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EXPERIMENTAL ARTICLES

A New *Bacillus licheniformis* Mutant Strain Producing Serine Protease Efficient for Hydrolysis of Soy Meal Proteins

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Abstract—Induced mutagenesis with γ-irradiation of the industrial strain *Bacillus licheniformis*-60 VKM B-2366 D was used to obtain a new highly active producer of an extracellular serine protease, *Bacillus licheniformis*-145. Samples of dry concentrated preparations of serine protease produced by the original and mutant strains were obtained, and identity of their protein composition was established. Alkaline serine protease subtilisin DY was the main component of the preparations. The biochemical and physicochemical properties of the Protolicheterm-145 enzyme preparation obtained from the mutant strain were studied. It exhibited proteolytic activity (1.5 times higher than the preparation from the initial strain) within broad ranges of pH (5–11) and temperature (30–70°C). Efficient hydrolysis of extruded soybean meal protein at high concentrations (20 to 50%) in the reaction mixture was the main advantage of the Protolicheterm-145 preparation. Compared to the preparation obtained using the initial strain, the new preparation with increased proteolytic activity provided for more complete hydrolysis of the main non-nutritious anti-nutritional soy proteins (glycinin and β-conglycinin) with the yield of soluble protein increased by 19–28%, which decreased the cost of bioconversion of the proteinaceous material and indicated promise of the new preparation in resource-saving technologies for processing soybean meals and cakes.

Keywords: Bacillus licheniformis, serine protease, induced γ-mutagenesis, soybean meal hydrolysis

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Wasteless processing of renewable materials is a general direction of modern industrial biotechnologies. One of the most pressing tasks is processing primary industry waste through the conversion of secondary protein-containing material into valuable protein products.

The use of enzyme preparations (EP) with proteolytic action for these purposes make it possible to obtain high-quality protein products with predetermined properties, also ensuring environmental safety of the technological process due to exclusion of aggressive reagents. Microbial serine proteases (EC 3.4.21) are among the most important groups of industrial enzymes. They are used in recycling protein-containing waste of vegetable raw material, as well as for unhairing in leather industry, in the food-processing industry for the preparation of protein hydrolysates, meat tenderization, cheese taste development, flour treatment, improvement of dough structure, and modification of wheat gluten, in the feed industry to increase feed assimilability, in the production of fish liver oil, silk degumming, etc. As the constituent parts of detergents, serine proteases not only remove body fat and increase the quality of laundry but also decrease the energy and water consumption, as well as the temperature and time of laundry (Kumar and Takagi, 1999; Bhunia et al., 2012 Mienda et al., 2014).

Considering the volumes of soybean protein processing, application of serine proteases for the production of soybean meal-based protein hydrolysates is an important direction. Soybean meal (SBM) is characterized by a high content of protein with a full-value amino acid composition and is a promising raw material for the manufacture of protein feed and food supplements. Soybean meal hydrolysates are widely used in the production of foodstuffs, feeds, and in dietetic, sport, and baby food (Dei, 2011; El-Shemy, 2011; McCarthy et al., 2013). At the same time, soy protein is a potent allergen. About 70% of soy protein is represented by glycinin and β-conglycinin, anti-nitritional proteins exhibiting antigenic properties and resistant

to heat treatment. That is why, with all the advantages of soy as a protein source of high nutritious and energy value, one of the most important steps of its processing is to decrease allergenic exposure to soy proteins. The enzymatic treatment with serine proteases combined with extrusion SBM pretreatment results in complete hydrolysis of anti-nutritional proteins with the formation of low-molecular weight hypoallergenic easily digested peptides (Fischer, 2006; Sun, 2011; Wang et al., 2014).

Bacteria of the genus *Bacillus* capable of secreting large amounts of extracellular enzyme proteins are the main industrial producers of serine proteases (Genckal and Tari, 2006; Bhunia et al., 2012; Azlina and Norazila, 2013). The efficiency of production of commercial EP is primarily determined by the level of activity of the producer strain. Therefore, studies are being conducted to search for the most productive microorganisms, to increase the activity of known industrial producers of serine proteases, and to optimize the conditions of their cultivation (Genckal and Tari, 2006; Nadeem et al., 2010; Sreedevi et al., 2014).

In order to improve the productive characteristics of the strains used in microbiological industry, methods of induced mutagenesis employing physical and chemical mutagens with subsequent screening of the mutant clones on selective media are widely used (Wang et al., 2007; Nadeem et al., 2010; Roja Rani et al., 2012; Javed et al., 2013; Venkata and Divakar, 2013; Sreedevi et al., 2014; Jeyasanta and Patterson, 2014). In this study, we used the techniques for obtaining second-level mutants on the basis of a highly productive industrial producer obtained, in turn, with methods of induced mutagenesis.

