ISSN 0003-6838, Applied Biochemistry and Microbiology, 2018, Vol. 54, No. 3, pp. 294–300. © Pleiades Publishing, Inc., 2018.
Original Russian Text © D.V. Zinchenko, T.A. Muranova, L.A. Melanyina, N.A. Belova, A.I. Miroshnikov, 2018, published in Prikladnaya Biokhimiya i Mikrobiologiya, 2018, Vol. 54, No. 3, pp. 277–285.

Soy and Rapeseed Protein Hydrolysis by the Enzyme Preparation Protosubtilin

D. V. Zinchenko[a], *, T. A. Muranova[a], L. A. Melanyina[a], N. A. Belova[a], and A. I. Miroshnikov[a]

[a] *Biotechnology Department, Moscow State University, Moscow, 119991 Russia*

**e-mail: zdv@bibch.ru*

Received October 31, 2017

Abstract—A comparative study of soybean and rapeseed protein hydrolysis by protosubtilin, an original Russian enzyme preparation widely used in animal feed production, has been performed. SDS-PAG electrophoresis, HPLC, and mass spectrometry have been employed to analyze the obtained products. The soybean protein isolate used for hydrolysate production was obtained from a commercial supplier, and rapeseed proteins were prepared from the meal by alkali extraction. Low molecular weight impurities were removed by ultrafiltration. The degree of protein hydrolysis has been shown to depend on the substrate-to-enzyme preparation ratio, hydrolysis time, and protein concentration. Rapeseed protein hydrolysis by protosubtilin at an enzyme/protein ratio of 1 : 20 and hydrolysis time of 20 h resulted in complete cleavage of the proteins present in the raw material and the accumulation of oligopeptides (molecular weight less than 14 kDa) and free amino acids, which accounted for 53 and 8% of the initial protein weight, respectively. In contrast to rapeseed proteins, soybean proteins showed considerable gelling at the initial stages of hydrolysis, and the formation of insoluble hydrolysis-resistant fragments was observed. The soluble part of the hydrolysate contained short oligopeptides and free amino acids, which accounted for 13% of the initial protein weight only.

Keywords: aquaculture, feeds, soybean proteins, rapeseed proteins, antinutrients, protosubtilin

DOI: 10.1134/S000368381803016X

INTRODUCTION

Soy and rapeseed have the highest potential as sources of a plant protein that is increasingly used for fishmeal replacement in feeds for animal, poultry, and fish farming. Since the reserves of fish used for fishmeal production are constantly decreasing, the development of alternative feeds required by aquaculture expansion all over the world is a task of increasing relevance. The oil-bearing crops named above are produced in enormous amounts, and this ensures a wide availability of derived products, such as meal, cake, flour, and protein concentrates, as well as isolates that are obtained from the above and characterized by a relatively high protein content [1–5]. Soy and rapeseed proteins have a balanced amino acid composition and contain all amino acids, even though the cysteine and methionine contents are characteristically low [2–5]. The production of protein hydrolysates from soy and rapeseed increases the availability of plant proteins for the animal digestive system and thus expands the possibilities of the use of these proteins in animal feed production [6, 7]. However, the introduction of soy and rapeseed protein isolates and concentrates into animal diets is hindered by the presence of antinutrients, which are not absorbed by the organism and can even pose a hazard to animal health and development, since they impair the digestion and absorption of feed components. This group of compounds includes digestive enzyme inhibitors, glucosinolates, phenol derivatives, saponins, phytic acid, and a range of other molecules [8, 9]. An additional purification stage is required for the removal of these compounds from protein isolates and concentrates. Another problem consists in the rather large size of proteins, their propensity to aggregation and gelling (especially in the case of soy proteins), and partial denaturation upon heat treatment during oil pressing and extraction from the seeds of these oil-bearing crops. Protein hydrolysate production acquires particular importance if protein additives of plant origin are intended for starting feeds for fish fry reared in aquaculture. Fishmeal hydrolysates are the conventional components of starting feeds for fish fry. Since the alkaline and acidic proteinase activity in fish larvae intestine is not sufficiently high, the feed administered at the postembryonic developmental stage should contain free amino acids, di-, oligo- and polypeptides, and low molecular weight soluble proteins in a ratio close to the composition of plankton organisms, which are the natural food of most fish, including fish fry [10, 11].

Soy protein use has long been the object of intensive studies. Soy protein hydrolysate manufacturers currently use mild enzymatic procedures of protein hydrolysis instead of strong acids in order to eliminate undesirable side reactions or products. However, the

major problems that emerge upon proteolytic hydrolysis of soy proteins are related to insoluble fragment formation, hydrolysate coagulation, and high enzyme costs. The number of studies related to the production of plant protein dispersions with a predefined hydrolysis depth is insufficient at present.

Soy and rapeseed protein hydrolysis by Flavourzyme 1000 L (Merck, Germany), Novozyme FM 2.0 L, and Alcalase 2.4 L (Novozymes, Denmark) commercial enzyme preparations produced in foreign countries was reported in [12–14]. Our earlier studies [15, 16] addressed the production of soy and rapeseed protein hydrolysates by treating the raw materials with an enzyme preparation from king crab hepatopancreas.

