodes camtschaticus*) captured in the Barents Sea by Antey-Sever LLC, which holds quotas for crab production, are delivered to the company's onshore facilities in the city of Murmansk for further processing. The limbs (containing meat) are separated from the carapace, while the gills and other inedible parts are removed. Subsequently, the limbs are boiled and frozen. During the processing of the crabs, waste is generated, including the cephalothorax, insides (such as the hepatopancreas), shell, and other leftover parts. The hepatopancreas constitutes approximately 8% of the crab's total mass.

The hepatopancreas of the red king crab (*Paralithodes camtschaticus*) was obtained from a large crab processing enterprise named Antey-Sever LLC (Murmansk, Russia). Crabs were caught by Antey-Sever LLC in three distinct periods: August–September, October–November, and January–February during 2024–2025; following harvest, the hepatopancreas samples were frozen and stored at $-20\text{ }^{\circ}\text{C}$. We purchased the frozen hepatopancreas from the enterprise and stored it at $-20\text{ }^{\circ}\text{C}$ until use.

2.2. Enzyme Preparation

Enzyme preparations were obtained from the hepatopancreas using a method that includes an acetone-based washing step [29]. Hepatopancreas samples were thawed, crushed, and homogenized using a laboratory homogenizer (model 1094, Tecator, Höganäs, Sweden) at $3\text{--}5\text{ }^{\circ}\text{C}$. For the autolysis study, the homogenate was stirred in a thermostatted vessel for 30 min. The homogenate was centrifuged using an Avanti J-25 centrifuge (Beckman Coulter, Indianapolis, IN, USA) in 400 cm^3 polypropylene tubes at 8000 rpm ($7500 \times g$), and the supernatant was collected. The aqueous enzyme preparation, containing a protein mixture, was lyophilized using a Heto FD 8 freeze dryer (Heto-Holten A/S, Allerød, Denmark). The lyophilized enzyme preparation was purified from lipids using acetone by mixing the preparation in acetone at $3\text{ }^{\circ}\text{C}$ for 2 to 3 min. The undissolved solid phase, containing the enzyme, was washed three times with acetone, separated by centrifugation at 5000 rpm for 10 min, and dried in an ShVS-45 vacuum drying oven (JSC "KMEP", Kazan, Russia) at $15 \pm 5\text{ }^{\circ}\text{C}$ for 3 h.

2.3. Content of Water, Proteins, Lipids and Minerals

The mass fractions of water, protein, lipids, and residue after ignition (mineral substances) were determined using standard methods [30]. Total nitrogen was determined by the Kjeldahl method; lipids by the Soxhlet method; moisture by the gravimetric method; and mineral substances by the combustion method. Each measurement was conducted three times ($n = 3$, biological replicates).

2.4. Enzyme Activities: Protease, Collagenase, and Endo- and Exochitinase

Enzyme preparations obtained from the hepatopancreas of the red king crab were analyzed for protease, collagenase, and endo- and exochitinase activities to determine proteolytic, collagenolytic, and chitinolytic activity, respectively. Spectrophotometric analysis was used: solution optical density and absorption spectra were recorded using a Shimadzu UV-3101PC spectrophotometer (Shimadzu, Kyoto, Japan). Each measurement was conducted three times ($n = 3$, biological replicates). The study is limited to assessing the total enzymatic activity without examining the individual enzymes present in the complex enzyme preparation.

2.4.1. Proteolytic Activity

Proteolytic activity (A_{prot} , $\mu\text{mol Tyr}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$) was determined using a modified Anson method [31,32]. The absorption of soluble peptides formed from sodium caseinate during a 10 min proteolytic enzyme reaction at 37 °C was measured spectrophotometrically at 280 nm. Casein sodium salt from bovine milk (Sigma-Aldrich C8654, Taufkirchen, Germany) was used. One unit of proteolytic activity was defined as the amount (in μmol) of tyrosine (Tyr) released per gram of enzyme per minute.

2.4.2. Collagenolytic Activity

Collagenolytic activity (A_{col} , $\text{mIU}\cdot\text{mg}^{-1}$) was determined spectrophotometrically by measuring hydroxyproline absorbance at 280 nm. Hydroxyproline was formed from collagen hydrolysis by collagenolytic enzymes for 30 min at 37 °C, followed by complete acid hydrolysis and subsequent 4-dimethylaminobenzaldehyde (Ehrlich's Reagent) colorimetric detection [32]. Bovine collagen type I from bovine Achilles tendon (Sigma-Aldrich C9879, Schnellendorf, Germany) was used. One unit of collagenolytic activity was defined as the amount (in μmol) of hydroxyproline (Hyp) released per milligram of enzyme (mIU/mg).

2.4.3. Chitinolytic Activity

Endochitinolytic activity (A_{endo} , $\mu\text{mol GlcNAc}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$) was determined by the enzymatic hydrolysis reaction of a colloidal chitin suspension (pH 7) after 30 min incubation with the enzyme preparation at 37 °C. The decrease in the optical density of the suspension at 700 nm was measured [33]. Exochitinolytic activity (A_{exo} , $\mu\text{mol GlcNAc}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$) was determined by the reaction of the N-acetyl-D-glucosamine hydrolysis product with 4-dimethylaminobenzaldehyde (Ehrlich's Reagent) to form a colored complex [34]. One unit of exochitinase activity was defined as the amount (in μmol) of N-acetyl-D-glucosamine (GlcNAc) released per gram of enzyme per minute during the reaction with colloidal chitin. Colloidal chitin was prepared from shrimp chitin (Sigma-Aldrich C7170, St. Louis, MO, USA) using a method involving chitin reprecipitation in concentrated hydrochloric acid [33].