The goals of the present work were (1) to develop, using methods of induced mutagenesis, a highly active and promising producer of serine proteases based on *Bacillus licheniformis*-60 (VKM B-2366 D), an industrial strain with a high-level secretion of proteolytic enzymes; (2) to obtain a concentrated enzyme preparation based on the new mutant strain; and (3) to carry out comparative analysis of the enzyme preparations from the new and the parent *Bacillus licheniformis* strains.

MATERIALS AND METHODS

Reagents. The study used the Reakhim (Russia), BioRad (United States), and Amresco (United States) reagents. The Protein Molecular Weight Markers, MW Range 6.5–200 kDa (Protea Biosciences Inc., United States), and PageRuler™ Prestained Protein Ladder, 10 to 170 kDa (Thermo Scientific, United States) kits were used as marker proteins to determine the molecular mass. Casein (Sigma, United States) and toasted soybean meal with 5.5% humidity were used as substrates.

Strains of microorganisms. *Bacillus licheniformis*-60, a producer of a complex of proteolytic enzymes, obtained using selection and mutagenesis and deposited under the number B-2366 D in the All-Russia Collection of Microorganisms (RF patent 2303066) was used as the parent strain.

Maintenance of the strain *B. licheniformis*. The parent strain was grown at 37°C until the sporulation stage for five days in test tubes on slant peptone agar (PA) of the following composition (%): peptone, 3; NaCl, 0.5; agar-agar, 2; tap water, pH 7.0–7.2. The grown cultures were then incubated for two weeks at 20°C and further maintained at 4°C. The strain was passaged after six to eight weeks.

Preparation of cell suspension. Sterile distilled water (5 mL) was poured into a test tube with *B. licheniformis* grown for five days at 37°C and mixed with a microbiological loop. The prepared spore suspension was transferred into a sterile test tube, stirred with a V-3 vortex-type shaker (ELMI Ltd., Latvia), and diluted with sterile distilled water to the titer of 10^6 cells/mL.

Mutagenesis. Co-mutagenesis of *B. licheniformis* bacteria was carried out on a GUT-200 chamber-type gamma unit with radioactive cobalt-60 M sources. SGD-8 glass dosimeters were used for measuring the radiation dose of the samples studied, considering that the irradiation power corresponded to the irradiation dose per unit time. The spore suspension of the strains studied was in 2-mL plastic test tubes, which were mounted during irradiation in a special unit assembled round the 190-mm irradiator in the radial direction to the irradiator center. Irradiation was carried out at 20–22°C. Peptone agar in petri dishes was inoculated with irradiated suspensions.

Determination of cell (spore) viability after mutagenic exposure. A series of sequential dilutions with sterile distilled water were made from γ-irradiated suspensions. PA in petri dishes was inoculated with the dilutions obtained (20 μL). The control (not exposed to irradiation) suspension was plated in a similar way. The plates were incubated at 37°C. The grown colonies were counted after 48 h. Taking the number of colonies in the control as 100%, spore viability (%) was calculated after treatment with the mutagen.

Submerged cultivation of the parent and mutant strains was carried out in shaken flasks in the fermentation medium of the following composition (%): wheat flour, 8; rye flour, 4; corn starch, 2; soybean flour, 2; $CaCO_3$, 0.25; $MgSO_4 \cdot 7H_2O$, 0.2; Na_2CO_3, 0.1. The fermentation medium was prepared in the following way: the flour and starch were stirred in tap water; the Termamyl SC enzyme preparation of thermostable α-amylase (Novozymes, Denmark) was introduced (2 units of amylolytic activity per 1 g of starch); the temperature was gradually increased to 85–90°C with the subsequent incubation for 40 min; magnesium sulfate was then added. To stabilize pH,

Na_2CO_3 was added to the medium as a 10% solution to the concentration of 0.1%. The weighed amount of $CaCO_3$ was introduced into each flask separately. The medium (50 mL) was dispensed into 750-mL shaker flasks and sterilized at 1 atm for 1 h.

The spore material obtained by cultivation in the test tubes with slant agar or on petri dishes for one to three weeks was introduced into the flasks with sterile medium using a microbiological loop. Cultivation was carried out on shakers (250 rpm) at 37°C for 72 h. The producer biomass was separated by centrifugation at 10750 *g* for 5 min. The culture liquid was used to determine proteolytic activity.

