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Hydrolysates of Soybean Proteins for Starter Feeds of Aquaculture: The Behavior of Proteins upon Fermentolysis and the Compositional Analysis of Hydrolysates

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Abstract—Currently, soybean occupies the first place as a global protein source for replacing fish meal in animal and aquaculture feeds. Since at the stage of fish postembryonic development, before the switch to active feeding, the efficacy of proteolysis in larvae is not high enough, soybean proteins in starter feeds should be hydrolyzed. The hydrolysate composition and behavior of the resulting protein fragments were shown to differ when soybean proteins were hydrolyzed by different enzyme preparations: the enzyme complex from the hepatopancreas of the Kamchatka crab (EC HPKC), protosubtilin, and the enzyme complex from pyloric appendage of cod (EC PAC). The most active enzyme preparation among them was EC HPKC, which demonstrated a high proteolytic activity at room temperature. Upon hydrolysis by EC HPKC, the yield of soluble hydrolysis products was 92% per weight of the initial protein material. Depending on the incubation time, the hydrolysates contained up to 60% of free amino acids (per weight of the hydrolyzed protein mixture) and short peptides less than 3 kDa. The use of protosubtilin or EC PAC at room temperature resulted in the intensive gelation and coagulation of the formed protein fragments resistant to further degradation. In order to achieve the yield of soluble hydrolysis products comparable with that for EC HPKC, it was necessary to increase the temperature. The yield of soluble products upon the EC PAC-induced hydrolysis of soybean proteins at 37°C achieved 82–88% of the initial protein material. The greatest part of the hydrolysate was represented by low-molecular-weight peptides with a molecular weight lower than 10 kDa and free amino acids (20.16% of the weight of the hydrolyzed protein mixture). Although the optimal temperature for the protosubtilin activity is 40–60°C according to the manufacturer's data we did not perform hydrolysis in the presence of protosubtilin at this temperature because of the hazard to sulfur-containing amino acids. The content of free amino acids and the size of protein fragments in the soybean protein hydrolysates obtained upon the EC HPKC-induced hydrolysis at room temperature and with EC PAC at 37°C met the requirements for the fish starter feeds. Manipulations with such parameters as the hydrolysis time and the enzyme complex/protein ratio for the used enzyme preparations allow to prepare soybean protein hydrolysates differing in their hydrolysis degree.

Keywords: cod-fish pyloric appendage, crab hepatopancreas, fermentolysis, soybean proteins, protosubtilin

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INTRODUCTION

In recent decades, the world segment of aquaculture has been growing fast and in 2014 reached 73.8 million tons versus the 93.4 million tons of commercial fishing (FAO report 2016, http://www.fao.org/3/a-i5555r.pdf). Commonly, fish meal is used as a protein component of feeds for aquaculture. Exhausted bioresources are the reason for the partial or full replacement of fish meal by plant proteins for the fish grown in aquaculture. The development of alternative feeds is an increasingly topical task due to a worldwide growing expansion of aquaculture.

Soybean occupies the first place as a global source of plant proteins intended for replacement of fish meal in animal and aquaculture feeds due to the wide availability of soybean derivative products and relatively high protein content (30 to 45%) in fat-free soybean meal [1]. The amino acid composition of soybean proteins is balanced well with all essential amino acids being present. However, they are characterized by a reduced concentration of cysteine and methionine if compared with fish meal [2–4]. Soybean derivative products as a protein source have been used in animal farming for a long time. However, they have been used in fish meal only recently and, mainly, for salmon, which are of commercial interest. The first attempts to replace fish meal proteins by plant proteins in fish feeds demonstrated that the consequences of such

Abbreviations: EC, enzymatic complex; HPKC, hepatopancreas of the Kamchatka crab; PAC, pyloric appendage of cod.

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replacement were much more many-sided than initially predicted. In many fish species including salmon, the protein augmentation dropped. The higher was the content of plant proteins in the ration, the higher was the reduction degree [5].

Several studies were conducted with the goal of evaluating the effect of soybean protein products in salmon meal [6–8]. The total replacement of fish meal in the ration was reported to be a success only in a limited number of publications. Most of the studies demonstrated disadvantageous results despite the apparent relevance of the nutritive value of the feeds. It was shown that introduction of low or moderate amounts of plant protein ingredients in the fish ration only insignificantly affected salmon growth, whereas at their high content, growth was inhibited [4]. It can be accounted for by the presence in soybean meal and protein isolates of "antinutrient" components, which are indigestible or even harmful, such as protease inhibitors, lectins, saponins, tannins, isoflavones, glucosinolates, phenol derivatives, phytic acid [6, 7], nonstarched polysaccharides, and oligosaccharides [8] causing different pathologies in fish. Negative effects included growth inhibition, pancreatic hypertrophy, hypoglycemia or liver disorders, formation of goiter as well as intestine dysfunction, changes of intestine microflora, which resulted in noninfectious subacute enteritis with low protein and lipid digestion, diarrhea, and neoplasia [8, 9]. It implies that before the use of soybean protein concentrates and isolates in animal and fish feeds the proteins should be purified from antinutrient components.