2.5. Statistical Analysis

Results were presented as mean $\pm$ standard deviation. In order to control the measurements' reproducibility, each measurement was conducted three times ($n = 3$, biological replicates). Experimental data were processed using Microsoft Excel LTSC Pro 2021. Data were subjected to one-way analysis of variance (ANOVA), and means were compared using Fisher's least significant difference (LSD) test. The confidence level was set at 95% ($p < 0.05$). When the ANOVA was significant ($p < 0.05$), Tukey's post hoc multiple comparison tests were applied. For enzyme activity analyses, optical absorption spectra were processed using MagicPlot (ver. 3.0.1, MagicPlot Systems, LLC, Saint

Petersburg, Russia). Statistical analysis was performed using the Statistical Package for Social Science (SPSS for Windows version 16.0, SPSS Inc., Chicago, IL, USA).

3. Results and Discussion

3.1. Chemical Composition of the Hepatopancreas

The chemical composition of the red king crab (*Paralithodes camtschaticus*) hepatopancreas used for enzyme preparation is shown in Table 1.

Table 1. Chemical composition of hepatopancreas from the red king crab (caught in January–February).

Components	Content, %
Moisture	72.3 ± 2.0
Protein	15.9 ± 0.3
Lipids	10.2 ± 0.4
Mineral Substances	1.6 ± 0.1

Means ± standard deviation (n = 3).

The raw material was characterized by high protein content (15.9 ± 0.3%) and significant lipid content (10.2 ± 0.4%). The latter required removal during enzymatic isolation.

3.2. Analysis of Methods for Obtaining Enzyme Preparations

Nearly all patents that propose methods for producing proteolytic and collagenolytic enzyme preparations from crab hepatopancreas and fish viscera utilize homogenization of the raw materials in water or a buffer solution. This process is followed by the fractionation of lipids, undissolved proteins, and a solution of water-soluble proteins (enzymes). The processing methods for enzyme-containing raw materials are diverse; therefore, it is challenging to classify all the presented methods into clearly defined groups. In this section, we have categorized the known methods based on key operations (Table 2), with a focus on lipid removal and the autolysis step of the raw materials.

Table 2. Methods for obtaining enzymes from the internal organs of crustaceans and fish.

Raw Materials	Enzyme	Basic Operations	References
Enzyme preparations obtained using lipid extraction with cold solvents			
Crab (<i>Paralithodes camtschaticus</i>) hepatopancreas	Collagenase	Destruction of hepatopancreas tissue in acetone at −20 °C, homogenization, washing of sediment with acetone and n-butanol at −20 °C	[29,35]
Crab (<i>Paralithodes camtschaticus</i>) hepatopancreas	Enzyme preparation	Homogenization of hepatopancreas in acetone (ratio 1:8, −20 ÷ −12 °C), then purification with acetone and n-butanol	[36]
Hepatopancreas of the Black Sea crustacean (<i>Palaemon adspersus</i>)	Collagenase	Homogenization of the hepatopancreas in acetone at −20 ÷ −15 °C, separation of the sediment, washing of the sediment with cold acetone, centrifugation; resuspension of the sediment with cold n-butanol and repeating the operations as for treatment with acetone	[37]
Enzyme preparations obtained using hepatopancreas homogenization (and autolysis)			
Methods involving centrifugation			
Crustacean (<i>Uca pugnator</i>) hepatopancreas	Collagenase	Homogenization of the hepatopancreas with 3 volumes of cold 0.05 M Tris-HCl (pH 7.4), centrifugation, re-extraction of the sediment by centrifugation	[38]