Enzyme preparations were obtained from the culture liquid (CL) of the parent and mutant strains. The strains were cultivated at the Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences (Pushchino), on a KF-108 unit (the working volume of reactor 1 L) with software for monitoring and control of the fermentation processes. The bacteria were cultivated for 70 h in the medium of the above-mentioned composition. Prior to sterilization, laprol (propylene glycol, 0.1%) was introduced into the medium as an antifoam agent. The cultivation was carried out at 37 ± 1°C, pH 6.5–6.8. In order to separate the biomass, the grown culture was centrifuged for 30 min at 4500 *g*. The culture liquid was concentrated in an AR 3.0-15 PS separating hollow-fiber ultrafiltration device with a cutoff threshold of 15 kDa (Kirishi, Russia). The enzyme protein was precipitated from the ultraconcentrate with acetone (two volumes of cooled acetone per one volume of ultraconcentrate). The suspension obtained was centrifuged for 15 min at 4500 *g*; the pellet was dried at 20–22°C for 72 h and homogenized using a laboratory mill (LZM, Russia).

Determination of the enzyme activity. Proteolytic activity was determined according to the universal method developed by Sigma-Aldrich (Cupp-Enyard, 2008) at 60°C, pH 9.0 using casein as a substrate. The amount of the enzyme catalyzing the cleavage of 1 μmol of tyrosine during 1 min under the experimental conditions (60°C, pH 9.0) was accepted as a unit activity. Proteolytic activity was expressed in U/mL; specific activity, in U/g protein.

The protein content in CL and EP was determined according to the method of Lowry using BSA as the standard (Peterson, 1979).

PAG electrophoresis of EP proteins. The EP proteins were separated in 12% PAG (25 mM Tris-glycine buffer, pH 8.3, with SDS at a concentration of 1 mg/mL) in a Mini Protein Cell system well for electrophoresis (Bio-Rad, United States). The gel was stained with G-250 Coomassie brilliant blue (Amresco, United States). The EPs were subjected to electrophoresis under denaturing conditions with preliminary inhibition of the proteases with 100 mM PMSF solution (Amresco, United States).

Mass spectrometric analysis. A slab with the protein studied was excised from PAG; the protein was treated with trypsin; the hydrolysate was analyzed using the MALDI-TOF mass spectrometry. The data obtained were processed using the Bruker Data Analysis software package (Brucker Corporation, United States). The search for the enzyme using the mass spectra in the NCBI and SWISS-PROTT protein databases was carried out using the Peptide Mass Fingerprint software package (Matrix Science Inc., United States).

Determination of the EP optimum pH and temperatures. The activity of EP in relation to casein within the 5.0–11.0 pH range was determined at 60°C using 0.1 M universal buffer.

The optimum temperatures during the action of EP on casein were determined in the 30–70°C range in 0.1 M universal buffer, pH 9.0.

Determination of the EP pH stability and thermostability. In order to determine pH stability, EP solutions in 0.1 M universal buffer with pH from 5.0 to 11.0 were incubated at 25°C for 24 h, and residual proteolytic activity on casein was determined under the standard conditions (60°C, pH 9.0).

EP thermostability was determined by incubating EP solution in distilled water at 30, 40, 50, 60, and 70°C and measuring residual proteolytic activity on casein under the optimal conditions (60°C, pH 9.0).

Hydrolysis of anti-nitritional SBM proteins. Extrusion pretreatment of toasted SBM was carried out using a Werner & Pleiderer Continua 37 twin-screw extruder (Germany) at 120°C.

The extruded SBM (ESBM) samples were hydrolyzed in 2-mL plastic test tubes at substrate concentrations of 20, 35, and 50% at 50°C, pH 6.0–6.2 (a natural pH value for the mixture of ESBM and tap water). The ESBM samples were mixed with the tap water containing EP at a concentration of 0.2 mg/g ESBM. The samples were vigorously mixed at the beginning of the process and then incubated without agitation for 5 h. After that, the hydrolyzed mixture was incubated at 85°C for 10 min in order to inactivate the enzymes. The hydrolysates were centrifuged for 10 min at 10750 *g*; the pellet was dried at 50°C for 2 h. The degree of protein hydrolysis in ESM was determined by the presence or absence in the electropherogram of the bands corresponding to the subunits of the main anti-nitritional soybean proteins, as well as by the yield of the soluble protein determined by the Lowry method.

PAG electrophoresis of ESM hydrolysates. Electrophoresis of ESM hydrolysates was carried out in 12% PAG with SDS in a Protean II xi Cell 20 chamber (BioRad, United States) according to the method of Laemmli (Peterson, 1970) with slight modifications in sample preparation: 20 mg of dry hydrolysate was dispensed into 2-mL plastic test tubes; protein extraction was carried out in 0.125 mL of SDS buffer (BioRad,

United States) for 1 h at 25°C under constant stirring followed by 5-fold dilution in SDS buffer with 5% β-mercaptethanol and incubation for 15 min at 100°C.

Statistical data processing was carried out using the Matlab 6.5 software package (The Mathworks. Inc., United States) with post-hoc Tukey's test at a level of significance $\alpha = 0.05$.

