UTILIZATION OF ENZYME-CONTAINING PRODUCTS OBTAINED FROM FISH WASTE IN LEATHER PRODUCTION PROCESSES

Olga Andreyeva[1], Anna Atamanova[1], Tetiana Maievska[2], Nataliia Raksha[3] and Olexiy Savchuk[3]

[1]*Kyiv National University of Technologies and Design, Nemirovich-Danchenko Street 2, Kyiv, Ukraine*
[2]*NGO 'Ukraine's Fishing Industry Cluster of Innovations'', Instytutska Street 18, section B, Kyiv, Ukraine*
[3]*Educational and Scientific Centre "Institute of Biology and Medicine" Taras Shevchenko National University of Kyiv, Volodymyrska Street 64/13, Kyiv, Ukraine*
wayfarer@ukr.net; forel2016@gmail.com; nkudina@ukr.net; olexiy.savchuk@ukr.net

Abstract: *The properties of enzyme-containing product obtained from fish-processing waste were studied. High enzymatic activity and structural features e.g. compatibility with collagen, as well as the results of organoleptic evaluation and analysis of biogenic fibrous material in the form of semi-finished leather products (pelt) after modification by this product indicate its possible use in biotechnological processes of leather production.*

Keywords: *fish waste, enzyme-containing product, leather production, biogenic fibrous material, modification, structure, properties.*

1 INTRODUCTION

Nowadays the main trends in the development of food, light and other various branches of industry are the production of competitive products along with the rational use of material and energy resources, as well as the reduction of the harmful pressure on the environment. The ways of implementation of these trends are the improvement of technological processes through the development and introduction of more sustainable processing methods of effective, hazardous chemical materials. Such materials include enzymes - specific compounds which due to their protein nature and catalytic action are becoming more popular due to their positive effects on the course of technological processes, the quality of products, and their ability to ensure the respect of the resource conservation principles and environmental protection. Enzymes have already been used for genuine leather production in various forms: from saliva, manure, pancreas, in ancient and outdated methods of past years to the new generation drugs synthesized in modern chemical and bio-industries [1-5]. Despite the undeniable advantages of these materials, the lacking of Ukrainian supplies along with the presence of expensive foreign-made products is considered to be the biggest challenge today. Therefore, the search for new promising sources and forward ways to produce affordable and effective domestic product is as relevant as essential as ever.

A number of studies conducted in the past several decades [6-11] revealed that during the fish processing a significant amount of waste is formed, which contains many valuable components *e.g.* minerals, proteins, fats, enzymes. As a result, fish waste has found its applications in animal feed, food for healthy nutrition, biodiesel/biogas, natural pigments, food packaging, cosmetics, enzymes purification, Cr immobilization, soil fertilization and food moisture maintenance [8].

While studying the mass composition of pond fish that dwell the Volga-Caspian basin (grass carps, carps, silver carps), it was found that the bulk of the fish wastes consist of bone tissue (about 62%) and internal organs (about 21%). Taking into consideration the high collagen content in the bone waste (up to 40% of the total amount of protein), it is advisable to obtain from them first structure-forming compounds, and later, due to the higher minerals content (25% of the total chemical composition), mineral supplements. The main part of the internal organs of pond fish is represented by the liver, intestines, swimbladder and roe (milt). The analysis of the pond fish entrails chemical composition revealed a high protein content in the intestines and swimbladder (11-28%), therefore it is desirable to use them to obtain protein products. However, the proteins of the intestine and liver, which include the tissues of the pancreas and characterized by the presence of various enzymes, is advisable to use for enzyme manufacturing. Due to the fact that the intestine of silver carp is characterized by a high fat content (20%), it is proposed to use it for biofuels and lipolytic enzymes manufacturing [9].

Enzymatic methods became an integral part of the modern food and feed industry processes namely the production of a wide range of products for human and animal consumption. Since a huge amount of diverse genetic material in the aquatic environment is present it is recognized as a huge enzymes resource. In recent years, the enzymes from fish and aquatic invertebrates have been isolated and their characteristics have been determined. Moreover, several interesting applications related to marine enzymes in the food industry have been obtained. In a review of Canadian scientists [10] the current information on the digestive and muscle enzymes of fish and aquatic invertebrates was summarized, as well as the important advances in the use of marine enzymes in food was reported.