Larvae of the genus (<i>Dermestes</i>)	Collagenolytic enzyme	Homogenization of raw materials in a 3% NaCl solution (pH 6–7), centrifugation; chromatography of the solution; purification of the enzyme solution by dialysis against a 0.005 M CaCl ₂ solution; lyophilization of the preparation	[39]
Methods including centrifugation and membrane filtration			
Crab (<i>Paralithodes camtschaticus</i>) hepatopancreas	Enzyme preparation	Homogenization of the hepatopancreas in a buffer solution (pH 6.0–9.5; 4 °C) of salt water; centrifugation, removal of the upper lipid layer and sediment; purification of the supernatant fractions containing the enzyme by microfiltration using membrane filters	[40–42]
Fish entrails (for example, pink salmon)	Enzyme preparation	Homogenization of raw materials and extraction of enzymes in a 3% NaCl solution at 35 °C; centrifugation; clarification by ultrafiltration of the liquid fraction (enzyme solution) on a membrane with a pass limit of 50 kDa; freeze-drying	[43]
Crab (<i>Paralithodes camtschaticus</i>) hepatopancreas	Enzyme preparation	Lipid removal without the use of a centrifugation step. Extraction of enzymes from raw materials in a NaCl solution at 19–21 °C; separation of insoluble particles by filtration (filter pore sizes of 10, 0.5, and 0.1 mm); removal of emulsified fat, cell debris, and microorganisms by microfiltration (pore size of 0.45 µm)	[44]
Methods involving precipitation of enzyme preparations with ammonium sulfate			
Fish entrails	Phosphodiesterase	Homogenization with water at 0–4 °C; extraction with an acid solution (pH 3); centrifugation and fractional fractionation of proteins with a solution of (NH ₄) ₂ SO ₄ ; the precipitate was dissolved and desalted by dialysis or gel filtration	[45]
Crab (<i>Paralithodes camtschaticus</i>) hepatopancreas	Collagenolytic protease	Isolation of three isoforms (A, B, C) of collagenolytic protease from the hepatopancreas of the Kamchatka crab by fractionation of the raw material with ammonium sulfate and chromatography on DEAE-Sephacrose 6B CL	[46]
Crab (<i>Paralithodes camtschaticus</i>) hepatopancreas	Enzyme preparation	Homogenization of crab hepatopancreas in a buffer solution, centrifugation, precipitation of proteins with ammonium sulfate; dissolution of the precipitate in Tris-HCl pH 7 and removal of ammonium sulfate by ultrafiltration	[47,48]
Crab (<i>Paralithodes camtschaticus</i>) hepatopancreas	Proteolytic enzymes	Homogenization of raw materials in a 0.1 M CH ₃ COONa solution at pH 6.0, filtration, removal of fat, microfiltration of the solution, then ultrafiltration; precipitation of proteins with ammonium sulfate; centrifugation, dissolution of the precipitate in water and desalination by dialysis; freeze-drying	[49]
Crab (<i>Paralithodes camtschaticus</i>) hepatopancreas	Collagenase	The use of ammonium sulfate to precipitate proteins and ultrafiltration to fractionate them	[50]
Fish entrails	Proteolytic enzymes	Extraction by homogenization of raw materials in 0.05 M Tris buffer, pH 7.4; centrifugation; precipitation of nucleic acids with polyethyleneimine; precipitation of proteolytic enzymes with ammonium sulfate; multi-stage purification by ion-exchange chromatography and dialysis	[51]
Hepatopancreas of snow crab <i>opilio</i> (<i>Chionoecetes opilio</i>)	Enzyme preparation	Homogenization of raw materials, autolysis in a solution of NaCl and NaHCO ₃ ; filtration, separation of fat from the aqueous phase; precipitation of protein with ammonium sulfate; dissolution of the precipitate, purification by microfiltration, ultrafiltration for desalination; lyophilization	[28]
Methods using chitosan lipid precipitation			

Hepatopancreas of commercial crabs	Collagenase	Homogenization of raw materials and autolysis 0–30 °C; precipitation of lipids with chitosan (pH 2.0–6.5); ultrafiltration of protein solution; lyophilization	[26,52]
Crab (<i>Paralithodes camtschaticus</i>) hepatopancreas	Enzyme preparation	Autolysis of raw materials and degreasing with chitosan salts; autolysis of raw materials at 21 °C (1:4 ratio); filtration; lipid precipitation with chitosan ascorbate; purification of the protein fraction by ultrafiltration; lyophilization	[27]
Methods using surfactants			
Crab (<i>Paralithodes camtschaticus</i>) hepatopancreas	Proteolytic enzymes	Homogenization in calcium chloride and sodium dodecyl sulfate solution; centrifugation, ultrafiltration, sterile filtration; freeze-drying	[53]
Crab (<i>Paralithodes camtschaticus</i>) hepatopancreas	Collagenase	Fractionation with an aqueous solution of ionic detergent (sodium dodecyl sulfate, sulfirol-8 (sodium 2-ethylhexyl sulfate), foaming agent (a mixture of sodium alkyl sulfates with a C-number of 10–13), alkyl sulfate paste (sodium alkyl sulfates based on primary alcohols with a C-number of 10–18)); concentration of the enzyme solution by dialysis	[54]
Methods including autolysis of raw materials			
Hepatopancreas of commercial crabs	Collagenase	Homogenization of raw materials and autolysis for 2–8 h at 0–30 °C and for 14–16 h at 19–21 °C	[43]
Fish entrails	Enzyme preparation	Preliminary autolysis of crushed raw materials in water at pH 6.2 for 1–5 h	[55]
Crab (<i>Paralithodes camtschaticus</i>) hepatopancreas	Enzyme preparation	Preliminary autolysis of varying duration at room temperatures and above: 6 h at 5–20 °C 14–15 h at 21 °C 1.5 h at 35 °C 6–15 h at room temperature	[26–28,43]

3.3. Effect of Different Lipid Removal Methods on Enzymatic Activity

Lipid removal is a critical and costly step in obtaining enzyme preparations from red king crab hepatopancreas.

Established methods for obtaining enzyme preparations from the hepatopancreas of red king crabs encompass four main production schemes (Table 2), each employing different defatting techniques:

1. Mechanical separation of lipids from an aqueous suspension of the hepatopancreas. This method represents the most advanced approach. To enhance lipid separation, the operation is conducted at room or elevated temperatures (ranging from 30 to 60 °C) for several hours to autolyze high-molecular-weight proteins and release water-soluble enzymes [27,28,42,51]. A notable disadvantage of this method is the reduction in activity of the resulting enzyme preparations during heat treatment. However, this method does not achieve complete lipid extraction from aqueous fractions, requiring subsequent defatting with organic solvents. Significantly, this approach reduces organic solvent requirements several-fold compared with the sequential extraction method that is used until complete defatting is achieved.
2. Complete extraction of lipids through three-fold washing with cold solvents (acetone and butanol) at −20 °C, using a hepatopancreas-to-solvent mass ratio of 1: (8–12) [29,34,36], to produce “acetone powder” [56]. For this three-fold washing process, the solvent consumption is approximately 60 kg of acetone and 30 kg of butanol per kilogram of hepatopancreas. However, this method cannot be regarded as environmentally friendly.