RESULTS AND DISCUSSION

Gamma-mutagenesis of the strain *B. licheniformis*-60. Ionizing gamma radiation has a strong mutagenic effect due to ruptures of one or two DNA strands with the resultant deletions or structural changes, formation of DNA–protein cross-linkages, and oxidation of the bases. Gamma-mutagenesis is successfully used for obtaining enzyme-producing strains and biologically active substances with improved properties (Wang et al., 2007; Modasrah et al., 2012; Shahbazi et al., 2014; Ottenheim et al., 2015).

The results of mutagenesis depend considerably on the parameters of mutagenic treatment: the mutagenic factor, its intensity, the exposure time, the mode of preparation of cell suspension, the physiological cell age (which depends on the growth phase), the composition of selective media, etc. Cell viability after treatment with a mutagen and the percentage of the variants with increased activity (plus-variants) upon growth on agar media are the main criteria of the efficiency of exposure to the mutagen. Both the viability and the frequency of emergence of positive mutations at a certain level of viability are individual for each strain and are determined experimentally.

In order to determine the optimum parameters of mutagenic treatment of the strain *B. licheniformis*-60, gamma-irradiation of the spore suspension was carried out in different modes (Table 1). Agar medium for subculturing irradiated cells was chosen on the basis of the data obtained for the parent strain *B. licheniformis*-60. When working with *B. licheniformis*-60, we established that the use of solid media containing casein or dry milk was not efficient for preliminary selection of the active variants using the diameter of the transparent zone with the protease activity exceeding 100–120 U/mL. All the clones obtained by plating of the isogenic population of *B. licheniformis*-60 were characterized by approximately 20-mm hydrolysis zones, and their activity in submerged cultivation varied between 220 and 270 U/mL. The literature data confirmed that the screening of productive variants by the casein hydrolysis zone during cultivation on appropriate solid media is not always suitable for *B. licheniformis* strains, because even highly active *B. licheniformis* clones form small hydrolysis zones (Kumar and Takagi, 1999). Therefore, the PA medium, on which the mutants with a high level of activity grew more rapidly and accumulated more biomass, i.e., formed larger colonies, was used for plating the cell suspension after mutagenic treatment. When the parent strain grew on PA at 37°C for 48 h, we observed the formation of irregular, mucous, smooth, nontransparent colonies with an elevated center and a beige pigment.

In the first series of experiments (Table 1), the radiation doses varied between 1500 and 5000 Gy with the exposure time of 8 h. The results obtained showed that the viability decreased from 9.5 to 0.8% when the exposure dose was increased, with the level of maximum activity of the mutant clones tending to increase. In order to further increase the efficiency of mutagenesis, the power (intensity) of irradiation was increased by reducing the exposure time to a dose of 5000 Gy. Reduction of the exposure time from 8 to 2 h resulted in the viability of bacteria decreasing to 0.11%. The maximal proteolytic activity in the culture liquid of selected clones was 412 U/mL in the variants exposed to irradiation for 2 h (Table 1).

Since an increase in the exposure dose to 5000 Gy due to the decrease of irradiation time to 2 h led to emergence of the clones with a higher activity compared to the parent strain, the mutagenic exposure in the subsequent experimental series was enhanced. The increase in the radiation dose to 5500, 6000, and 6500 Gy over 2 h of exposure caused the viability to decrease to 0.09, 0.08, and 0.06%, respectively. The most active clones with a proteolytic activity of 463 and 486 U/mL were obtained with the radiation doses of 5500 and 6000 Gy, respectively (Table 1). It is worth noting that, among the variants exposed to doses of 5000–6000 Gy for 2 h, the greatest number of highly active clones were selected against a small number of clones with decreased proteolytic activity.

In order to test the stability of the mutant bacteria, which is an essential characteristic of industrial strains, fourteen clones with a level of activity exceeding 400 U/mL were selected during cultivation in flasks (Fig. 1). Clone 145, obtained by exposure of *B. licheniformis*-60 to a dose of 5000 Gy for two hours, was selected for further work based on the data concerning the stability of maintenance of proteolytic activity during transfers (three passages). The level of its activity during submerged cultivation in liquid media (in flasks) varied in the 407–422 U/mL range and did not decrease after passages on PA.

Properties of *B. licheniformis*-60- and *B. licheniformis*-145-based EP. The dry Protolicheterm-60 and Protolicheterm-145 EP were obtained by cultivation in reactors as described in the Materials and Methods section. Proteolytic activity in the CL of the mutant strain after completion of the fermentation process was approximately 1.5 times higher (the specific protease activity was 1.3 times higher) than that of the parent strain. The same ratio was also observed for dry EP: the proteolytic activity of Protolicheterm-145 was 1.5 times higher (the specific protease activity was

Table 1. Viability and proteolytic activity of *B. licheniformis*-60 selected variants depending on the dose and intensity of γ-irradiation