According to Arvanitoyannis and Kassaveti [8], fish waste should be recycled to obtain valuable enzymes that are beneficial to the world, and not improperly disposed, causing environmental pollution. It is useful to use fish waste as fish silage (a liquid product made from waste with the addition of acid or, less frequently, alkali), fishmeal for livestock farming, pharmaceuticals etc.

To convert fish waste into useful products, a group of Nigerian scientists used three sources (*Pseudomonas fluorescens, Enterobacter cloacae, and Bacillus megaterium*) for protease production. The most effective activity was detected at a temperature of 45°C and pH 9, thus promoting the fish stock decomposition. The protease enzymes can be used in the industry of baby food production, laundry detergents manufacturing, as well as in medicine to control blood clotting and pathogenic proteins decomposition [11].

The review of Ukrainian fish market in 2019 [12] as an optimal scenario for 2020 has maintained the fish and seafood imports within 400 thousand tons while increasing the total value of goods that are imported by expanding the range of imported fish products; the production volumes of own aquatic bioresources will be remained within the limits of 90-100 thousand tons. At the same time, an increase in fish products exports by 10-15% was expected due to the opening of new markets and an increase in shipping through the already existing contracts. The data from the State Statistics Service [13] showed that in 2020 some 76.5 thousand tons of own aquatic bioresources was extracted, 48.2 tons of which is fish. Although this result is lower than expected, it reveals a real source of fish waste generator in the country which could be used to produce secondary material resources for different industries.

In view of these facts, the aim of the present research was to study the properties of enzyme-containing product from fish-processing industry waste to identify its possible use as a secondary material resource in the production of genuine leather. For this purpose, the following objectives have been pursued:

- to perform the analysis of the product from fish-processing industry waste;
- to determine the enzymatic activity of the product;
- to find out the structural features of this product;
- to determine the suitability of the enzyme-containing product for the modification of biogenic fibrous material in the form of semi-finished leather products (pelt).

2 MATERIALS AND METHODS

The enzyme-containing product (ECP) was obtained from the waste of *Oncorhynchus mykiss*. ECP is a brown fine-fibrous substance that is readily soluble in warm water. Both common and advanced techniques - chromatographic, spectrophotometric, electrophoretic, infrared spectroscopy - were used to study the obtained product. The frozen mass of fish processing waste was suspended in the extraction buffer (0.1 M Na-phosphate (pH 7.0), containing 0.15 M NaCl, 1.5 mM ethylenediamine tetraacetic acid (EDTA), PEG-6000, and 0.1% Triton X-100) at the ratio 1:3, w/v and stirred continuously at 4°C for 1 hour. After that, the sample was centrifuged (Allegra 64 R Centrifuge, Beckman Coulter, USA) at 10 000 g for 30 min at 4°C.

The supernatant was purified by size exclusion chromatography on a Sephadex G25 column (GE Healthcare). After that, the supernatant was lyophilized (Telstar LyoQuest) and stored at 4°C until use. The lyophilized samples (50 $mg.mL^{-1}$) were dissolved in 10 mM Tris-HCl (pH 8.0) containing 5 mM $CaCl_2$. After centrifugation at 10.000 g for 5 min, the supernatant was loaded to a benzamidine sepharose column (flow rate of 180 mL per hour), which was pre-equilibrated with the same buffer. The bound fraction was eluted with 50 mM glycine (pH 3.0) containing 5 mM $CaCl_2$, and 1 M NaCl at a flow rate of 180 mL per hour. The obtained fraction was immediately neutralized to pH 7.4 to prevent loss of enzyme activity. Then the fraction was subjected to size exclusion chromatography on Sephadex G25 column pre-equilibrated with distilled water buffered with NaOH to pH 8.0. The fraction was loaded and the peak was collected at a flow rate of 45 mL per hour. The obtained fraction was lyophilized and used as the enzyme-containing product for further research.