3. Use of sorbents, such as chitosan or its salts, to remove lipids from aqueous solutions of enzyme preparations [27,52,57]. A significant disadvantage of these methods is the potential contamination of the enzyme preparation with impurities from the sorbents if they are not completely removed from the solution.
4. A method for precipitating a dissolved enzyme preparation from solution using precipitants such as salts or water-miscible organic solvents. Precipitation (salting out) with ammonium sulfate is commonly used in laboratory practice, though it is sometimes described in patents [28,50]. The complexity of this method arises from the need for subsequent purification of the enzyme preparation to remove salt residues. Additionally, precipitation with acetone and subsequent washing of the precipitate with acetone are employed by some researchers, though this practice is typically limited to laboratory settings.

After comparing all the methods described above, we conducted an investigation into centrifugation for rapid fractionation, specifically focusing on the separation of the lipid fraction from the aqueous hepatopancreas suspension using a separation factor attainable with equipment ($7500 \times g$). The moisture and lipid contents of both the aqueous fraction and the centrifuge sediment are presented in Table 3.

Table 3. Moisture and lipid contents of the lipid fraction, dried enzyme preparation from the aqueous fraction, and dried sediment after hepatopancreas dispersion fractionation. Initial wet hepatopancreas mass: $2.00 \cdot 10^2$ g.

Fraction Type	Mass of Fraction, g	Moisture, %	Lipids, %	Proportion of Fat from the Total Fat Content of Raw Material, %
Lipid fraction	33.7 ± 0.8^a	$< 0.5^a$	98.0 ± 0.5^a	90.1 ± 1.2^a
Enzyme preparation (dried) obtained from the aqueous fraction	17.9 ± 0.5^b	8.8 ± 0.2^b	1.6 ± 0.2^b	1.4 ± 0.6^b
Insoluble precipitate (dried)	5.6 ± 0.2^c	6.1 ± 0.3^c	29.6 ± 0.5^c	8.4 ± 0.9^c

Different superscripts indicate significant differences in the tested fractions ($p < 0.05$). Means $\pm$ standard deviation ($n = 3$).

The results presented in Table 3 indicate that $90.1 \pm 1.2\%$ of the total hepatopancreas fat mass was transferred to the separated lipid fraction, with $8.4 \pm 0.9\%$ precipitating and $1.4 \pm 0.6\%$ remaining in the aqueous enzyme preparation. These values demonstrate incomplete lipid removal during the fractionation step, necessitating additional post-centrifugation lipid extraction from the aqueous protein fraction. This requirement for further purification is a significant drawback of the method.

To address this issue, we developed two methods for complete lipid extraction during enzyme preparation, based on the previously described techniques. These methods vary in certain details; however, the most critical aspect to emphasize is the use of low temperatures during the separation (centrifugation) process. This approach represents a novel strategy not previously discussed in the literature.

The first method involved homogenizing the raw material (hepatopancreas) in water at a 1:1 ratio. The resulting aqueous protein (enzyme) solution was then separated from the mixture and subsequently freeze-dried. The lyophilized proteins were washed with acetone to remove impurities, and the washed enzyme preparation was dried under vacuum. For every 1 kg of hepatopancreas, approximately 0.5 kg of acetone was used, resulting in a complex enzyme preparation with a yield of 10%.

The second method also involved homogenizing the hepatopancreas in water at a 1:1 ratio. After separation of the aqueous protein (enzyme) solution from the mixture, the enzymes were precipitated with acetone [58]. These precipitated enzymes were then separated by centrifugation. Next, the precipitate (the resultant enzyme preparation) was washed with acetone and dried under vacuum. For every 1 kg of hepatopancreas, approximately 3 kg of acetone was utilized, resulting in a yield of 1% for the complex enzyme preparation.

For comparison, we obtained the enzyme preparation through lipid extraction using acetone and n-butanol at $-20\text{ }^{\circ}\text{C}$ to produce “acetone powder.” The yields of the enzyme preparations ranged from 10% to 12%.

No lipids were detected in enzyme preparations obtained by complete acetone and n-butanol extraction at $-20\text{ }^{\circ}\text{C}$ (acetone powder), nor in those produced via the first and second methods (lipid mass fraction was below detection limits).

The hepatopancreas of Kamchatka crabs (*Paralithodes camtschaticus*) is a rich source of various proteases that are essential for digestion and metabolism. Several classes of proteases may predominate, including serine proteases (such as trypsin and chymotrypsin, which are commonly found in the digestive tract and play a crucial role in breaking down proteins into peptides) [59], as well as cysteine, aspartic, and metalloproteinases [60,61].

The resulting enzyme preparations were evaluated for proteolytic activity (A_{prot}) and exochitinolytic activity (A_{exo}), with the values for each defatting method presented in Table 3. The enzyme preparation obtained through lipid extraction using solvents (acetone and n-butanol) exhibited low activity, with proteolytic activity measured at $144 \pm 5\text{ }\mu\text{mol Tyr}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$ and exochitinolytic activity measured at $0.05 \pm 0.01\text{ }\mu\text{mol GlcNAc}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$. Overall, the data presented in Table 3 are consistent with previously published enzymatic activities from the hepatopancreas of white shrimp (*Litopenaeus vannamei*) [62].

A comparison of the enzyme activities resulting from the two proposed methods indicates that, although the second method required more acetone, it yielded an enzyme preparation with higher activity (Table 4).