Irradiation dose, Gy	Irradiation time, h	Viability, %	Maximal proteolytic activity of selected variants, U/mL
Control (without irradiation)		100	271 ± 9^{a}
1500	8	9.53 ± 0.49^{a}	324 ± 6^{b}
2000	8	2.50 ± 0.66^{b}	317 ± 6^{b}
2500	8	1.23 ± 0.42^{c}	343 ± 6^{bc}
3000	8	1.07 ± 0.29^{cd}	332 ± 12^{bd}
4000	8	1.03 ± 0.67^{cd}	339 ± 8^{bc}
5000	8	0.77 ± 0.31^{cd}	366 ± 13^{cd}
Control (without irradiation)		100	275 ± 6^{a}
5000	4	0.25 ± 0.17^{cd}	361 ± 15^{cd}
	2	0.11 ± 0.04^{d}	412 ± 13^{e}
Control (without irradiation)		100	267 ± 8^{a}
5500	2	0.09 ± 0.06^{d}	463 ± 7^{f}
6000	2	0.08 ± 0.04^{d}	486 ± 9^{f}
6500	2	0.06 ± 0.04^{d}	432 ± 12^{e}

* The table shows the mean values of three measurements indicating the standard deviation.
** The data in each column with different letter indexes are significantly different at the level of significance $\alpha = 0.05$.

1.3 times higher) compared to Protolicheterm-60 (Table 2).

The protein compositions of concentrated Protolicheterm-145 and Protolicheterm-60 were studied. The electrophoresis data in the presence of SDS (Fig. 2a) showed that the proteins of both the main and minor components in both EPs studied had the same molecular masses. Mass spectrometry established that the main component of the EP obtained using the parent and mutant strains was alkaline serine protease of the subtilisin DY type from *B. licheniformis* (Fig. 2b). Protolicheterm-145 was active at pH from 5 to 11 with the optimum activity at pH 7–10 (Fig. 3a) and in the 30–70°C range with the optimum at 60–

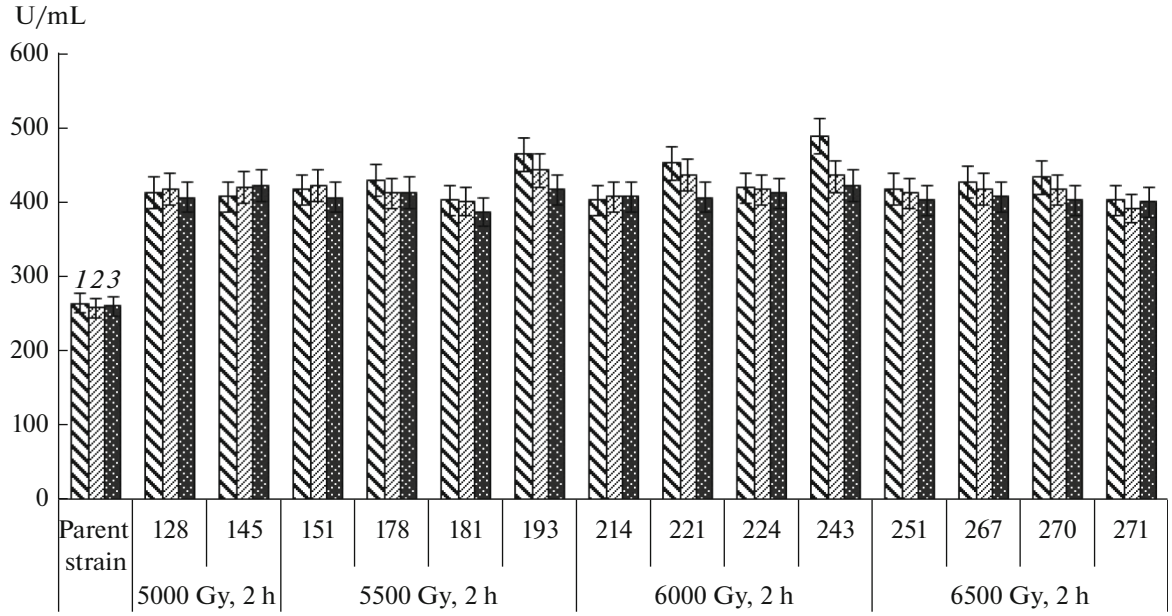

Fig. 1. Preservation of the proteolytic activity (U/mL CL) (stability) of *B. licheniformis*-60 mutant clones during three transfers on the PA medium: the first passage (*1*); the second passage (*2*); and the third passage (*3*).