The goal of the present study was a comparative analysis of soy and rapeseed protein hydrolysis by protosubtilin, a commercial enzyme preparation developed in Russia, and an analysis of the composition of the obtained hydrolysates.

METHODS

Shan'sun-90 soy isolate (the Yanta and Atlant group of companies, Russia) with a raw protein content of 92% (as reported by the manufacturer), rapeseed meal (Greinlyuks, Russia) with a raw protein content of 37.3% (GOST (State Standard) 11048-95), protosubtilin G3x enzyme preparation (Sibbiofarm, Russia), and chemicals from Sigma (United States) were used in the present study.

Protosubtilin. The preparation was rinsed with distilled water (1 : 10, wt/vol) prior to use, insoluble material was separated by centrifugation at 25000 *g* for 15 min in a J2-21 centrifuge (Beckman, United States), and the supernatant was concentrated by ultrafiltration through a membrane (MWCO 3 kDa) in a Millipore cell (Millipore, United States) and lyophilized.

Total protein extraction from rapeseed meal. The starting material was pounded with a pestle for 15 min to obtain a fine powder. The powder was rinsed with 60% ethyl alcohol for 30 min with stirring and centrifuged at 25000 *g* for 15 min. The pellet was air-dried. Proteins were extracted from the air-dry preparation with 0.5 M NaCl for 2 h at a pH of 10.5 and room temperature with intensive stirring. The powder-to-liquid ratio was 1–3% (wt/vol). The suspension pH was adjusted to 10.5 with 2 M NaOH. Insoluble plant debris was removed by centrifugation at 35000 *g* for 20 min. The supernatant was subjected to ultrafiltration through a membrane (MWCO 10 kDa) for the removal of low molecular weight compounds extracted together with the proteins. The protein solution was diluted with water (pH of 8.0) or buffer (pH of 8.0) several times during ultrafiltration in order to maintain a protein concentration close to that in the starting extract. The proteins were precipitated with 80% ethyl alcohol (2 h, low temperature) for desalting, and the pellet was collected by centrifugation and resuspended in water (pH 8.0) for further lyophilization or in 0.1 M tris-HCl buffer, pH of 7.5, for further enzymatic hydrolysis. The Kjeldahl method [17] was used to assess the total protein content in the starting material and the protein preparations.

Polyphenol detection in the protein preparations. A qualitative color reaction with iron(III) chloride was used to characterize the polyphenol content of the preparations [18]. The color of the obtained protein preparation solutions did not change after the addition of 2–3 drops of a 5% $FeCl_3$ solution, which is indicative of the absence of phenolic compounds.

Hydrolysis of the protein preparations. Hydrolysis of the preparations (10–15 mg) by protosubtilin G3x was performed without prior denaturation at a pH of 7.5 and room temperature, and the hydrolysis time was varied. The protein concentration in the reaction mix was 3 mg/mL, and the enzyme/substrate ratio was 1 : 20 or 1 : 100 (wt/vol). The reaction medium pH was readjusted to 7.5 as acidification of the medium occurred during hydrolysis. The reaction was stopped by heating at 90°C for 2 min. The hydrolysates were cooled and stored frozen at –20°C.

The Bradford method [19] was used to determine the protein concentration.

Polyacrylamide gel electrophoresis. Protein preparations and hydrolysates were subjected to electrophoresis in 15% PAG in the presence of 10% SDS according to the procedure of Laemmli [20].

Amino acid analysis. Proteins and peptide were hydrolyzed with 5.6 M HCl for 24 h at 110°C, and amino acid analysis was performed on a SYKAM S430 amino acid analyzer (Sykam, Germany). The amino acid analyzer was also used to assess the total content of free amino acids in the hydrolysates.

Mass spectrometric analysis of protein hydrolysates. The peptides were fractionated in an EASY-nLC 1000 nanoflow chromatographic system (Thermo Scientific, United States) with an OrbiTrap Elite high-resolution mass spectrometer (Thermo Scientific, United States) as the detector. Intact peptide spectra were registered in the m/z range of 500–2000 at a resolution of 240000, the ions were fragmented in an HCD high-pressure dissociation chamber, and fragment spectra were recorded at a resolution of 60000. The peptides were separated on a capillary column (150 mm × 75 μm) (Phenomenex, United States).

Chromatographic analysis of the hydrolysates. Hydrolysates were analyzed in a BREEZE HPLC system (Waters, United States) equipped with a Phenomenex Luna C18(2) column (100 Å, 5 μm, 250 × 4.6 mm) equilibrated with 0.1% trifluoroacetic acid, in an acetonitrile concentration gradient (2 → 95% for 30 min) at a flow rate of 1 mL/min and column temperature of 30°C. The volume of the sample injected onto the column was 20 μL. The peptides were detected at a wavelength of 215 nm.