Table 4. Proteolytic (A_{prot}) and exochitinolytic (A_{exo}) activity of enzyme preparation (at $37\text{ }^{\circ}\text{C}$) obtained via different lipid removal methods from January–February red king crab hepatopancreas.

Method Used to Obtain the Enzyme Preparation	A_{prot} , $\mu\text{mol Tyr}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$	A_{exo} , $\mu\text{mol GlcNAc}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$
Lipid extraction with solvents (acetone powder)	$144 \pm 5^{\text{a}}$	$0.05 \pm 0.01^{\text{a}}$
Lyophilization of an aqueous solution (first method)	$252 \pm 8^{\text{b}}$	$0.94 \pm 0.05^{\text{b}}$
Precipitation with acetone from an aqueous solution (second method)	$883 \pm 9^{\text{c}}$	$2.74 \pm 0.07^{\text{c}}$

Different superscripts indicate significant differences ($p < 0.05$) in the tested fractions. Means $\pm$ standard deviation ($n = 3$).

The data presented in Table 4 demonstrate that acetone precipitation (the second method) significantly enhances the resulting enzyme preparation’s proteolytic and chitinolytic activities. Specifically, the proteolytic activity obtained using the second method is $883 \pm 9\text{ }\mu\text{mol Tyr}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$, compared to $252 \pm 8\text{ }\mu\text{mol Tyr}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$ for the first method. However, the second method requires a substantial amount of acetone.

Additionally, the acetone precipitation method has a notable drawback associated with the separation of the precipitate from the water–acetone solution. The protein

precipitate is loose, and due to the presence of water in the solution, it forms a viscous substance during subsequent acetone washes that is difficult to separate. In contrast, the first method avoids this issue, as the lyophilized enzyme preparation is washed with acetone.

Although the activity of the enzyme preparation obtained by the second method is higher, its yield is nearly ten times lower than that of the first method. Furthermore, the second method is complicated by the challenges associated with the separation and washing of the precipitate.

The first method, which combines freeze-drying with acetone washing of the dried product, appears to be the most suitable, as it can be integrated into a single process chain following the separation of the hepatopancreas and the removal of lipid-insoluble substances.

Therefore, we selected the first method, which is novel compared with previously published methods and patents [29,34,36,56] for the following advantages:

- Low-temperature homogenization to preserve the activity of all hepatopancreas enzymes;
- Mechanical separation of lipids and insoluble proteins through the use of a flow separator (tricanter), which enables convenient and continuous operation;
- Freeze-drying the protein (enzyme) solution to completely remove water from the preparation;
- Washing the lyophilized preparation with anhydrous acetone to eliminate residual lipids, followed by drying under vacuum. The dry (anhydrous) preparation can be easily washed with anhydrous acetone, reducing acetone consumption to only 0.5 kg per 1 kg of the starting material (hepatopancreas).

The proposed method significantly reduces acetone consumption and exclusively employs mechanical techniques for isolating the enzyme preparation, aligning with the principles of green chemistry.

Based on our findings, we propose the following process flowsheet for producing a complex enzyme preparation from the hepatopancreas of red king crab (*Paralithodes camtschaticus*) (Figure 1). The production process comprises seven steps:

1. Thawing the red king crab hepatopancreas, previously stored at $-20\text{ }^{\circ}\text{C}$, at $0\text{ }^{\circ}\text{C}$, followed by 1 min of homogenization (Tecator 1094, Tecator, Höganäs, Sweden), maintained below $0\text{ }^{\circ}\text{C}$;
2. Mixing the resulting suspension at $0\text{ }^{\circ}\text{C}$ with cooled distilled water ($3\text{--}5\text{ }^{\circ}\text{C}$) at a 1:1 ratio and stirring for 30 min (autolysis time and temperature varied from 3 to $37\text{ }^{\circ}\text{C}$ during process development; pH 6.0–6.5; see Section 3.4);
3. Centrifugation of the suspension at 8000 rpm and $4\text{ }^{\circ}\text{C}$ for 60 min (Avanti J-25 centrifuge, Beckman Coulter, Indianapolis, IN, USA);
4. Collection and cheesecloth filter of the middle protein-rich fraction (protein solution) to remove residual fat;
5. Lyophilization of the aqueous protein solution (Heto FD 8, Heto-Holten A/S, Allerød, Denmark); condenser temperature of $-40\text{ }^{\circ}\text{C}$; initial temperature of shell of $-25\text{ }^{\circ}\text{C}$ with stepwise gradient increase in temperature up to $25\text{ }^{\circ}\text{C}$; drying time of 20 h; residual pressure of 0.04 hPa;
6. Washing the dried enzyme preparation with acetone (3–4 times) at $3\text{--}5\text{ }^{\circ}\text{C}$ to remove residual lipids; the criterion for “complete” lipid removal is a lipid content below the detection sensitivity of the Soxhlet method;
7. Final drying of the acetone-washed powder in a ShVS-45 vacuum drying oven (JSC “KMEP”, Kazan, Russia) at room temperature (residual pressure $< 100\text{ hPa}$).

The final enzyme preparation remains stable at $-20\text{ }^{\circ}\text{C}$ and at room temperature for one year.