Other issues are a rather large size of soybean proteins, their tendency to aggregation and gelation as well as their partial denaturation upon heating in the process of soybean fat wringing and extraction. This leads to the formation of insoluble protein material, which hampers functioning of digestive enzymes, and causes a decrease in the protein yield upon the preparation of protein isolates.

The use of protein hydrolysates for feed production increased the availability of protein material for the digestive system of fish and animals [10–12]. The introduction of various enzyme protein hydrolysates in starter feeds of larvae and baby fishes in aquaculture [13, 14], which is implemented with the consideration of specific features of larvae digestion in comparison with that of young or adult fish, is becoming increasingly common [15, 16]. Traditionally, hydrolysates of fish meal are used in starter feeds for aquaculture. As described in [17–19], the hydrolysis of fish meal proteins to a degree of 65% provided the optimal content of such final protein products as free amino acids, oligopeptides with a range of molecular weights from 600 to 8000 Da (about 70% of the protein material) and not more than 15% of larger protein fragments. Unfortunately, the number of studies describing the preparation of soybean hydrolysates for the inclusion into starter aquaculture feeds with the given hydrolysis degree was insufficient [20–22].

In this work, we performed a comparative analysis of methods of preparation and composition of soybean hydrolysates obtained in the presence of the following enzyme preparations: commercial preparation "protosubtilin" of bacterial origin [23] and cheap enzyme preparations, which are extracts from subproducts of crab and fish pyloric appendage [24, 25]. The EC HPKC and EC PAC displayed a high proteolytic activity due to the presence of proteases of different specificity.

RESULTS AND DISCUSSION

The major soybean proteins are globulins glycinin and β-conglycinin at a ratio of 75/115, which constitute 80% of the protein material [26, 27]. Both proteins have a complex quaternary structure: glycinin is composed of five subunits with a sedimentation coefficient 11S, whereas β-conglycinin contains three subunits (76, 72, and 53 kDa) and its sedimentation coefficient of 7S. β-Conglycinin is characterized by a low content of histidine, tryptophan, methionine, and cysteine; its β-subunit (53 kDa) contains one cysteine residue of about 470 aa and does not contain methionine residues [4]. In addition to globulins insoluble in water but soluble in neutral salt solutions, soybean bears water-soluble proteins characterized by a lower molecular weight, namely, minor γ-conglycinin, and a relatively large amount of other proteins including serum proteins comprising in total 9 to 15% of the protein weight [28].

The yield of proteins upon extraction from soybean meal depended on the reaction conditions and storage. When protein isolates were obtained from the meal subjected to intense heating resulting in changes in the protein solubility, the protein yield considerably decreased [4]. The inclination of soybean proteins to denaturation and gelation required a careful selection of isolation conditions. Many studies on soybean solubility were performed [26]. Possessing a complex quaternary structure formed by large polypeptides, soybean globulins can rapidly denature under certain conditions to unfold polypeptide chains and the following aggregation initiates their gelation. Protein aggregation is stimulated by heating, freezing, acids, high pressure, and enzymes. The gelation rate depends on the protein concentration in solution. Although gelation of linear molecules (such as polysaccharides and fibrillation proteins) has been studied well, gelation of globular proteins is poorly understood due to their complex structures [29]. Gelation upon fermentolysis of soybean proteins was observed by many researchers [22, 30]. It was shown that gelation occurred due to the formation of protein fragments (including large enough) with isoelectric points close to pH values of the hydrolysis reaction. In the course of the hydrolysis a protein set can vary. The studies

demonstrated that the starting proteins were absent in the hydrolysate gels. It implies that gelation was a result of the formation of peptides with higher p*I* points than p*I* of the starting proteins. This difference caused a change in the balance between electrostatic and hydrophobic interactions, which may lead to the formation of aggregates close to peptide p*I* values. To summarize, hydrolysate gelation is a complex and nonspecific process affected by the peptide structure formed upon hydrolysis [31].