RESULTS AND DISCUSSION

The products of oil-bearing crop processing attract increasing interest as sources of easily available protein. Soy is currently the primary source of plant protein for the food industry and animal feed production on a global scale. This is due to the wide availability of soy processing products characterized by a relatively high protein content. Rapeseed is among the five oil-bearing crops cultivated at the largest scale worldwide, even though it is less common than soy, cotton, peanuts, or sunflower. Rapeseed can thrive at lower temperatures than the crops listed above, and it therefore acquires particular importance as a plant protein source as the climatic conditions of Russia are considered. Rapeseed [21] and soy proteins [22, 23] have a balanced amino acid composition characterized by a sufficiently high content of sulfur-containing amino acids and lysine.

Rapeseed proteins were extracted from defatted meal according to a previously described procedure [15] that allowed for the production of protein preparations free of the antinutrients present in rapeseed meal. Rapeseed meal proteins were extracted with 0.5 M NaCl at a pH of 10.5 and a meal concentration of 3% (wt/vol) at most. Some of the undesirable components were removed by rinsing the meal with 60% ethyl alcohol. Low molecular weight antinutrient compounds, which were extracted together with the protein, were separated by extract ultrafiltration through membranes that retained compounds with a molecular weight higher than 10 kDa. The proteins were precipitated with ethyl alcohol at a 1 : 9 ratio (vol/vol) at 4°C to remove NaCl, resuspended in water, and lyophilized. Amino acid analysis showed that the protein content in the obtained preparations was close to 92%. Reaction with iron(III) chloride [18] did not cause a color change in the protein solutions, which is indicative of the absence of phenolic compounds. The amino acid composition of the proteins preparations was comparable to that of fishmeal, even though the levels of certain amino acids differed [15].

A commercial protosubtilin preparation was used for rapeseed and soy protein hydrolysis. According to the manufacturer's information, the preparation included cooking salt, chalk, and corn flour, and the insoluble material was therefore separated before use, and the solution was concentrated and lyophilized. Protosubtilin is a complex enzyme preparation of bacterial origin. The proteolytic enzyme subtilisin produced by *Bacillus subtilis* bacteria is the major component of the preparation. The enzymatic activity of the preparation is mediated by a complex of neutral and alkaline proteases and the associated minor components, including alpha-amylase, beta-glucanase, xylanase, and cellulase. As reported by the manufacturer, protosubtilin shows maximal enzymatic activity at a pH of 4–6 and 40–60°C. The total rapeseed protein includes globulins (~70% of the total protein) with a pI in the range of 4–7 [24] and albumins with a pI of ~10 [25], whereas the pI of soy proteins ranges from 4.5 to 6.4. Therefore, hydrolysis was performed at a pH of 7.5 and room temperature, that is, the conditions were close to optimal but allowed better protein solubility.

A commercial soy protein isolate and rapeseed proteins (3 mg/mL) were hydrolyzed with protosubtilin under the conditions described above at a protosubtilin/protein ratio of 1/20 or 1/100 for 1, 4, and 20 h. Denaturation of the proteins prior to hydrolysis did not affect hydrolysis depth. The obtained hydrolysates were analyzed by SDS-PAG electrophoresis (Fig. 1), HPLC (Fig. 2), and mass spectrometry (Figs. 3 and 4), and the free amino acid content of the hydrolyzates was measured.

Globulins, the major rapeseed proteins, are soluble in saline solutions. Globulins (MW ~300 kDa) consist of six subunits that form a quaternary structure. Polypeptide chains of the subunits are partially linked by disulfide bridges. A low molecular weight (~12–14 kDa) and high solubility in water is characteristic of albumins. An electrophoregram of rapeseed protein hydrolysate is shown in Fig. 1a. As evident from Fig. 1a, virtually all high molecular weight proteins were completely hydrolyzed within 1 h at a protosubtilin/protein ratio of 1/20 or 1/100. The molecular weight of peptides formed at a protosubtilin/protein ratio of 1 : 20 was lower than 14 kDa. The cleavage rate of the peptides with molecular weights of 10 to 14 kDa was reduced at a protosubtilin/protein ratio of 1 : 100. The reaction mix became slightly cloudy at the initial stage of hydrolysis. One can assume that large peptide fragments with an isoelectric point of approximately 7.5 formed at that time and were destroyed during the subsequent hydrolysis. As a result, rapeseed protein hydrolysate mostly contained components soluble at a pH of 7.5.

Thus, rapeseed protein hydrolysis by protosubtilin for 20 h at an enzyme/protein ratio of 1 : 20 resulted in complete destruction of the proteins and accumulation of low molecular weight oligopeptides (molecular weight lower than 14 kDa) that accounted for 53% of the starting protein material (by weight) and free amino acids that accounted for 8% of the starting material. Chromatograms of the obtained rapeseed protein hydrolysates after 20 h of protosubtilin treatment at different enzyme/protein ratios are shown in Figs. 2a and 2b. The hydrolysis of starting protein material was apparently deeper at a ratio of 1 : 20, as the content of smaller oligopeptides was higher than in the hydrolysate obtained at a protosubtilin/protein ratio of 1 : 100. Thus, the depth of rapeseed protein hydrolysis could be controlled by varying the enzyme/protein ratio (other conditions being equal). The results of mass spectrometric analysis of rapeseed protein hydrolysates are shown in Fig. 3. The mass chromatogram (Fig. 3b) shows that the molecular