The ECP was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as reported [14] using 4% (w/v) stacking gel and 15% (w/v) separating gel. SDS-PAGE was performed using Mini-Protean Tetra System (Bio-Rad, USA) at 19 mA for stacking and 36 mA for separating gels. The samples were prepared by mixing with sample buffer (0.005 M Tris-HCl, pH 8.8, 2% SDS, 5% sucrose, and 0.02% bromophenol blue) at the ratio

of 1:1 (v/v). The samples were heated at 95°C for 1 min before loading in the gel. The total amount of proteins applied per well of gel was 20 μg. The gels were stained with 2.5% Coomassie brilliant blue R-250 in 10% (v/v) ethanol, 10% (v/v) acetic acid, 15% (v/v) isopropanol and the background of the gel was destained with 7% (v/v) acetic acid for 30 min. Apparent molecular weights of proteins were estimated using a protein calibration mixture (Bio-Rad, USA).

Zymography was carried out according to the method [15]. The separating gel solution (15%) was polymerized in the presence of fibrinogen (1 mg·mL^{-1}). The samples were not heated before loading in the gel. After electrophoresis, the gels were soaked in 2.5% Triton X-100 solution with shaking (30 min at 25°C) to remove SDS and renaturation of proteins. The gels were washed with distilled water for 10 min to remove Triton X-100 and then were incubated in 50 mM Tris-HCl (pH 7.5) at 37°C for 12 h. The digested bands were visualized as the unstained areas on the dark background of the gel. TotalLab 2.04 program was used to analyze the obtained electropherograms and zymograms. The represented electropherogram and zymogram are typical for the series of the repeated experiments (at least three in each series).

The enzymatic activity of ECP was analyzed by two different methods - using a chromogenic substrate Phe-Pip-Arg-pNA and by determining the caseinolytic activity. The activity against Phe-Pip-Arg-pNA was measured as described [16]. The reaction mixture consisted of 50 mM Tris-HCl (pH 9.0) and a sample of ECP (20 μg of total protein). The reaction was initiated by the addition of Phe-Pip-Arg-pNA (Renam, RF) (0.3 mM). The production of p-nitroaniline was monitored at regular intervals at 405 nm. The amount of p-nitroaniline realized from the substrate was calculated using a molar extinction coefficient of 10.000 M-1 x cm^{-1} for free p-nitroaniline. The caseinolytic activity of ECP was measured using casein as a substrate according to the method [17]. Casein (2%) in 50 mM Tris-HCl buffer (pH 7.4) containing 0.13 M NaCl was incubated in the presence of the sample of ECP (50 μg of total protein) at 37°C for 30 min. The reaction was stopped by the addition of trichloroacetic acid (7%) and the sample was kept for 15 min at 4°C. Then the sample was centrifuged at 15.000 g for 30 min. The absorbance of the supernatant was measured spectrophotometrically (SmartSpecPlus, Bio-Rad, USA) at 280 nm against the blank in which the test sample was replaced with an equal volume of 50 mM Tris-HCl buffer (pH 7.4) containing 0.13 M NaCl. The concentration of proteins in the ECP was determined by the method of Bradford [18], using bovine serum albumin as a standard.

Infrared spectroscopy was exploited to study the structural features of the ECP. IR-spectra were recorded in transmission mode by FT-IR spectrometer Tensor-37 (Bruker, Germany). The lyophilized samples and spectrally pure potassium bromide were pressed into thin pellets at a ratio of 2:100. The obtained spectrogram was processed using the baseline and internal standard methods; the interpretation of individual groups and bonds was performed according to [19-21].

The technological properties of ECP were studied taking into account the results of the use of ECP in the amount of 0.20, 0.35, 0.50 and 0.65% during modifying (softening) biogenic fibrous material in the form of a pelt. To exclude the influence of topographic areas of the skins, the experimental groups were composed using the method of asymmetric fringe [22]. Each group consisted of three samples. The control group #1 contained an unmodified limed hide. The modifications of groups #2-6 were carried out according to the standard method of the production of natural leather. The samples of group #2 were treated with 0.35% industrial enzyme preparation Enzymas 1072 while the samples of groups #3-6 - 0.20-0.65% ECP.

The effect of ECP consumption on the properties of the pelt was determined based on organoleptic assessment, welding temperature, moisture content, mass fraction of nitrogen, and gelatin melting. Sample preparation was carried out in accordance with ISO 2418. The welding temperature was determined as the temperature at which the pelt shrinks when heated in water in accordance with the requirements of DSTU 3177: Determination of welding temperature. The moisture content in the pelt was determined gravimetrically at a temperature of 100-105°C in accordance with ISO 4684. The mass fraction of nitrogen was determined by the Kjeldahl method. The Kjeldahl method was used to assess the state of a skin semi-finished product before and after its treatment with enzymes. The analysis was carried out in accordance with the requirements of DSTU ISO 5397.