In order to obtain the soybean protein preparation we used commercial fat-free soybean meal using the method described in [24].The meal was preliminary washed with 60% alcohol for the removal of the alcohol-soluble compounds. This procedure supported the removal of 75% of phenol derivatives and about 50% of carbohydrates and oligosaccharides [32–34], which are in the list of "antinutrient" compounds [6–8]. Isoelectric points of soybean proteins are in the range of 4.5 to 6.4, which determines their solubility. Therefore, alkaline conditions (pH 10.5) were used for the extraction of soybean proteins. Also, alkaline conditions were taken due to the presence in soybean meal of phytic acid (included in the group of antinutrient compounds), which can form complexes with proteins at acidic pH and modifies their properties [35, 36].

Proteins from soybean meal were extracted with 0.5 M NaCl at pH 10.5 and room temperature under intensive stirring. It was shown that 0.5 M NaCl protected the protein quaternary structure from alkaline denaturation followed by gelation resulted from glycinin dissociation [4]. The yield of the protein extract was 26% per the protein content in soybean meal. The protein content in the resulting protein preparations was 87–92%.

For the removal of low-molecular-weight antinutrient compounds from the protein extracts, we used ultrafiltration through membranes with pores allowing passage of the molecules with a molecular weight less than 10 kDa. A polyphenol analysis demonstrated the absence of phenol-derived compounds in the obtained protein isolate. The amino acid composition in the protein preparation was close to that in fish meal but with a slightly reduced content of cysteine and methionine.

EC HPKC was obtained from frozen preparations by protein extraction using the Rudenskaya method [37]. Collagenases from Dekapoda crabs form a specific subgroup of chymotrypsin-like serine proteases (EC 3.4.21.32) according to the NC-IUBMB classification. A general name of crab collagenolytic proteases is brachyurines. Currently, crab serine proteases of three types are isolated: brachyurines Ia possessing a wide specificity close to that of chymotrypsin, trypsin, and elastase and displaying collagenolytic activity; Ib, with a wide specificity but decreased activity towards trypsin substrates; and brachyurines II, trypsin-like proteases [38]. In this work, for the preparation of rape protein hydrolysates we used a total EC HPKC.

The purified protein preparation obtained by extraction at pH 10.5 of commercial fat-free soybean meal was hydrolyzed by lyophilized EC HPKC, which was a mixture of several proteases, whose combined effect determined its high proteolytic activity. The hydrolysates were analyzed by HPLC, SDS PAGE, mass-spectrometry, and assays of proteins and free amino acids in the hydrolysate soluble fractions.

The experiments on fermentolysis of soybean proteins under varied conditions (an enzyme complex/protein ratio, room temperature and 37°C, hydrolysis time of 2, 4, and 18 h) at pH 8.0 supported the conclusion that an increase in temperature to 37°C did not affect the hydrolysis degree, which depended on the protein/EC HPKC ratio and the fermentolysis time. Neither noticeable gelation nor stable formation of insoluble products was observed during the treatment. The HPLC analysis demonstrated a high degree of soybean hydrolysis under the selected conditions, even at a 1 : 100 EC/protein ratio (Fig. 1a). It can be seen on the electropherogram that a marked hydrolysis of starting proteins followed by accumulation of low-molecular-weight fragments was observed at a 1 : 20 EC/protein ratio as early as nearly in 1 h (Fig. 2). In 18 h, the hydrolysate contained mainly low-molecular-weight oligopeptides and amino acids beyond the gel borders.

It is noteworthy that hydrolysates obtained at different EC/protein ratios differed in their composition (Fig. 2). In an hour, more complete degradation was observed at a 1 : 20 EC/protein ratio. However, in 18 h at the same ratio the hydrolysis process slowed down and the hydrolysate contained more oligopeptides with a molecular weight ranging from ~5 to 20 kDa than was found at a 1 : 100 EC/protein ratio in the same time period. It implied that the protein hydrolysis proceeded to a greater extent at a lower enzyme load.

A high hydrolysis degree of EC HPKC soybean proteins was also confirmed by mass spectrometry: most of the hydrolysis products were found to be soluble protein fragments with a molecular weight less than 3 kDa. According to the data of the amino acid analysis, the yield of soluble hydrolysis products was 92% per weight of the initial protein material including 60.2% of free amino acids (room temperature, 18 h, initial protein concentration 3 mg/mL) (Table 1).

To summarize, the use for the hydrolysis of soybean proteins of EC HPKC, which was a mixture of proteolytic enzymes with different specificity, provided both a good hydrolysis degree and a yield of soluble hydrolysis products meeting the requirements of the development of starter feeds for aquaculture. It is noteworthy that EC HPKC is a cheap source of enzymes being almost a waste in crab processing in Russia.