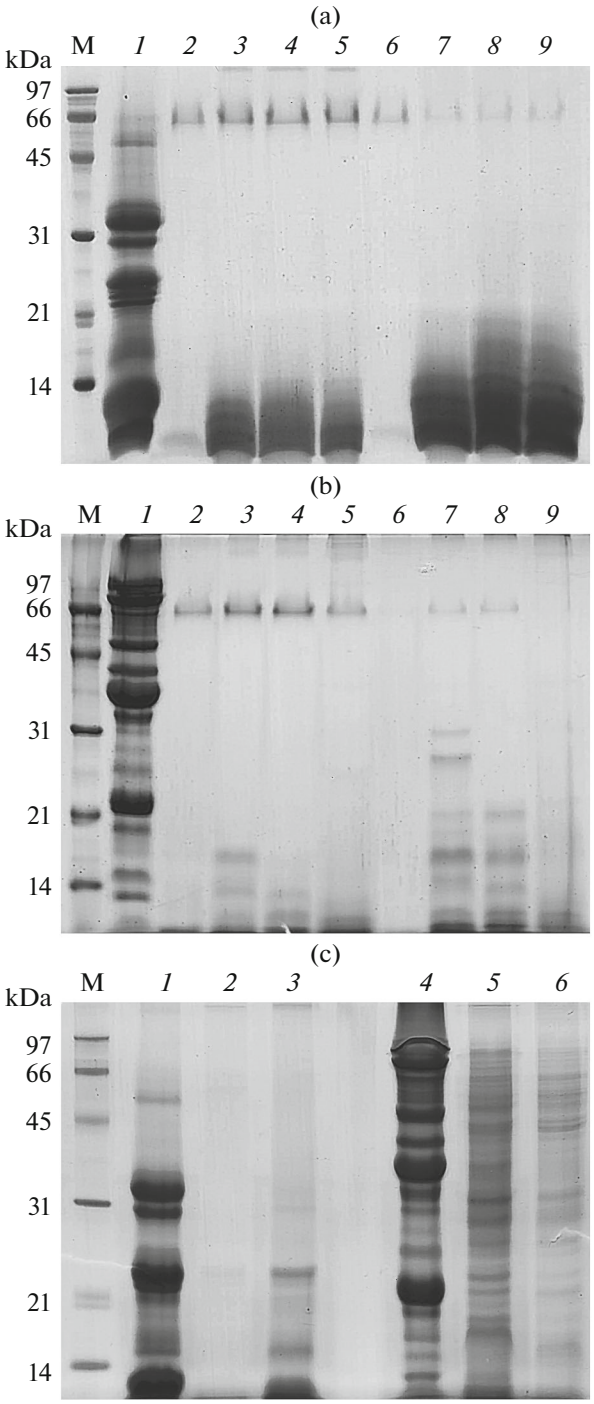

Fig. 1. SDS-PAG electrophoresis of rapeseed (a) and soy (b) protein hydrolysates obtained by protosubtilin treatment at an enzyme/protein ratio of 1 : 20 (*3*–*5*) or 1 : 100 (*7*–*9*) during 1 (*3*, 7), 4 (*4, 8*), and 20 h (*5*, 9): *1*—starting protein preparation from rapeseed or soy; *2*, *6*—5 and 1% (of protein weight) protosubtilin, respectively. (c)—SDS-PAG electrophoresis of the intact rapeseed protein extract (*1*), soy protein isolate (*4*), and the insoluble fractions of rapeseed (*2*, *3*) and soy (*5*, *6*) hydrolysates formed after treatment with the enzyme preparation at an enzyme/protein ratio of 1 : 20 (*2*, *5*) and 1 : 100 (*3*, *6*).