Table 2. Proteolytic activity and protein content in the EP based on the parent and mutant *B. licheniformis* strains

Producer strain	EP	Protease activity, U/g (mL)	Protein, mg/g (mL)	Specific activity, U/mg protein
B. licheniformis-60 (parent strain)	CL	278 ± 12.17	19.13 ± 0.38	14.53 ± 0.86
	Acetone-precipitated CL ultraconcentrate	5790 ± 93.07	261.00 ± 8.19	22.20 ± 0.79
B. licheniformis-145 (mutant strain)	CL	427 ± 7.81	22.77 ± 0.76	18.77 ± 0.83
	Acetone-precipitated CL ultraconcentrate	8654 ± 70.94	298.33 ± 8.14	29.03 ± 0.97

* The table shows the mean values of three measurements indicating the standard deviation.

65°C (Fig. 3b). The pH or temperature range within which the EP exhibited at least 80% of its maximum activity was taken to be the optimum for EP action.

At 25°C, the EP was characterized by high stability in a broad pH range (Fig. 4): proteolytic activity in its aqueous solutions after 24-h incubation at pH 4.5 was observed to decrease by 30%; at pH 10.0, by 7%; at pH 11.0, by 42%.

It is equally important that the Protolicheterm-145 EP possessed relatively high stability. Figure 5 shows that the dynamics of the EP activity decrease during incubation for 2 h at increased temperatures. In the range up to 40°C, its activity almost did not change; at 50°C, 80% of activity was retained; at 60°C, 25%; an increase in temperature to 70°C caused the enzyme to be completely inactivated.

Note that the above characteristics of the Protolicheterm-145 enzyme preparation did not differ from those of Protolicheterm-60.

Assessment of the efficiency of the EP obtained on the basis of the parent and mutant strains of *B. licheniformis* on hydrolysis of the anti-nitritional proteins of extruded soybean meal. The conditions for ESBM hydrolysis by proteases were chosen considering the parameters of a real technological process: high substrate concentrations (20, 35, and 50%); the EP dose, 0.2 mg/g ESBM; the duration of enzymatic treatment, 5 h; the temperature, 50°C; no pH correction; natural initial pH of an aqueous ESBM suspension (pH 6.0–6.2). The efficiency of hydrolysis was assessed by the disappearance in the electropherogram of the bands corresponding to the subunits of the main anti-nitritional soybean proteins glycinin and β-conglycinin (Fig. 6). The glycinin molecule is known to consist of the main polypeptide B with a molecular mass (Mm)

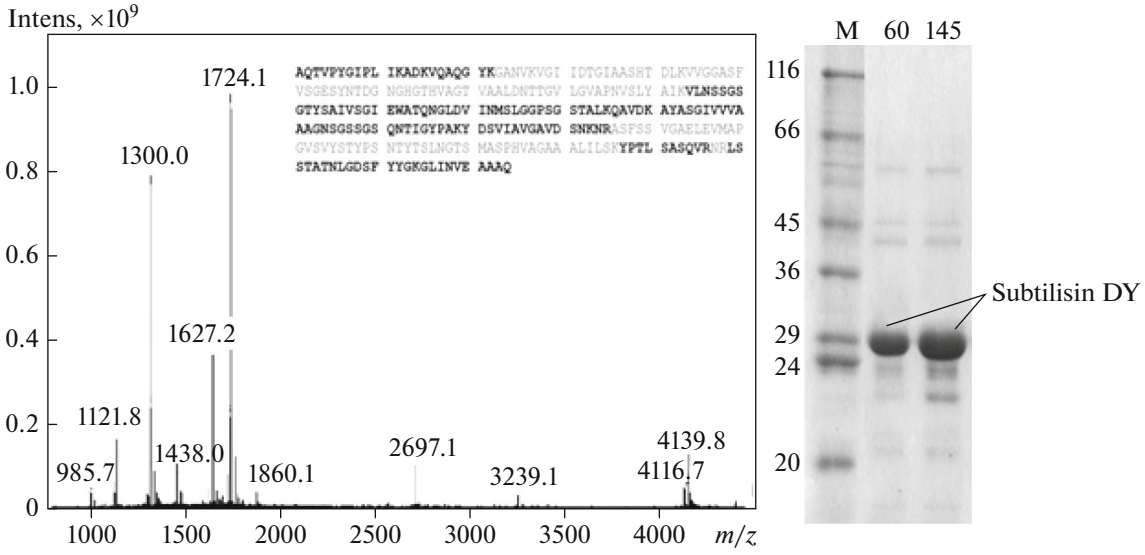

Fig. 2. MALDI-TOF mass spectrum of trypsin hydrolysate of the component corresponding to subtilisin DY (a) and the electropherogram of the Protolicheterm-60 and 145 EP (b). Designations: M, molecular mass marker proteins: β-galactosidase (116 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), and the soybean trypsin inhibitor (20 kDa).