Gelatin melting was estimated according to the protocol [22, 23]. The concentration of gelatin was determined using a calibration curve built on the basis of the results of determining the optical density of gelatin solutions of various concentrations (from 0.10 to 0.75 mg/mL).

3 RESULTS AND DISCUSSION

A product in the form of a brown fine fibrous substance soluble in warm water and containing various proteolytic enzymes was obtained by the primary sorption and further lyophilization of fish processing wastes. To purify the fraction of trypsin-like enzymes, which constitute a significant part of the proteases of the feedstock, and to stabilize the obtained enzymes, the chromatography on a benzamidine-sepharose column followed by lyophilization was carried out (Figure 1).

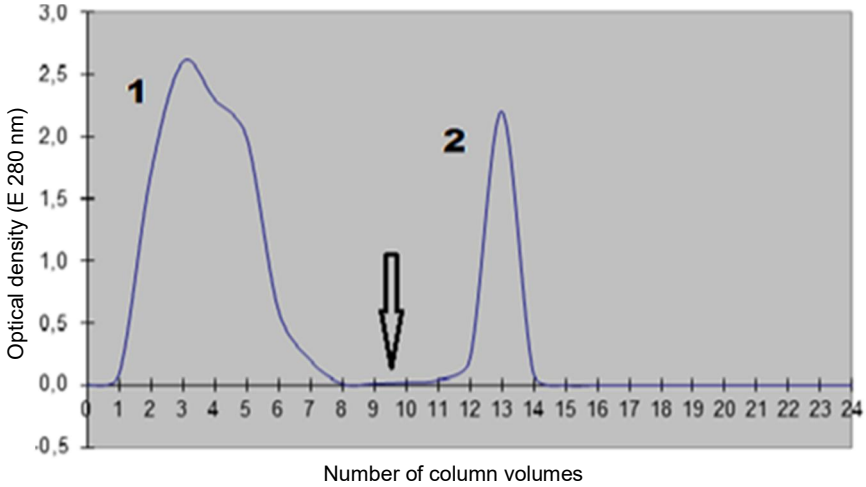

Figure 1 Chromatogram of the obtaining of ECP from the fish processing wastes a benzamidine-sepharose column: 1 - the fraction containing unbound proteins; 2 - the fraction of ECP the arrow indicates the point where the working buffer (10 mM Tris-HCl (pH 8.0) containing 5 mM $CaCl_2$) was replaced with the elution buffer (50 mM glycine (pH 3.0) containing 5 mM $CaCl_2$, and 1 M NaCl)

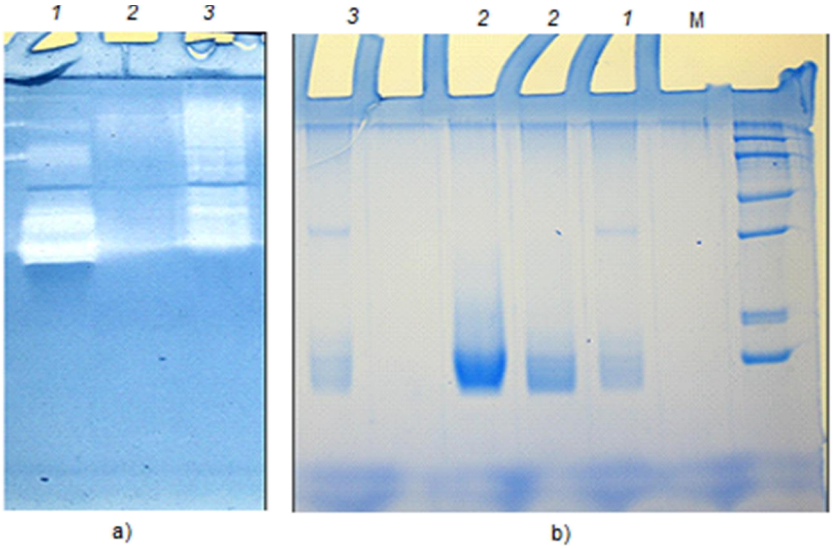

Figure 2 Substrate SDS-PAGE (a) and SDS-PAGE (b) analysis of ECP obtained by affinity chromatography on benzamidine-sepharose: 1 - starting material; 2 - the unbound fraction (10 and 20 μg of proteins per line); 3 - the fraction of ECP; M - molecular weight markers (97, 66, 45, 31, 21, 14 kDa)