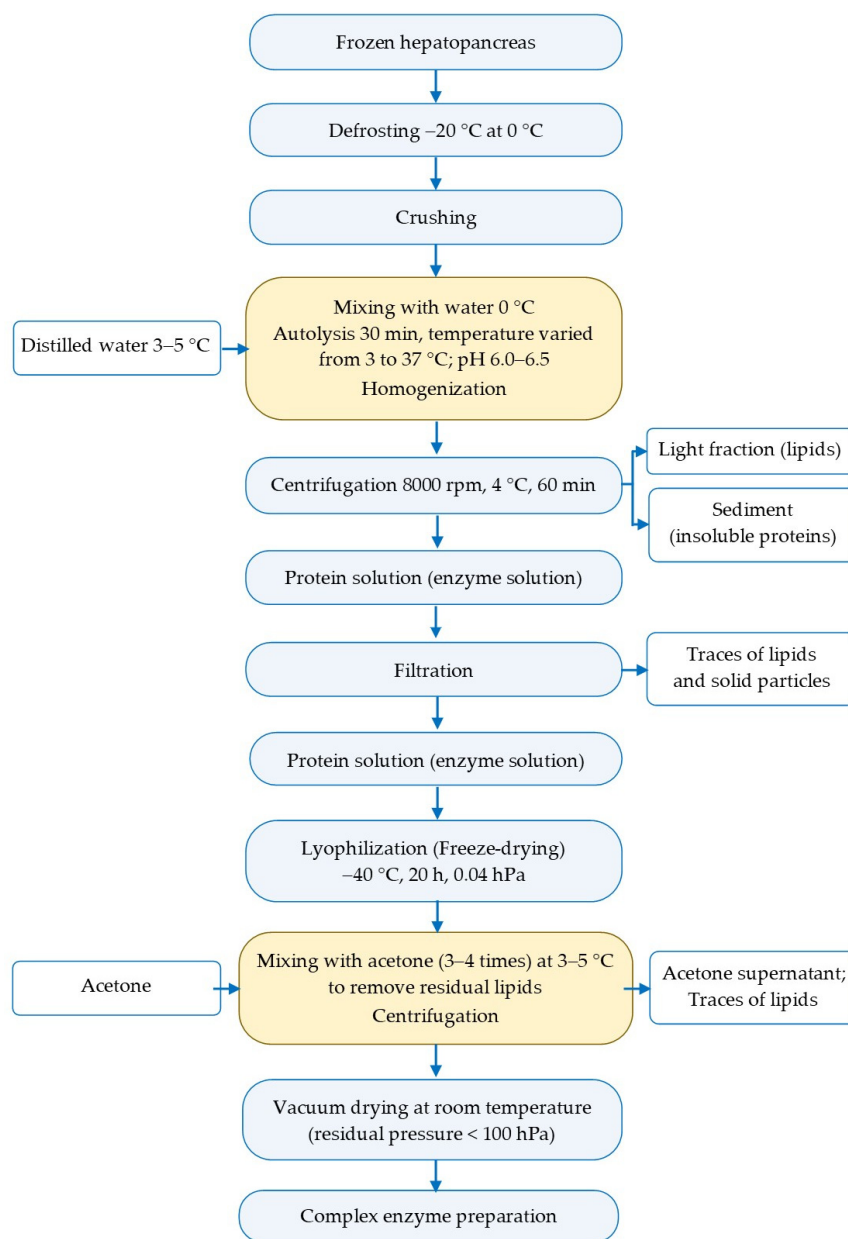


Figure 1. Flowchart for the preparation of a complex enzyme extract from the hepatopancreas of the red king crab, following the proposed method.

3.4. Influence of Autolysis Temperature on Enzymatic Activity

Autolysis of raw materials is commonly employed in enzyme isolation and addresses several key issues [25]. First, it enhances lipid yield through the enzymatic hydrolysis of various lipoproteins and other lipid–protein compounds. Second, it increases the yield of the water-soluble protein fraction by breaking down high-molecular-weight proteins and releasing low-molecular-weight enzymes from the insoluble precipitate.

We studied the dependence of enzymatic activities of the resulting preparation on the autolysis temperature, which occurs during raw material homogenization before centrifugation for 30 min (see Section 3.3., Figure 1). Autolysis of red king crab

hepatopancreas involves the enzymatic breakdown of raw components, forming peptides, amino acids, and fatty acids. Precise control of autolysis conditions (temperature, pH, time) enables production of products with desired properties for food, medical, and other applications.

Our study investigated autolysis temperature effects on enzyme preparation properties from hepatopancreas samples. The results presented in Figure 2 indicate that the autolysis temperature is a critical factor in maintaining high enzymatic activity for all enzymes in the final product. It has been shown that significant decreases in proteolytic and chitinolytic activities occurred as temperatures rose from 3 to 37 °C (at pH 6.0–6.5). Proteolytic activity decreased 7.5-fold and exochitinolytic activity 1.6-fold (Figure 2); collagenolytic activity decreased the least (1.1-fold). These findings question the feasibility of patented enzyme productions using high-temperature hepatopancreas autolysis to enhance lipid yields [26–28].