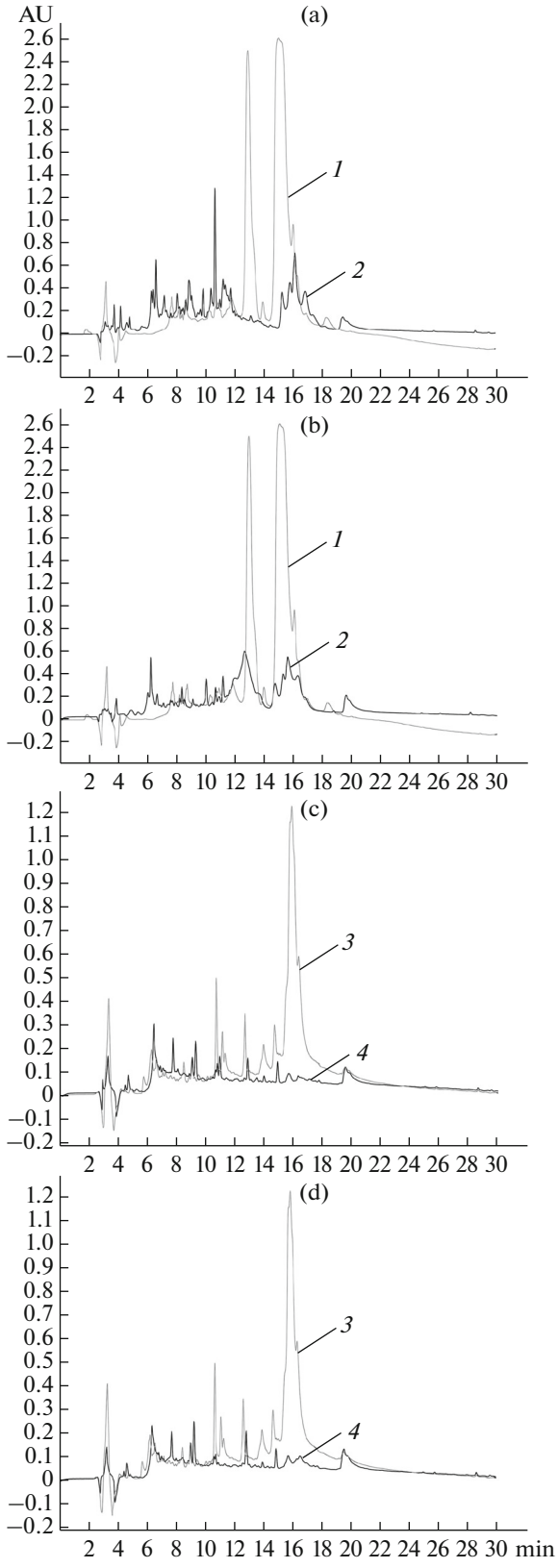

Fig. 2. HPLC of the soluble fractions of rapeseed protein (a and b) and soy protein isolate (c and d) hydrolysates obtained by protosubtilin treatment at an enzyme/protein ratio of 1 : 20 (a and c) and 1 : 100 (b and d): *1*—intact rapeseed proteins, *2*—rapeseed protein hydrolysate, *3*—soy protein isolate, and *4*—soy protein hydrolysate.

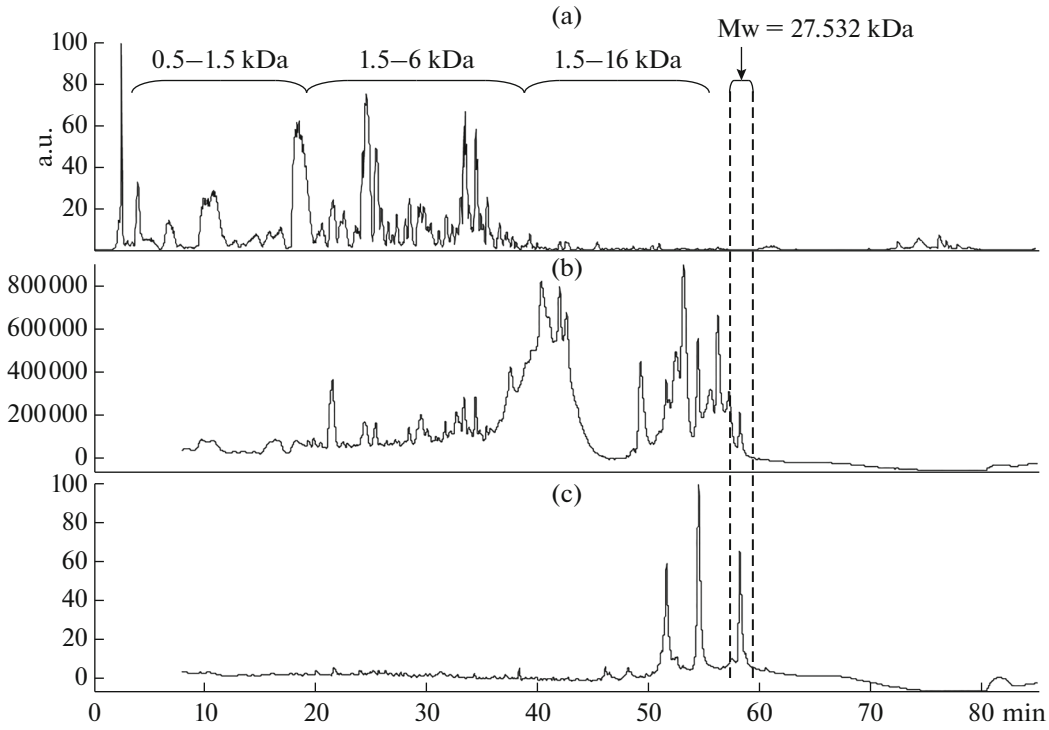

Fig. 3. Mass chromatogram (full ion currents) of a rapeseed protein hydrolysate obtained after 20 h of protosubtilin treatment at an enzyme/protein ratio of 1 : 100 (a) and results of extract HPLC on an ACQUITY UPLC Protein BEH C4 Waters 300 Å column equilibrated with 0.05% formic acid in an acetonitrile concentration gradient (10–80%) with peptide detection at 226 (b) and 280 nm (c). Molecular weight ranges for the hydrolysis products that corresponded to the retention times indicated are shown above the mass chromatogram.

weights of most peptides in the hydrolysate were within a range of 1.5–16 kDa. Similar results were reported in a study [8] that addressed rapeseed protein hydrolysis by Alcalase and Flavourzyme, commercial enzyme preparations produced in foreign countries. The serine protease subtilisin A is the major component of the Alcalase preparation. Flavourzyme contains endo- and exoproteases produced by *Aspergillus oryzae* bacteria. The final hydrolysate was characterized by a 60% degree of hydrolysis and complete solubility at pH values between 2.5 and 7.