The presence of active proteolytic enzymes in the fraction eluted from the benzamidine-sepharose column was confirmed by enzyme electrophoresis using fibrinogen as a substrate (Figure 2a). The result of the SDS-PAGE assay indicates the presence of several proteins in the fraction obtained after the affinity chromatography step: 60, 30, 21, 18 and 14 kDa (Figure 2b). Next, the enzymatic activity of ECP was evaluated using a specific chromogenic substrate Phe-Pip-Arg-pNA for serine proteases. According to the obtained data, the activity of ECP was 260 μmol $pNA.min^{-1}.mg^{-1}$ protein; the level of protein was 0.41 $mg.mg^{-1}$ extract. The caseinolytic activity of ECP was estimated to be 450 $IU \cdot g^{-1}$ product.

Since the treatment of biogenic fibrous leather materials during the production of natural leather is carried out in a wide range of temperature and pH, to which the enzymes are very sensitive, the influence of these factors on the enzymatic activity of ECP has been investigated. For this purpose, the enzymatic activity of ECP was estimated at temperatures of 25, 35, 45 and 55°C, and the range of pH from weakly acidic (pH 5.56) to basic (pH 12.45). As can be seen from Figure 3, the enzymatic activity of the ECP increased with increasing temperature. The maximum enzymatic activity was found at 55°C.

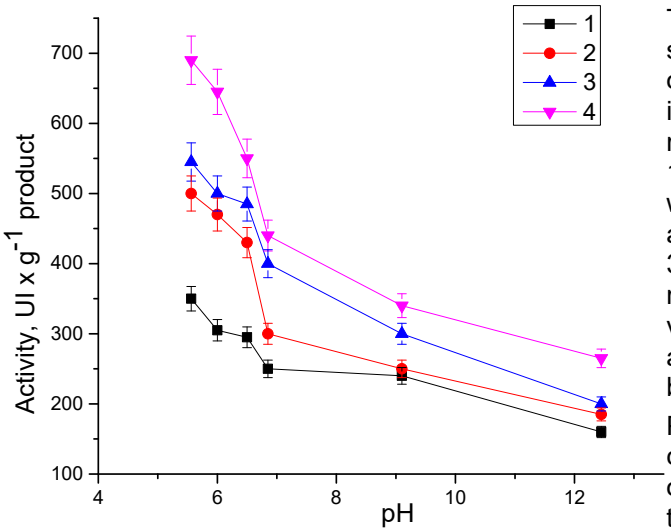

Figure 3 The influence of temperature and pH on the enzymatic activity of ECP: 1 - 25°C; 2 - 35°C; 3 - 45°C; 4 - 55°C

However, the increase in pH caused a decrease in the enzymatic activity of ECP. On the whole, it can be said that the enzymatic activity of ECP was more pronounced at pH of 6.0-7.0 and at a temperature not lower than 35°C. The obtained data indicate the possibility of using ECP in the leather industry, in particular in processes that occur at pH close to neutral and at increased temperatures, for example, during bating.

The compatibility of the materials used with the fibrous collagen, which is the major component of the skin, is important for the effective modification of biogenic objects such as animal skin. It is known from the theory and practice of leather production that not only the uniform diffusion of chemical material but also its fixation in the microstructure of the dermis, is important for achieving the necessary technological effect. This largely depends on the structure of the material used.

To identify the structural features of ECP, an IR spectroscopic study was carried out. The analysis of the spectrogram (Figure 4) revealed that the most intense absorption bands were in the frequency range from 3700 to 3100 cm^{-1} and from 1750 to 1000 cm^{-1}, while less intense bands of absorption were in the range from 900 to 400 cm^{-1}. The wide absorption band was observed in the range of 3700-3100 cm^{-1} with peaks at 3333 and 3505 cm^{-1}. These results indicate the presence of bonded and free valence NH-groups, bonded OH-groups, and amides, which involved in the formation of hydrogen bonds.