Protosubtilin is a complex enzyme preparation produced by *Bacillus subtilis*. A proteolytic enzyme

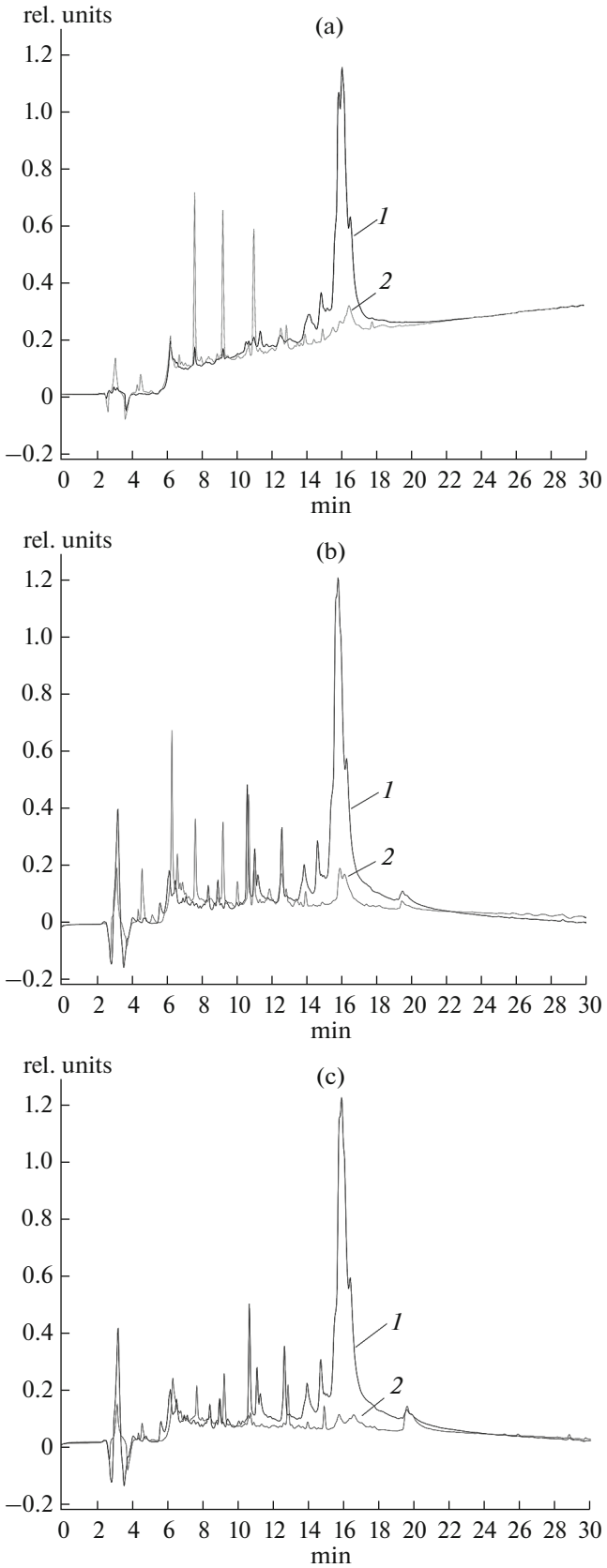

Fig. 1. HPLC of the starting soybean protein extract (1) and its hydrolysates (a soluble part) (2) resulting from the 18-h hydrolysis at a 1 : 100 EC/protein ratio in the presence of EC HPKC (a), EC PAC (b), and protosubtilin (c).

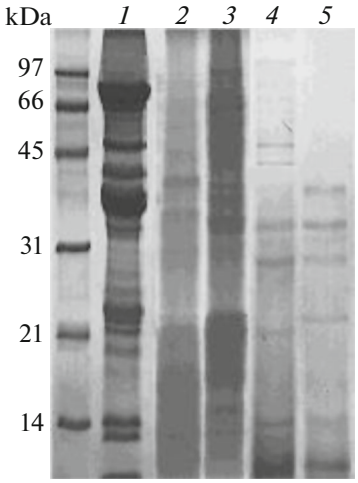

Fig. 2. SDS-PAGE of the soybean protein isolate (lane *1*) and its hydrolysates resulted from the hydrolysis at room temperature in the presence of EC HPKC at 1 : 20 (*2, 4*) and 1 : 100 (*3*, *5*) EC/protein ratio for 1 (lanes *2, 3*) and 18 h (lanes *4, 5*).

subtilisin is the protosubtilin principal component. The preparation also contains small amounts of the complex of neutral and alkaline proteases and related minor components, such as alpha-amylase, beta-glucanase, xylanase, and cellulase. Preliminarily purified lyophilized protosubtilin was used for the hydrolysis of soybean proteins [23]. According to the supplier's data the optimal conditions for manifestation of enzymatic activity are a pH range of 4 to 6 and a temperature 40 to 60°C. However, based on the properties of soybean proteins (their isoelectric points are within 4.5–6.4) the hydrolysis was performed at pH 7.5 and room temperature.

