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Purification and Characterization of a Subtilisin-Like Proteinases Secreted in the Stationary Growth Phase of *Bacillus amyloliquefaciens* H2

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Abstract—Proteinases secreted during the early and late stationary phases have been isolated from the culture liquid of *Bacillus amyloliquefaciens* H2 using CM-cellulose ion-exchange chromatography with subsequent FPLC on a Mono S column. Considering the character of hydrolysis of specific chromogenic substrates and the type of inhibition, these enzymes were identified as subtilisin-like proteinases. The molecular weight of both proteinases is 29 kD. The proteolytic activity of the proteinases secreted during the early and late stationary phases towards the synthetic substrate Z-Ala-Ala-Leu-pNA was maximal at pH 8.5 and 9.0, respectively. The maximal activity of both proteinases was observed at 37°C, and the proteins were stable within the pH range of 7.2-9.5. The subtilisin-like proteinases from *B. amyloliquefaciens* were shown to catalyze synthesis of peptide bonds.

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Key words: subtilisin-like proteinases, *Bacillus amyloliquefaciens*, purification, properties

Bacteria produce different extracellular enzymes, among which the most studied group is the family of serine proteinases—subtilases, or subtilisin-like proteinases [1, 2]. The genes of many bacterial subtilases have been cloned and sequenced; the structure and properties of the enzymes have been studied in detail and for some proteins, and the spatial structure has been determined [2-7]. However, the functional role of the subtilisin-like proteinases in the cell remains unclear. Usually, the proteinases secreted in the beginning of the stationary phase of bacterial growth are investigated. There is virtually no information in the literature on the synthesis of these enzymes in the late growth phases. Previously, it was shown that during the growth of *Bacillus intermedius*, the cells release into the culture liquid a subtilisin-like proteinase whose content is maximal in the beginning of the stationary growth phase and at the late stationary growth phase (stages V and VI of spore formation) [8, 9]. Both fractions of the enzyme have been isolated in the homogeneous state, and their physicochemical and enzymatic properties have been determined [10-12]. The proteinases isolated in the early and late stationary growth phases are virtually identical in terms of their specificity and physicochemical properties. The comparative characteristic of the subtilisin-like proteinases secreted in different growth phases of different species of bacteria may reveal the functions of these proteins in the metabolism of the bacteria.

The goal of the present study was isolation of the subtilisin-like proteinases secreted by the cells of *B. amyloliquefaciens* H2 in the early and late stationary growth phases and comparison of their physicochemical properties and the ability to synthesize peptide bonds.

MATERIALS AND METHODS

Bacterial strain. The culture of *B. amyloliquefaciens* H2 was from the collection of the Department of Microbiology of Kazan State University. The bacterium was grown on the medium described earlier [8] at 30°C and 200 rpm. The medium volume/flask volume ratio was

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1 : 5. The culture liquid was freed from the cells by centrifugation (60 min, 4500*g*, 4°C).

Proteolytic activity was determined with the use of the synthetic chromogenic substrate Z-Ala-Ala-Leu-pNA [13] synthesized using Houmard's method [14] at the Chemical Faculty of Moscow State University. The unit of the enzyme activity was determined as the amount of the enzyme hydrolyzing 1 μmol of the substrate per minute.

Protein concentration was determined spectrophotometrically assuming that the concentration of 1 mg/ml corresponds to $A_{280} = 1$ in a 1-cm cuvette.

Isolation of the enzyme. The enzyme was isolated from the culture liquid of *B. amyloliquefaciens* as described earlier for the subtilisin-like proteinase from *B. intermedius* [10]. The culture liquid (2 liters), pH 6.3, was diluted ten-fold with water and added to CM-cellulose (Sigma, USA) equilibrated with 0.02 M Na-acetate buffer, pH 6.3, containing 0.5 mM $CaCl_2$. Then the CM-cellulose was poured into a column (17 × 1.5 cm) and washed with the same buffer. The proteins were eluted with 0.2 M Na-acetate buffer, pH 6.3, containing 0.5 mM $CaCl_2$. The eluate was diluted 10-fold with water and applied to a Mono S 5/5 FPLC column (Pharmacia, Sweden) equilibrated with 0.02 M Na-acetate buffer, pH 6.3, containing 0.5 mM $CaCl_2$. The proteins were eluted with a 0-0.5 M linear NaCl gradient in the same buffer at 1 ml/min. The fractions, that were active towards the substrate Z-Ala-Ala-Leu-pNA, were collected and used in the subsequent work. The protein was rechromatographed on the Mono S column in a similar way.