Peaks in the frequency range of 2857 and 2928 cm^{-1} correspond to symmetric and asymmetric valence oscillation of CH_2-groups. The oscillation in the range of 3100-3000 cm^{-1} with peak at 3068 cm^{-1} characterizes the valence and deformation vibrations of the N-H bond in the NH_3^+ group and valence vibrations of the CN-group of the Amide II overtone. The presence of peaks at 1750-1480, 1743, 1663, 1629 and 1527 cm^{-1} could be a result of the oscillation of NH-bonds (Amide I and Amide II). This suggests the valence oscillation of the bond (O=C-NH-), as well as β- and α-conformation of molecules. A significant band of absorption with a peak at 1234 cm^{-1} was observed in the frequency range of 1280-1000 cm^{-1}, which indicates the valence oscillation of CN-groups in Amide (Amide III) and deformation oscillation of the O-H bond. In the same region of the spectrum, there are bands at 1167-1064 cm^{-1}, which are typical for the valence oscillation of CN, C-O, and C=C groups and bonded NH groups. A wide band at the range of 850-450 cm^{-1} characterizes the valence oscillation of bounded NH-groups with rather intense peaks at 707 and 603 cm^{-1} (Amide V) and a less intense peak at 530 cm^{-1} (Amide VI). The obtained data show the multifunctional nature of ECP and its ability to interact with the functional groups of other chemicals, such as dermis collagen.

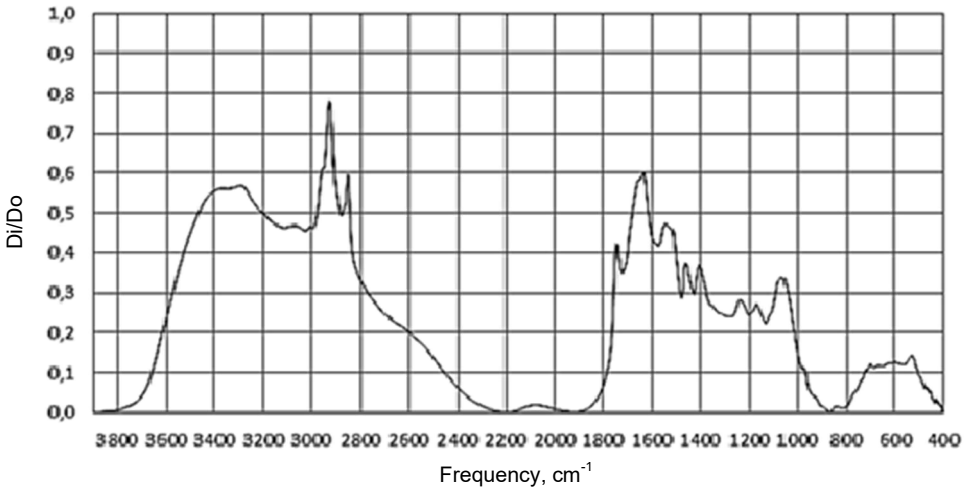

Figure 4 IR-spectrogram of ECP

Table 1 Effects of the modification conditions on the physicochemical properties of biogenic fibrous material (M±m; n=5)

Parameter	Group					
	*1**	*2***	*3*	*4*	*5*	*6*
	Product consumption [%]***					
	0	*0.35*	*0.20*	*0.35*	*0.50*	*0.65*
Welding temperature [°C]	60.00±3.0	61.00±3.0	56.00±2.8	58.00±2.8	59.00±3.0	59.00±3.0
Moisture content [%]	83.00±4.0	79.60±3.0	79.60±3.0	81.20±3.0	77.80±3.8	78.10±4.0
Mass fraction of nitrogen [%]	15.93±0.8	14.74±0.5	14.24±0.5	16.50±0.6	16.85±0.5	16.79±0.6
Gelatin melting [%]	10.95±0.5	14.40±0.6	15.40±0.5	23.60±1.0	26.20±1.2	25.20±1.2

*Note: *Starting material after liming, without modification (without bating); **Modification (bating) of the material with softening agent Enzymas 1072; ***by the weight of the sample.*

The next series of experiments was devoted to the study of the technological characteristics of the obtained enzyme-containing product. Given the results of our previous research, the modification of biogenic material in the form of a semi-finished leather product (pelt) from sheepskin during softening was investigated. According to the typical technique [24], softening is carried out at a pH close to the neutral value and a temperature of 36-38°C. The experiment was performed according to the conditions shown in Table 1. No difficulties were met in processing the samples of experimental groups #3-6; the treated samples were soft; they had a clean front surface and an imprint retained when pressed on the front surface. The samples of group #5 (ECP consumption was 0.50% of the sample weight) were the best in the terms of organoleptic evaluation. The face surfaces of the samples were more plastic and silkier.