Commercial soybean protein isolate was hydrolyzed with protosubtilin under the above conditions at a protein concentration of 3 mg/mL, a protosubtilin/protein ratio of 1 : 20 and 1 : 100 for 1, 4, and 20 h. The hydrolysates were analyzed by HPLC (Fig. 1c), SDS PAGE (Fig. 3), mass spectrometry, as well as by the content of proteins and free amino acids in the hydrolysate soluble fragments (Table 1).

It is noteworthy that the protosubtilin-induced fermentolysis of a commercial soybean protein isolate caused considerable coagulation of the protein material. It could be due to the appearance of insoluble protein fragments, which essentially decreased the yield of soluble hydrolysis products. According to the amino acid analysis, at a 1 : 20 protosubtilin/protein ratio and the hydrolysis time of 20 h the total yield of the soluble peptide material achieved 12.8% including 2.4% of free amino acids per the starting protein material. The mass spectrometry analysis of the soluble part of soybean protein hydrolysates after the protosubtilin-induced hydrolysis demonstrated that the data spread of peptide weights in the hydrolysates was

Table 1. Analysis of soybean protein hydrolysates obtained in the presence of EC HPKC, protosubtilin, and EC PAC after 18–20 h hydrolysis. The yields are given in % per the weight of the hydrolyzed protein preparation

Enzyme preparation	Fermentolysis temperature, °C	Yield of soluble hydrolysis products, %	Yield of free amino acids, %	Molecular weights of major soluble hydrolysis products, kDa
EC HPKC	20	92.0	60	1–3
Protosubtilin	20	12.8	2.4	1.5–12
EC PAC	37	82.7–88	20.16	1–10
	20	10.16	n/a	<16

within the range of 1.5–12 kDa. It is seen at the HPLC chromatogram that large protein fragments and the intact protein were absent in the soluble hydrolysate fraction (Fig. 1c). The lack of high-molecular-weight fragments may indicate both the hydrolysis degree and that the hydrolysate insoluble part is composed from large protein fragments formed during the fermentolysis. The comparison of the electropherograms of soluble parts and insoluble precipitates obtained at different enzyme/protein ratios showed that the major part of the protein material was represented by insoluble protein fragments with a large data spread of molecular weights (Fig. 3).

Intensive gelation and coagulation of soybean proteins during fermentolysis were observed by many researchers when studying Flavourzyme 1000L, Novozyme FM 2.0 L, and Alcalase 2.4 L preparations containing subtilisin [20, 22, 27, 39–42]. In the presence of subtilisin Carlsberg coagulation of soybean proteins occurred at the hydrolysis degree of 10% [43]. At pH ≈7.5 gelation took place at the hydrolysis degree of 5%. Changes of ionic strength (0.03, 0.2, and 0.5 M) only insignificantly affected the aggregation pH, which implied that aggregation is not only a balance of electrostatic and hydrophobic interactions but a much more complicated process. The SDS PAGE demonstrated that degradation of β-conglycinin and glycinin occurred in all the cases. However, the major glycinin component was highly stable towards the abovementioned preparations.

For hydrolysis of soybean proteins we used first time an enzyme preparation from a codfish pyloric appendage [25]. Fish pyloric appendages are saccular outgrowths in the gastrointestinal tract. Their number in different fish species is different. For example, salmons have 300 to 400 appendages, whereas in sturgeons they coalesced and formed one glandular organ. The studies of pyloric appendages of cod fish, salmon, and some perch species confirmed that they played an important role in digestion [44]. In all the four studied fish species, sugars, amino acids, and dipeptides were found to be located mainly in pyloric appendages,