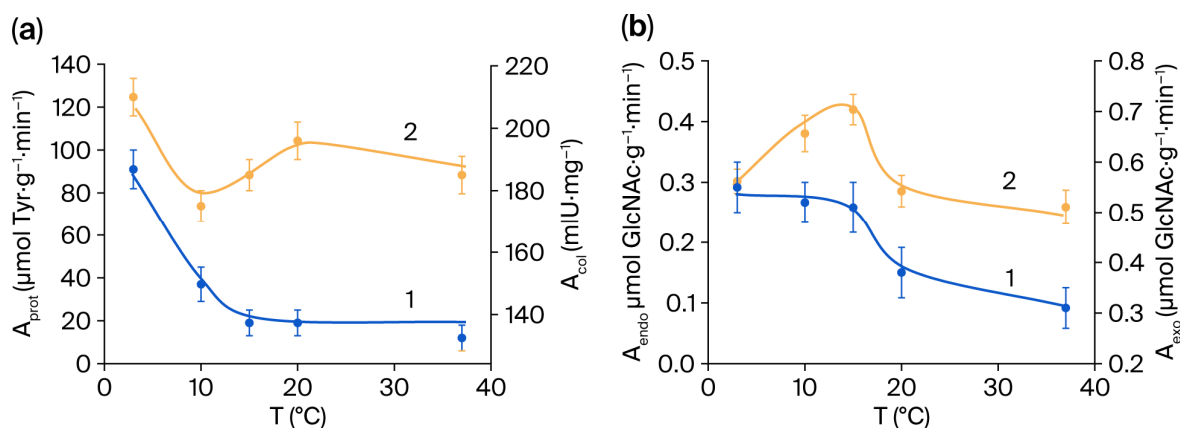


Figure 2. Enzyme activities from hepatopancreas samples as a function of autolysis temperature (30 min): (a) proteolytic, A_{prot} , (1) and collagenolytic, A_{col} , (2); (b) exochitinolytic, A_{exo} , (1) and endochitinolytic, A_{endo} , (2); $T = 37$ °C.

Clearly, low temperatures during hepatopancreatic autolysis can preserve enzyme activity, likely because of several factors. At high temperatures, proteins, including enzymes, can denature rapidly. In contrast, at low temperatures, this process slows down, allowing enzymes to maintain their active form for a longer period.

Although the rate of autolysis is higher at elevated temperatures, these conditions can also promote undesirable reactions and lead to the degradation of the enzymes themselves. Furthermore, along with the target proteases, other proteolytic systems may be involved in the reaction, or advanced degradation products may accumulate, which can inhibit enzyme activity or lead to uncontrolled degradation of the enzyme complex. Low temperatures reduce the likelihood of these adverse reactions and help maintain the stability of the enzyme's active site, which is critical for their effective function.

Thus, performing autolysis at low temperatures not only preserves but may also enhance enzyme activity.

The obtained results (Figure 2) suggest that to preserve hepatopancreas enzyme activity, aqueous dispersion processing, including autolysis, should be conducted at temperatures near 0 °C (not exceeding 5 °C). Autolysis requires monitoring as it can reduce isolated enzyme activities. Given the time required for thawing, homogenization, and separation, minor autolysis is unavoidable; therefore, all enzyme isolation operations should proceed at low temperatures.

3.5. Influence of Harvest Time on Enzyme Preparation Activity

The characteristics of enzyme preparations are significantly influenced by the properties of the raw materials used. Consequently, understanding the impact of the crab harvesting season on the activity of proteases present in the hepatopancreas is a critical factor in processing the hepatopancreas for the production of enzyme preparations.

Proteolytic enzyme activity in the hepatopancreas varies by season, with minimum values in summer (July–August) and maximum values in winter (January–February) [10,62]. Proteolytic enzyme activity in January–February is, on average, 2.2–2.7 times higher than in August–September.

In our study, the proteolytic activity of the enzyme preparation from hepatopancreas collected in August–September was $91 \pm 9 \mu\text{mol Tyr}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$, while proteolytic activity from winter-caught crabs reached $252 \pm 12 \mu\text{mol Tyr}\cdot\text{g}^{-1}\cdot\text{min}^{-1}$ (Figure 3).