The character of enzymatic hydrolysis was different when a commercial soy protein isolate was treated with protosubtilin under the conditions used for rapeseed protein hydrolysis (pH of 7.5, room temperature, protein concentration 3 mg/mL). The reaction mix became rather cloudy during the hydrolysis of soy proteins, and clearing of the mix did not occur afterwards, which is probably due to the formation of insoluble protein fragments. An electrophoregram of the soluble part of the soy hydrolysate is shown in Fig. 1b. The absence of high molecular weight fragments could be indicative of considerable depth of hydrolysis or an accumulation of large protein fragments in the insoluble component of the hydrolysate (Fig. 1c). The soluble part of the hydrolysate of the soy protein isolate was subjected to HPLC analysis (Figs. 2c and 2d). As evident from the chromatograms, neither large peptide fragments nor the intact proteins were found in the soluble part of the hydrolysate. The total area of peptide peaks was significantly less than in the case of rapeseed protein hydrolysate. Amino acid analysis showed that the soluble part of the hydrolysate contained short oligopeptides and free amino acids, which together accounted for 13% of the starting protein material only. The results of mass spectrometric analysis of the soluble fraction of soy protein hydrolysate are illustrated in Fig. 4. As evident from the mass chromatogram (Fig. 4b), molecular weights of most peptides in the hydrolysate were within a range of 1.5–12 kDa. However, protein fragments of a high molecular weight were also found in the hydrolysate.

The globulins glycinin and β-conglycinin present in soybean at a ratio of 75/115 are the major soybean proteins that account for 80% of soy protein material together [26, 27]. Both proteins have a complex quaternary structure. Glycinin consists of five subunits and has a sedimentation coefficient of 11S, whereas β-conglycinin (sedimentation coefficient 7S) consists of three subunits (76, 72, and 53 kDa) [4, 28]. In addition to globulins that are insoluble in water but soluble in neutral saline solutions, soy contains water-soluble proteins characterized by a lower molecular weight. This group of proteins includes the minor γ-conglyci-

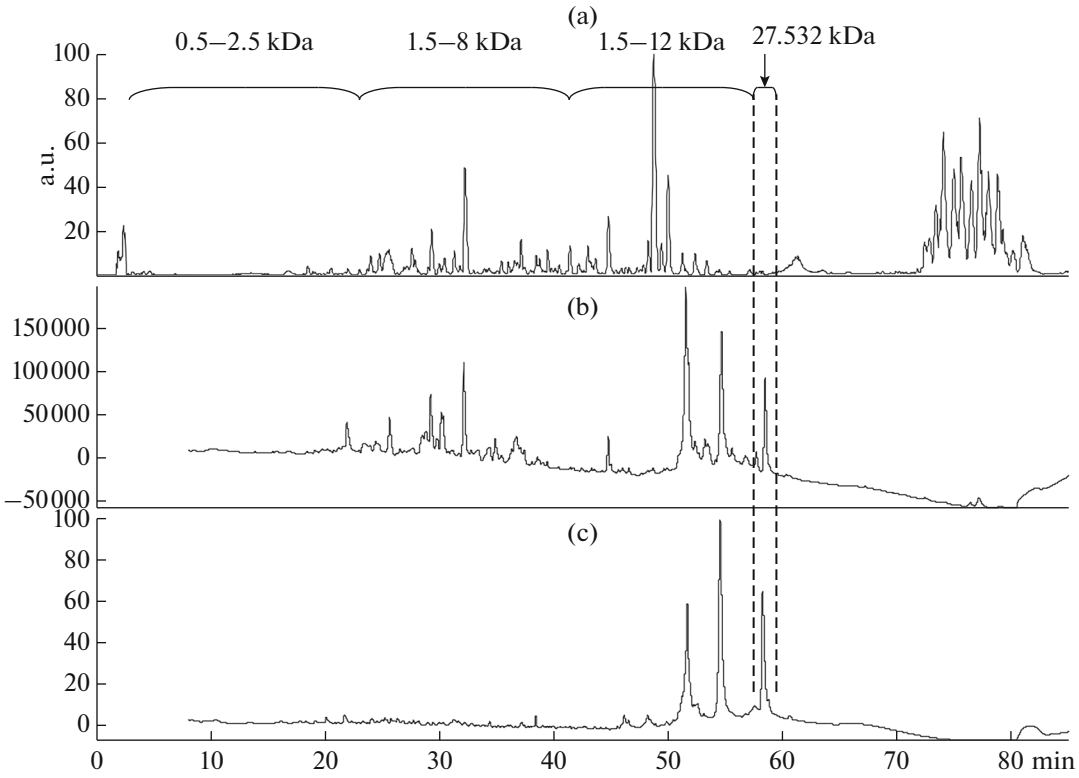

Fig. 4. Mass chromatogram (full ion currents) of a soy protein hydrolysate obtained after 20 h of protosubtilin treatment at an enzyme/protein ratio of 1 : 100 (a), and results of extract HPLC on an ACQUITY UPLC Protein BEH C4 Waters 300 Å column equilibrated with 0.05% formic acid in an acetonitrile concentration gradient (10–80%) with peptide detection at 226 (b) and 280 nm (c). Molecular weight ranges for the hydrolysis products that corresponded to the retention times indicated are shown above the mass chromatogram.