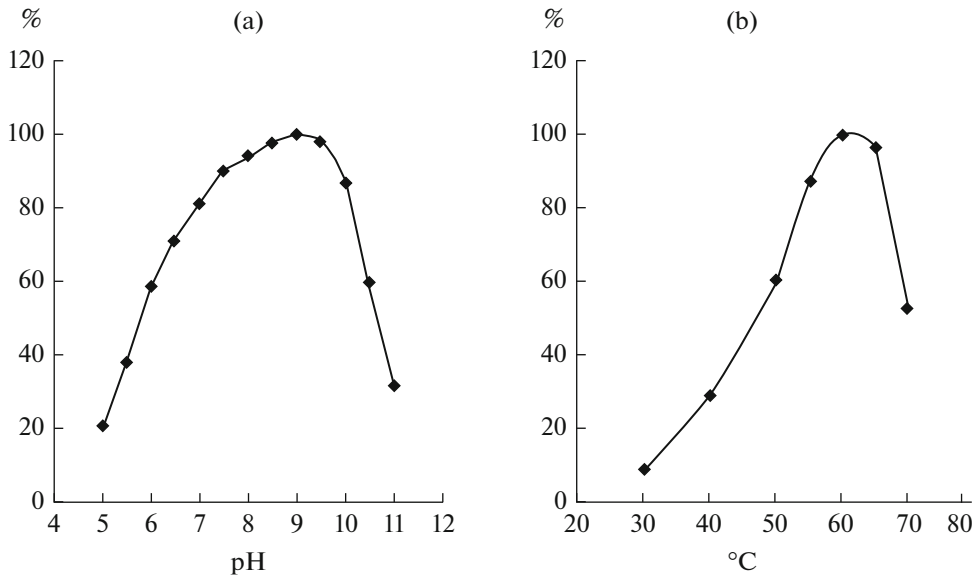

Fig. 3. Proteolytic activity of Protolicheterm-145 depending on pH (a) and temperature (b).

of 20 kDa and the acidic polypeptide A with Mm of about 38 kDa connected by a disulfide bond. β-Conglycinin is a glycoprotein trimer consisting of three subunits: α' (57–72 kDa), α (57–68 kDa), and β (45–52 kDa) (Fischer, 2006). Thus, the completeness of hydrolysis of anti-nitritional proteins can be determined by the absence of protein bands with a molecular mass higher than 15 kDa in the electropherogram.

The data shown in Fig. 6 provide ample evidence that, under the experimental conditions, both EP efficiently hydrolyzed anti-nitritional soybean proteins. The difference between the Protolicheterm-145 and Protolicheterm-60 enzyme preparations was that, when the parent strain-based EP was used, the hydrolysates contained residual amounts of difficultly hydrolysable glycinin subunits as well as the intermediate hydrolysis products with Mm higher than 15 kDa. The amount of non-hydrolyzed high-molecular weight peptides increased with an increase in the substrate concentration to 50%. Owing to its higher proteolytic activity, the EP based on the mutant *B. licheniformis*-145 completely hydrolyzed the ESBM

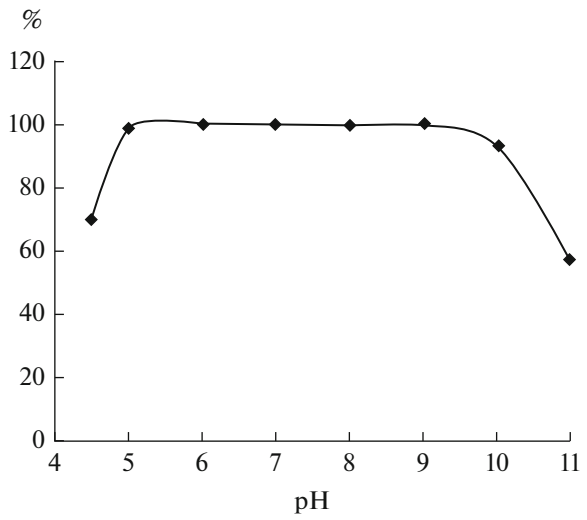

Fig. 4. pH-Stability of the proteolytic activity of the Protolikheterm-145 EP after incubation of its aqueous solutions for 24 h at 25°C.

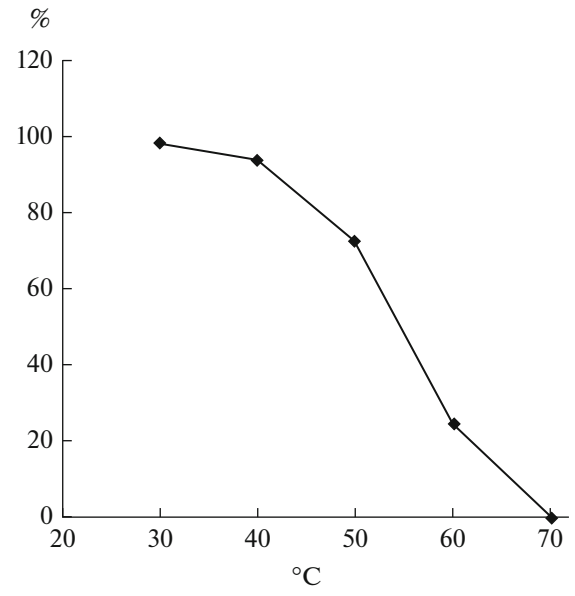

Fig. 5. Effect of temperature on stability of the proteolytic activity of the Protolicheterm-145 EP during incubation of its aqueous solutions (pH 6.0) for 120 min.