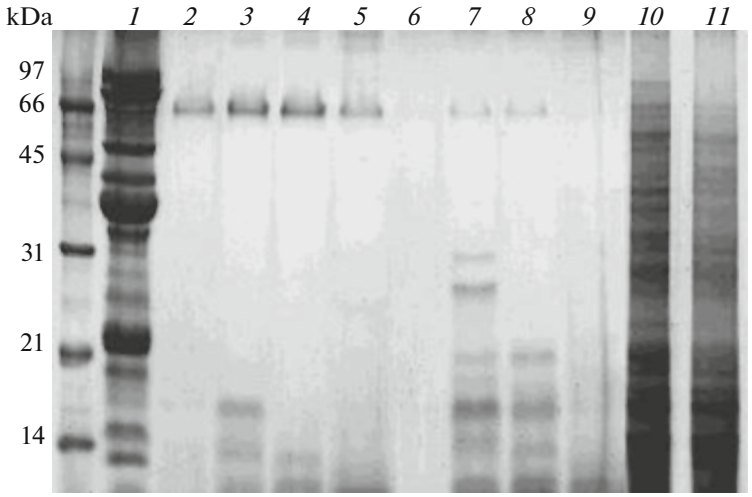

Fig. 3. SDS-PAGE of the soybean protein isolate (lane *1*) and the soluble part of its hydrolysates obtained in the presence of protosubtilin at room temperature at an enzyme/protein ratio of 1 : 20 (*3–5*) and 1 : 100 (*7–9*) after hydrolysis for 1 (*3, 7*), 4 (*4, 8*), and 20 h (*5, 9*): *2, 6,* the protosubtilin preparation quantitatively equivalent to that in samples *3, 4, 5* and *7, 8, 9* respectively. *10, 11,* insoluble fractions of soybean protein 1 : 100 hydrolysates formed in the presence of protosubtilin at an enzyme/protein ratio of 1 : 20 (*10*) and 1 : 100 (*11*) for the hydrolysis time of 20 h.

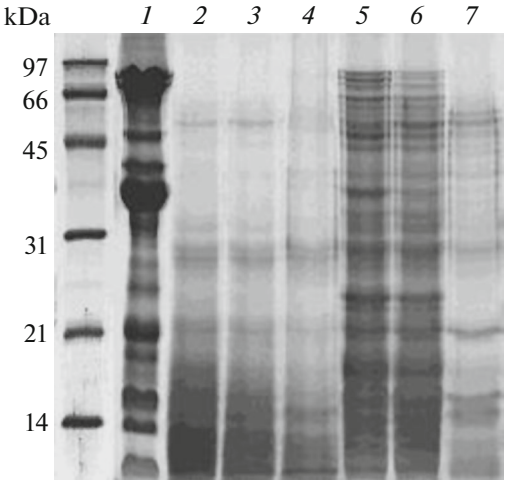

Fig. 4. SDS-PAGE of soybean isolate (*1*) and the soluble part of the hydrolysate in the presence of EC PAC (*2–7*) at room temperature and an enzyme/protein ratio of 1 : 20 (*2–4*) and 1 : 100 (*5–7*) and a hydrolysis time of 1 (*2, 5*), 3 (*3, 6*), and 20 h (*4, 7*).

where their concentrations were much higher than those in the gut. It was unambiguously confirmed that fish pyloric appendages contain enzymes capable of cleaving proteins, carbohydrates, and fats [45–48]. Currently, fish internal organs are being intensely studied as a potential source of various enzymes. Trypsin and trypsine-like enzymes from pyloric appendages were isolated and characterized for a large spectrum of cold- and warm-water fish [49–57]. Cod pyloric appendages used in this work are a cheap source of highly active enzymes, since codfish is one of the major commercial fish.

A commercial soybean protein isolate (3 mg/mL) was treated with an extract of cod pyloric appendages (EC PAC) with 0.1% NaCl. A ratio of the enzyme preparation to the hydrolyzed protein was calculated on the basis of the protein content in the preparations. Since p*I* values of soybean proteins were in the range of 4.5 to 6.4, the hydrolysis was carried out at pH 8.0.

A commercial soybean protein isolate (3 mg/mL) was hydrolyzed with EC PAC at room temperature or at 37°C at a 1 : 20 or 1 : 100 EC PAC/protein ratio (per protein) for 1, 3 and 20 h. Hydrolysates were analyzed by HPLC (Fig. 1b), SDS-PAGE (Fig. 4), and mass spectrometry. Also, the content of free amino acids was determined.

During the hydrolysis at room temperature, the reaction mixture grew turbid and remained as such until the process was completed, which could be explained by the formation of insoluble protein fragments. A soluble part of the hydrolysate obtained at a maximal enzyme loading was analyzed by HPLC (Fig. 1c). A comparison with the chromatogram of the starting protein preparation clearly showed that the soluble portion did not contain the starting protein or large protein fragments after 20 h of hydrolysis. The compositional changes in the hydrolysate soluble parts after 1, 3 and 20 h of hydrolysis can be seen in Fig. 4. The soybean protein hydrolysis at a 1 : 100 EC PAC/protein ratio proceeded much slower: in 3 h a considerable amount of high-molecular-weight fragments was still present in the hydrolysate. However, in 20 h the hydrolysis degree was approximately the same as it was for the 1 : 20 EC PAC/protein ratio. However, according to the results of the amino acid analysis, it was found that, upon hydrolysis at room temperature and a 1 : 20 EC PAC/protein ratio, the soluble part at hydrolysate part contained only 10.16 wt % of the protein material, whereas at a 1 : 100 ratio this amount was 22%. The mass spectrometry data of the soluble part showed that it was composed of very short peptides with a molecular weight of less than 1 kDa and a set of 1–10 kDa oligopeptides.