nin and a relatively small number of other proteins, including the serum proteins, which account for 9 to 15% of total soy protein weight together. Soy globulins have a complex quaternary structure formed by large polypeptides and undergo rapid denaturation with polypeptide chain unfolding under certain conditions, with gelling induced by subsequent aggregation. Protein aggregation is triggered by heating, freezing, medium acidification, high pressure, and enzymes. The gelling rate depends on the protein concentration in the solution. The gelling of linear molecules, such as polysaccharides and fibrillar proteins, is thoroughly characterized, but globular protein gelling still remains enigmatic because of the complex structure of these molecules [29]. Coagulation of soy proteins upon enzymatic hydrolysis was also observed by other researchers [28, 30–32]. Polypeptide coagulation at a 10% degree of hydrolysis occurred when soy proteins were hydrolyzed by subtilisin Carlsberg (CS) [33], and aggregation was already observed at a 5% degree of hydrolysis at a pH of ~7.5. Intensive gelling of soy protein fragments was also observed in the case of processing with Novozyme FM 2.0 L and Alcalase 2.4 L enzyme preparations, which contain subtilisin as the major component [12]. Variation of ionic strength of the medium (0.03, 0.2, and 0.5 M) had only a slight effect on aggregation pH. This indicated the complex nature of aggregation and the existence of factors other than the equilibrium between electrostatic and hydrophobic interactions. SDS-PAG electrophoresis showed that all enzyme preparations destroyed β-conglycinin and glycinin. However, the major glycinin component showed high resistance to the action of the enzyme preparations listed above.

Analysis of a large number of studies on the enzymatic hydrolysis of soy proteins revealed the low accessibility of these proteins for enzymatic hydrolysis due to their compact tertiary and quaternary structure, which renders many protein fragments shielded and poorly accessible for enzymes [34–36].

CONCLUSIONS

Thus, the conditions for enzymatic hydrolysis of rapeseed proteins by protosubtilin, a commercial enzyme preparation developed in Russia, have been selected. The hydrolysate obtained did not contain antinutrients, and the hydrolysis depth was characterized by several methods. The content of free amino acids and oligopeptides (MW < 10 kDa) in the hydrolysates was dependent on the hydrolysis conditions, as was the content of protein fragments with a higher molecular weight. The content of free amino acids and

oligopeptides (MW < 14 kDa) in a rapeseed protein obtained after 20 h of processing at a protosubtilin/protein ratio of 1 : 20 was 8 and 53% of the starting protein material, respectively.

Insoluble fragment formation and coagulation of cleavage products is among the major problems in soy protein proteolysis. The present study revealed intensive coagulation of hydrolysis products and insoluble pellet formation upon protosubtilin treatment of a commercial soy protein isolate and a related considerable decrease of the yield of soluble hydrolysis products. The free amino acid content in the soluble fraction of soy protein hydrolysate (obtained after 20 h of treatment at a protosubtilin/protein ratio of 1 : 20) was 2.4% of the starting soluble protein material only, oligopeptides with a molecular weight lower than 14 kDa accounted for 10.4% of the starting material, and the total yield of soluble peptides was 12.8% only.

ACKNOWLEDGMENTS

The present work was supported by the Russian Science Foundation (project no. 16-14-00133).