As can be seen from Table 1, an increase in the ECP consumption in groups #3-6 from 0.20 to 0.65% does not significantly affect the hydrothermal stability of the samples. However, an increase in the mass fraction of nitrogen and melting of gelatin was observed. In determining the melting of gelatin, the color and the intensity of the solution in flasks differed significantly (Figure 5).

Figure 5 Flasks containing analytical reagents for spectrophotometric evaluation

The increase in the yield of gelatin could be explained by the removal of interfiber protein residues from the structure of biogenic material under the influence of ECP; the increase in the softness, the plasticity of the skin - due to the peptization of non-collagen components, and loosening of the dermis structure as the result of removal of residues of microfibrillar proteins; the increase in nitrogen levels - due to the formation of a certain number of nitrogen-containing groups as a result of enzymatic modification of proteins in the dermis.

4 CONCLUSIONS

The analysis of scientific and technical literature revealed the need for an affordable and effective domestic enzyme-containing remedy for biotechnological processes of leather production. The additional source for the manufacture of such preparations may be fish-processing waste that is rich in minerals, proteins, fats, enzymes, and other biologically active compounds. The enzyme-containing product ECP was hence obtained from the fish-processing waste in the form of a finely fibrous substance of brown color, which is readily soluble in warm water. Both common and advanced techniques - chromatographic, spectrophotometric, electrophoretic, infrared spectroscopy - were used to study the obtained product.

Electrophoretic fractionation of the product showed the presence of enzymes with a molecular weight in the range from 93 to 14 kDa. High proteolytic activity of enzymes was identified, which, in a recalculation on a specific chromogenic substrate, was 260 μmol pNA·min^{-1}.mg^{-1} protein. The high activity of the studied object was also confirmed by the estimation of caseinolytic activity (450 IU.g^{-1} product). According to the Bradford method, which is based on the ability of proteins to bind to Coomassie Brilliant Blue G-250, it was identified that the protein content in 1 mg of the extract is 0.41 mg.

The results of infrared spectroscopy showed the multifunctional nature of the product with the presence in Bradford the structure of various groups and bonds: bounded and free NH-groups, bounded OH-groups, and amides, which take part in the formation of hydrogen bonds; NH-bond in the NH_3^+ group, CN-group of the Amide II overtone, as well as NH-bonds of Amide I and Amide II, which provides an argument to speak about the valence fluctuations of the -CO-NH- bond, as well as the presence of β- and α- conformations of enzyme molecules.

While studying the effect of temperature and pH on the enzymatic activity of the product, it was found that the increase in the temperature for every 5°C results in the increase of enzymatic activity by 30-50 units; the pH factor acted in the opposite direction. Generally speaking, the highest enzymatic activity of the product was recorded in the pH range of 6-7 and at a temperature of at least 35°C. The data allowed to predict the possibility of using the product in the processes that will take place at a pH close to neutral and at elevated temperatures.

After modification of biogenic fibrous material in the form of semi-finished leather products (pelt) with an enzyme-containing product at the stage of bating, the semi-finished product became soft-touch, flexible, and had a clean front surface. The best result was achieved while using 0.50% ECP when a deeper gelatin melting (26.21%), a higher total nitrogen content (16.85%), and a better organoleptic evaluation were applied, compared to the results of modification with an industrial enzyme remedy at consumption of 0.35% by weight of samples, when the organoleptic assessment and the same indicators were worse (14.40 and 14.72%, respectively).

This effect can be explained by the ability of enzymes contained in the product obtained from fish-processing waste to loosen the structure of the dermis and remove interfiber proteins from it. The results indicate the potential technological capabilities of this product and the feasibility of further research in the chosen direction.

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