Obviously, at room temperature (which is economically more beneficial), the EC PAC-induced soybean protein hydrolysis was incomplete and resulted in forming of insoluble fragments. As we showed in our experiments, a much higher degree of hydrolysis was achieved if the temperature was increased to 37°C. According to the data of the amino acid analysis, the yield of soluble hydrolysis products at this temperature was 82.7 wt % per the starting protein including 20.16% of free amino acids. At a 1 : 100 ratio the soluble part of hydrolysate constituted 88.4% (Table 1). The results imply that EC PAC contains the enzymes with a high proteolytic activity, which provides a high hydrolysis degree, even of such complex proteins as soybean proteins.

The analysis of our studies allows a conclusion that the behavior of soybean proteins and the protein fragments formed in the process of hydrolysis induced by EC HPKC, protosubtilin, and EC PAC is different and the yield of soluble hydrolysis products in the case of protosubtilin and EC PAC depends on the temperature (Table 1). Of the enzymes used, the most active one was EC HPKC, which displayed a high proteolytic activity at room temperature. The analysis of fermentolysis products demonstrated a high hydrolysis degree of soybean proteins: the yield of soluble hydrolysis products achieved 92% per weight of the starting protein material. Depending on the hydrolysis time, the hydrolysate contained up to 60% (18 h) of amino acids (per weight of the hydrolyzed protein material) and short peptides, whose weight did not exceed 3 kDa. Another pattern was observed for protosubtilin or PAC extract used for hydrolysis of soybean proteins at room temperature. In the process of hydrolysis intensive gelation and coagulation of the resulting protein fragments unsusceptible to further hydrolysis took place. In order to achieve the yield of soluble hydrolysis products comparable with that for EC HPKC, it was necessary to increase the temperature to 37–40°C. For EC PAC, the yield of soluble hydrolysis products at 37°C was 82–88% per weight of the starting protein

material. A major hydrolysate portion was composed by free amino acids, low-molecular-weight peptides with a mass less than 10 kDa, and a small amount of protein fragments with a higher molecular weight. Although the optimal temperature for the protosubtilin enzymatic activity is 40–60°C according to the supplier's recommendations, the protosubtilin-induced hydrolysis of soybean proteins was not performed in this temperature range due to the potential hazard for sulfur-containing amino acids.

To summarize, we obtained hydrolysates of soybean proteins, the composition of which met the requirements of starter feeds for aquaculture [17–19, 58, 59]. Variations of such parameters as hydrolysis time, a ratio of the enzyme preparation/protein, and temperature allowed the hydrolysis to proceed to various degrees.

EXPERIMENTAL

Soybean meal Soyanta™-200 (Irkutskii MZhK) with the raw protein content of ~52% (supplier's data) and the soybean isolate SHANSUN-90 with the raw protein content of ~92% (supplier's data) were received from a group of companies YANTA and Atlant LLC (Yekaterinburg); the enzyme preparation protosubtilin G3x was from Sibbiofarm LLC (Russia); frozen HPKC, from Arktikservise Ltd (Murmansk); the frozen EC PAC, from the Murmansk trawl fishing marine (Russia). Chemical reagents were purchased from Sigma (United States).

Preparation of the enzyme complex from soybean meal [24]. Meal was ground in a mortar for 15 min to have a finer powder. The powder was washed with 60% ethanol under stirring for 30 min and centrifuged at 25000 g for 20 min on a J2-21 centrifuge (Beckman, United States). The precipitate was dried in air and the proteins were extracted with 0.5 M NaCl at pH 10.5 and room temperature and vigorous stirring for 2 h at a 1–3% (w/v) soybean meal/liquid ratio. Insoluble plant residuals were removed by centrifugation at 35000 g for 20 min on a J2-21 centrifuge. For the removal of low-molecular-weight compounds coextracted with proteins, the supernatant was subjected to ultrafiltration with the membranes allowing passage of the molecules with a molecular weight less than 10 kDa. For desalting, the proteins were precipitated with cold 80% ethanol for 2 h. After centrifugation, the proteins from the precipitate were suspended in either water (pH 8.0) followed by lyophilization or 0.1 M Tris-HCl buffer, pH 8.0, and used for enzymatic hydrolysis.

The content of the total protein in the starting material and in the protein preparations were evaluated by the Kjeldahl method [60]. The protein preparations were tested for the polyphenol presence using a qualitative color reaction with iron(III) chloride [61]. The results evidenced the absence of phenol derivatives in the samples.