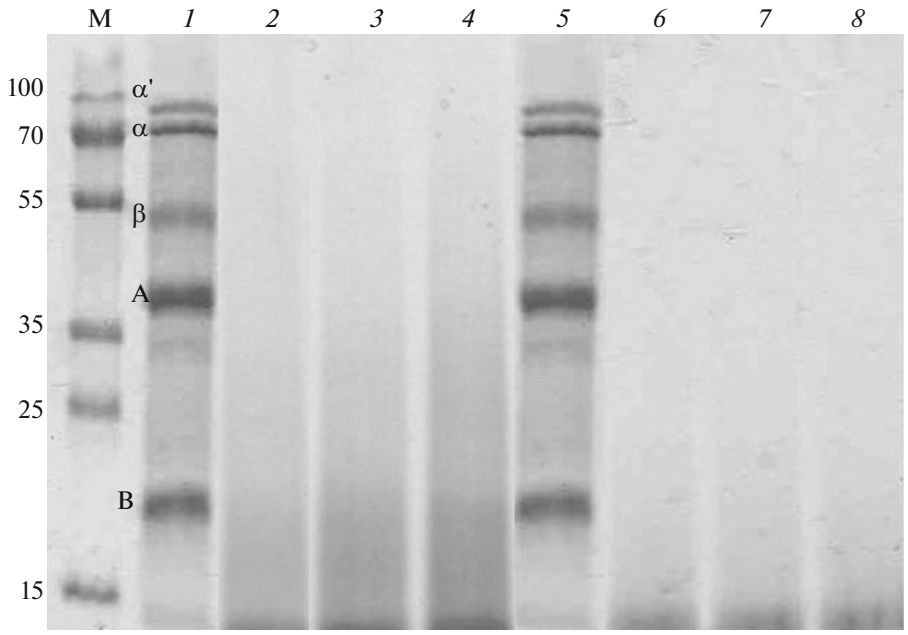

Fig. 6. Electropherogram of the hydrolysates obtained by the action of the Protolicheterm-60 EP on ESBM suspensions at a concentration of 20% (*2*), 35% (*3*), and 50% (*4*) and by the action of Protolicheterm-145 on suspensions at a concentration of 20% (*6*), 35% (*7*), and 50% (*8*). Control: ESBM without enzymatic treatment (*1*, *5*). The protein molecular mass markers PageRuler™ 10–170 kDa (M).

protein to peptides with Mm below 15 kDa, irrespective of the substrate concentration. These data are confirmed by the results of determination of the soluble protein content in hydrolysates (Table 3). The soluble protein yield in the hydrolysates obtained increased 6.2–7.3-fold in relation to non-hydrolyzed ESBM when the parent strain-based EP was used and 8.0–8.7-fold in the case of the mutant strain-based EP. Compared to the preparation obtained using the parent strain, Protolicheterm-145 increased the soluble protein yield by 19–28% (depending on the substrate concentration).

Thus, the serine protease EP obtained from the new highly productive mutant strain *B. licheniformis*-145 exhibited a higher protease activity compared to the parent strain-based Protolicheterm-60. Importantly, an obvious advantage of Protolicheterm-145 in hydrolysis of highly concentrated ESBM suspensions makes its use promising in resource-saving technologies for processing soybean meals and cakes.

The use of the highly active Protolicheterm-145 EP is most promising in the processes of recycling secondary protein-containing material for the production of valuable products with predetermined properties in the food and feed industries.

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Table 3. Soluble protein yield upon ESBM hydrolysis with *B. licheniformis*-60- and *B. licheniformis*-145-based enzyme preparations

ESBM concentration	Soluble protein, mg/g		Increase in the soluble protein yield due to the use of the mutant strain-based EP, %
	parent *B. licheniformis*-60-based EP (control)	mutant *B. licheniformis*-145-based EP	
20%	186 ± 6.25[a]	221 ± 7.21[a]	119 ± 3.6[a]
35%	175 ± 3.60[a]	217 ± 8.19[ab]	124 ± 2.7[ab]
50%	158 ± 7.55[b]	203 ± 4.36[b]	129 ± 4.0[b]
EP-free ESBM (control)	25.4 ± 0.46		

* The table shows the mean values of three measurements indicating the standard deviation.
** The data in each column with different letter indexes are significantly different at the level of significance $\alpha = 0.05$.

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