REFERENCES

1. Salunkhe, D.K., Adsule, R.N., Chavan, J.K., and Kadam, S.S., *World Oilseeds. Chemistry, Technology and Utilization*, New York: Springer, 1992.
2. Slawski, H., Adem, H., Tressel, R.P., Wysujack, K., Koops, U., and Schulzet, C., *Aquaculture*, 2011, vol. 63, no. 2, pp. 605–611.
3. Ravindran, V., Abdollahi, M.R., and Bootwalla, S.M., *Poultry Sci.*, 2014, vol. 93, no. 10, pp. 2567–2577.
4. Kinsella, J.E., *J. Am. Oil Soc.*, 1979, vol. 56, no. 3, pp. 242–258.
5. Shahidi, F., *Canola and Rapeseed. Production, Chemistry, Nutrition and Processing Technology*, Shahidi, F., Ed., New York: Springer Science and Business Media, 1990.
6. Vioque, J., Sanchez-Vioque, R., Clemente, A., Pedroche, J., Bautista, J., and Millan, F., *J. Am. Oil Soc.*, 1999, vol. 76, no. 7, pp. 819–823.
7. Xiang Dong Sun, *Int. J. Food Sci. Technol.*, 2011, vol. 46, no. 12, pp. 2447–2459.
8. Francis, G., Makkar, H., and Bekker, K., *Aquaculture*, 2001, vol. 199, no. 3, pp. 197–227.
9. Rodrigues, I.M., Coelho, J.F.J., and Carvalho, M.G., *J. Food Eng.*, 2012, vol. 109, no. 3, pp. 337–346.
10. Grozesku, Yu.N., Bakhareva, A.A., and Shul'gina, E.A., *Rybovod. Rybn. Khoz.*, 2011, no. 4, pp. 49–52.
11. Alamdari, Kh., Dolganova, N.V., Ponomarev, S.V., and Vinnov, A.S., Vestnik Astrakhan. Gos. Tekhn. Univ., Ser. Rybn. Khoz., 2013, no. 2, pp. 172–177.
12. Hrĉková, M., Rusňáková, M., and Zemanoviĉ, J., *Czech. J. Food Sci.*, 2002, vol. 20, no. 1, pp. 7–14.
13. Caldéron De La Barca, A.M., Ruiz-Salazar, R.A., and Jara-Marini, M.E., *J. Food Sci.*, 2000, vol. 65, no. 2, pp. 246–253.
14. Kuipers, B.J., van Koningsveld, G.A., Alting, A.C., Driehuis, F., Gruppen, H., and Voragen, A.G., *J. Agric. Food Chem.*, 2005, vol. 53, no. 4, pp. 1031–1038.
15. Muranova, T.A., Zinchenko, D.V., Kononova, S.V., Belova, N.A., and Miroshnikov, A.I., *Appl. Biochem. Microbiol.*, 2017, vol. 53, no. 6, pp. 680–687.
16. Muranova, T.A., Zinchenko, D.V., Melanina, L.A., and Miroshnikov, A.I., *Appl. Biochem. Microbiol.*, 2018, vol. 54, no. 1, pp. 76–82.
17. *Kolorimetricheskie (fotometricheskie) metody opredeleniya nemetallov* (Colorimetric (Photometric) Methods for Determining Non-Metals), Busev, A.I., Ed., Moscow: Izd. Inostr. Lit., 1963.
18. Zaprometov, M.N., *Biokhimiya katekhinov* (Biochemistry of Catechins), Moscow: Nauka, 1964.
19. Bradford, M.M., *Anal. Biochem.*, 1976, vol. 72, no. 7, pp. 248–254.
20. Laemmly, U.K., *Nature*, 1970, vol. 227, no. 8, pp. 680–685.
21. Cheftel, J.C., Cuq, J.L., and Lorient, D., *Protéines alimentaires. Biochimie—propriétés fonctionnelles—valeur nutritionnelle–modifications chimiques*, Paris: Technique et Documentation—Lavoisier, 1985.
22. Slawski, H., Adem, H., Tressel, R.P., Wysujack, K., Koops, U., and Schulzet, C., *Aquaculture*, 2011, vol. 63, no. 4, pp. 605–611.
23. Ravindran, V., Abdollahi, M.R., and Bootwalla, S.M., *Poultry Sci.*, 2014, vol. 93, no. 10, pp. 2567–2577.
24. Chabanon, G., Chevalot, I., Framboisier, X., Chenu, S., and Marc, I., *Process Biochem.*, 2007, vol. 42, no. 10, pp. 1419–1428.
25. Krause, J.P. and Schweke, K.D., *Colloids Surf.*, 2001, vol. 21, nos. 1–3, pp. 29–36.
26. Nielsen, N.S., in *Structure of Soy Protein*, Altschul, A.M. and Wilcke, H.L., Eds., New York: Academic Press, 1985, pp. 26–66.
27. Nishinary, K.Y., Fang, S.Guo., and Philips, G.O., *Food Hydrocolloid*, 2014, vol. 39, pp. 301–318.
28. Sexton, P.J., Paek, N.C., and Shibles, R.M., *Field Crops Res.*, 1998, vol. 59, no. 1, pp. 1–8.
29. Clark, A.H., *Functional Properties of Food Macromolecules. Gelation of Globular Proteins*, 2nd ed., Hill, S.E., Ledward, D.A., and Mitchell, J.R., Eds., New York: Springer, 1998.
30. Inouye, K., Nagai, K., and Teisuke, T., *J. Agric. Food Chem.*, 2002, vol. 50, no. 5, pp. 1237–1242.
31. Bernardi Don, L.S., Pilosof, A.M.R., and Bartholomi, G.B., *J. Am. Oil Soc.*, 1991, vol. 68, no. 2, pp. 102–105.
32. Tang, C-H., Wu, H., Yu, H-P., Li, L., Chen, Z., and Yang, X.Q., *J. Food Biochem.*, 2006, vol. 30, no. 1, pp. 35–55.
33. Bas J.H. Kuipers, Gerrit A. van Koningsveld, Arno C. Alting, Frank Driehuis, Harry Gruppen, and Alphons G.V. Voragen, *J. Agric. Food Chem.*, 2005, vol. 53, no. 4, pp. 1031–1038.
34. Tsumura, K., Saito, T., Kugimiya, W., and Inouye, K., *J. Food Sci.*, 2004, vol. 69, no. 5, pp. 363–367.
35. Chen Lin, Jianshe Chen, Jianyan Ren, and Mouming Zhao, *Food Hydrocolloid.*, 2011, vol. 25, no. 5, pp. 887–897.
36. Xiang Dong Sun., *Int. J. Food Sci. Technol.*, 2011, vol. 46, no. 12, pp. 2447–2459.

Translated by S. Semenova