The EC HPKC was isolated from frozen HPKC by the protein extraction at pH 6.0 in buffer A (0.1 M sodium acetate, pH 6.0, 100 mM NaCl, 2 mM $CaCl_2$) followed by centrifugation at 22000 *g* on a J2-21 centrifuge [37]. The upper fat layer and the precipitate were removed, and the supernatant proteins were precipitated with ammonium sulfate (80% saturation). After centrifugation at 35000 *g* on a J2-21 centrifuge the proteins were dissolved in buffer A, dialyzed versus the same buffer and stored at −20°C.

Hydrolysis of the protein preparations without the preliminary protein denaturation was performed for a given time in the presence of EC HPKC under the following conditions: room temperature, pH 7.5–8.0, protein concentration 3 or 5 mg/mL, 1 : 20 or 1 : 100 EC/substrate ratio per the protein weight detected by the Bradford method [62]. pH value of reaction mixture was kept at the level of 8.0. The reaction was terminated by heating at 90°C for 5 min. Hydrolysates were cooled and stored frozen at −20°C.

Commercial protosubtilin was washed with distilled water (1 : 10 w/v) prior to use. The insoluble material was separated by centrifugation at 25000 *g* for 15 min on a J2-21 centrifuge and the supernatant was concentrated by ultrafiltration through a membrane with a pore size allowing passage of the molecules with a molecular weight less than 3 kDa (Millipore) on an Amicon Ultra cell (Merck, United States) followed by lyophilization.

The protosubtilin-induced hydrolysis of the commercial protein isolate was carried out without prior protein denaturation at room temperature, pH 7.5, the protein concentration 3 mg/mL, and a 1 : 20 or 1 : 100 EC/substrate ratio per protein weight for various time periods [23]. As the reaction mixture was acidified, its pH vaIue was kept close to 7.5. The reaction was terminated by heating at 90°C for 2 min. The hydrolysates were cooled and stored frozen at −20°C.

For the preparation of EC PAC, frozen PAC were homogenized in 0.1% NaCl at a 1 : 20 (w/v) ratio and the homogenate was filtered through a membrane with a pore size allowing passage of the molecules with a molecular weight less than 100 kDa. The filtrate was lyophilized and the lyophilizate was homogenized in 40 mM Tris-HCl buffer, pH 7.5, at a 1 : 25 (w/v) ratio. The insoluble material was separated by centrifugation at 25000 *g* for 15 min on a J2-21 centrifuge. The supernatant was concentrated by ultrafiltration through a membrane with a pore size allowing passage of the molecules with a molecular weight less than 3 kDa to a protein concentration of 2.5 mg/mL and used for soybean protein hydrolysis.

The EC PAC-induced hydrolysis of the commercial protein isolate was carried out at the protein concentration of 3 mg/mL, pH 8.0, room temperature or 37°C, and 1 : 20 or 1 : 100 ratio per the protein weight

for 1, 3 and 20 h. The reaction was terminated as described above. The hydrolysates were cooled and stored frozen at −20°C.

SDS PAGE of the protein preparations and hydrolysates was carried out by the Laemmli method [63] in 12 or 15% PAG in the presence of 10% SDS.

The amino acid composition of the samples hydrolyzed by 5.6 M HCl at 110°C for 24 h was detected on a SYKAM 430 amino acid analyzer (Sykam GmbH, Germany). The tryptophan concentration was determined after alkaline hydrolysis of the samples according to GOST 32201-2013 on an Agilent 1200 chromatograph supplied with a fluorescent detector. The total concentration of free amino acids in the enzymatic hydrolysates was determined by the amino acid analysis without acid hydrolysis of the samples.

The mass spectrometry analysis of the protein hydrolysates was performed after the peptides were separated on a nano-flow EASY-nLC 1000 chromatograph (Thermo Scientific, United States); high resolution OrbiTrap Elite mass spectrometer (Thermo Scientific, United States) was used as a detector.

Panoramic spectra were registered in the m/z range of 500 to 2000 at a 240000 resolution; the ion fragmentation was conducted in a high pressure dissociation chamber HCD; fragmentation spectra were recorded at a resolution of 60000. The peptides were separated on a capillary column 150 mm in length and 75 μm in diameter (Phenomenex, United States).

The hydrolysates were chromatographed on a high pressure BREEZE chromatographic system (Waters, United States) on a Phenomenex Luna C18(2) column (100 Å, 5 μm, 250 × 4.6 mm) equilibrated with 0.1% trifluoroacetic acid in a gradient of acetonitrile concentration (2→95% in 30 min) at a flow rate 1 mL/min and a column temperature 30°C. The peptides were detected at a wavelength of 215 nm.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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