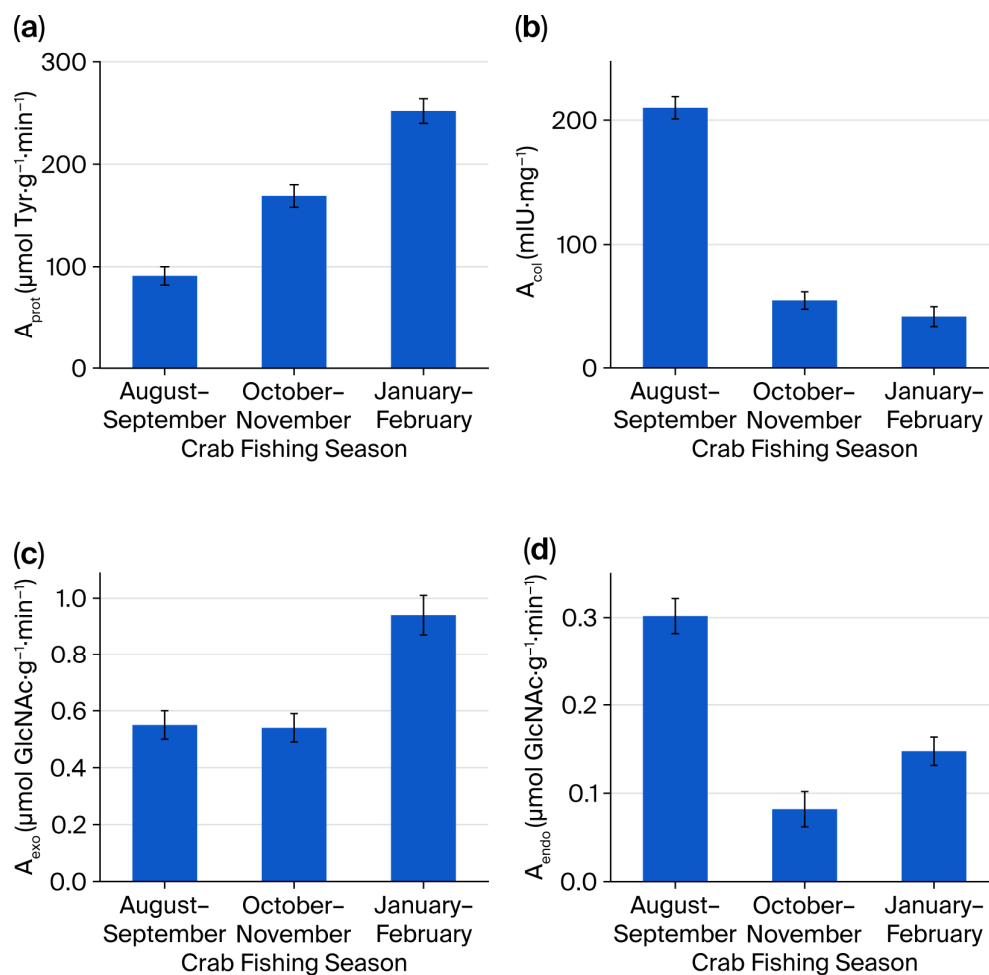


Figure 3. Enzymatic activities in the complex preparation from red king crab hepatopancreas collected at different times of year: (a) proteolytic activity, A_{prot} ; (b) collagenolytic activity, A_{col} ; (c) exochitinolytic activity, A_{exo} ; (d) endochitinolytic activity, A_{endo} ; $T = 37^\circ\text{C}$.

The proteolytic activity of enzyme preparations from winter-caught crabs was 2.77 times higher than that in the summer, consistent with previous reports on Kamchatka crab [10]. Conversely, collagenolytic activity was lower in February ($42 \pm 8 \text{ mIU}\cdot\text{mg}^{-1}$) compared with August ($210 \pm 9 \text{ mIU}\cdot\text{mg}^{-1}$). These seasonal enzymatic variations reflect changing metabolic requirements, with proteolytic activity supporting digestion during

winter food shortages and collagenolytic activity supporting growth and molting in summer [6].

In winter, food availability decreases, necessitating increased proteolytic activity to facilitate efficient digestion of scarce food resources and to maximize the extraction of available amino acids [7]. Under starvation conditions, the organism may initiate controlled proteolysis of its own proteins, such as contractile muscle proteins or storage fractions. The resulting amino acids are then utilized for the synthesis of vital proteins, including enzymes, transport proteins, and immune factors. Many species of crustaceans enter their reproductive season in the spring, and the accumulation of amino acids during winter serves as a reserve for synthesizing egg and milk proteins during the pre-spawning period.

During summer, crabs undergo molting, a process essential for growth that involves periodically shedding their old exoskeleton (cuticle), composed of chitin and structural proteins, including collagen [10]. Collagenases are responsible for breaking down the old cuticular matrix at the sites of future rupture. Collagenolytic enzymes play a critical role in connective tissue remodeling, facilitating the subsequent growth of limbs and internal organs. Enhanced collagenolytic activity also allows for more efficient digestion of collagen-rich foods, such as mollusks, fish, and other crustaceans, which are more readily available during the summer months.

These changes in enzyme activity do not occur in isolation; rather, they are closely linked to key environmental factors, including seasonal temperature variations, daylight hours, and food availability and composition. Seasonal fluctuations in proteolytic and collagenolytic activities in crabs represent a complex, regulated adaptive strategy that enables optimization of metabolism: conserving energy for survival during winter and promoting growth and reproduction during summer [6,7].

This understanding is crucial when preparing raw materials for enzyme preparations derived from commercial crabs.

4. Conclusions

Red king crab (*Paralithodes camtschaticus*) hepatopancreas is a valuable secondary raw material with high protein and lipid contents and can be used as a source for the extraction of complex hydrolytic enzyme preparations. In this study, a low-temperature process for producing a complex enzyme preparation from red king crab hepatopancreas was proposed and experimentally validated. The process involves the removal of most lipids from an aqueous dispersion by low-temperature mechanical centrifugation, followed by lyophilization of the protein fraction and subsequent purification of the dried preparation with a small amount of acetone. This sequence of operations substantially reduces the volume of material subjected to solvent treatment. Compared with conventional acetone powder production, the proposed method reduces acetone consumption and eliminates the need for butanol.

A comparative study of protein precipitation from aqueous solution using acetone was also performed. Although this approach provided the highest specific enzymatic activity, it was associated with a low preparation yield and technological difficulties in separating the precipitate. In contrast, the method proposed in this study, which combines lyophilization of the aqueous protein fraction with subsequent acetone washing of the dried preparation, provides a more favorable combination of characteristics: high preparation yield, sufficiently high enzymatic activity, reduced acetone consumption, and improved technological feasibility.

The effect of short-term autolysis temperature on the proteolytic, collagenolytic, endochitinolytic, and exochitinolytic activities of the enzyme preparation was established. Increasing the temperature to 20–37 °C significantly reduced proteolytic activity.

Therefore, the proposed isolation procedure should be carried out at low temperatures, preferably within the range of 0–5 °C.

A seasonal dependence of the enzymatic profile of both the hepatopancreas and the resulting enzyme preparation was demonstrated. Raw material obtained in winter was characterized by high proteolytic activity, whereas summer raw material exhibited high collagenolytic activity. Thus, the harvesting season of red king crab should be considered an important parameter for standardizing the raw material and, consequently, the enzyme preparation obtained from it.

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