Purity and molecular weights of the enzymes were determined electrophoretically in 12.5% polyacrylamide gel in the presence of 0.1% SDS using the following proteins standards: hen egg ovalbumin (45 kD), carboanhydrase from bovine erythrocytes (29 kD), soybean trypsin inhibitor (23 kD), and α-lactalbumin (16.2 kD).

Influence of inhibitors. Diisopropyl fluorophosphate (DFP) (5 mM), *o*-phenanthroline (1 and 3 mM), phenylmethylsulfonyl fluoride (PMSF) (1 and 3 mM), mercury chloride (1, 3, and 5 mM), *p*-chloromercuribenzoate (p-CMB) (1 and 3 mM), duck ovomucoid, soybean trypsin inhibitor (Sigma), sea anemone inhibitor, ovalbumin, potato inhibitor, and leupeptin (2 mg/ml) were used as the inhibitors. The proteinases were incubated with the inhibitors at 23°C for 1 h, and then the residual activity towards Z-Ala-Ala-Leu-pNA was determined under standard conditions.

Substrate specificity. Specificity of the enzymes was determined with the synthetic di- and tripeptides Z-D-Ala-Leu-pNA, Glp-Ala-Phe-pNA, Glp-Ala-Ala-pNA, Z-Gly-Ala-Phe-pNA, Z-Ala-Ala-Phe-pNA, and Pyr-Phe-Ala-pNA using the method for determination of the proteolytic activity with the substrate Z-Ala-Ala-Leu-pNA [13]. The substrate specificity of the subtilisin-like proteinases was also determined using natural substrates ovalbumin, globin, azocasein, and azoalbumin. The activity of the proteinases towards 1% ovalbumin and 1% globin (Serva, Germany) was determined in 0.1 M Tris-HCl buffer, pH 9.0. The reaction mixture contained 0.5 ml of the substrate, 0.25 ml of 0.1 M Tris-HCl buffer, pH 9.0, and 0.25 ml of an enzyme solution. The mixture was incubated 20 min at 37°C, and then supplemented with 0.1 ml of 50% trichloroacetic acid. After filtration, A_{280} of the resulting filtrate was measured. The control sample (0.25 ml of the enzyme solution, 0.25 ml of the buffer, and 0.1 ml of 50% trichloroacetic acid) was incubated 20 min at 37°C, and then A_{280} of the resulting filtrate was measured.

The activity of the enzymes towards azocasein and azoalbumin was determined by hydrolysis of 0.5% substrate solution in 0.05 M Tris-HCl, pH 7.0, containing 1 mM $CaCl_2$. The reaction mixture contained 0.5 ml of the substrate, 0.495 ml of 0.1 M Tris-HCl buffer, pH 9.0, and 5 μl of the enzyme solution. After 5-min incubation at 37°C, 0.1 ml of 50% trichloroacetic acid was added to the reaction mixture. The solution was filtered, and 0.5 ml of the filtrate was supplemented with 2.5 ml of 6% Na_2CO_3. The absorption of the resulting solution was measured at 410 nm. The unit of the activity was determined as the amount of the enzyme that was necessary to increase A_{410} by 1 unit per minute.

The specificity of the enzyme towards B-chain of oxidized insulin was determined as described earlier [11]. A solution of the substrate (1 mg/ml in 0.02 M $NaHCO_3$, pH 8.5) was supplemented with 10 μl of the enzyme solution (1 mg/ml in the same buffer) and incubated for 4 h at 37°C. The dried hydrolyzates were separated by HPLC on an Ultrasphere Octyl column (4.6 × 250 mm) using a 0-70% gradient of acetonitrile containing 0.1% CF_3COOH. Absorption of the eluates was measured at 215 and 280 nm, and their composition was analyzed using a Hitachi 835 amino acid analyzer (Hitachi, Japan).

Enzymatic characteristic. Enzymatic properties of the proteins were investigated using the synthetic chromogenic substrate Z-Ala-Ala-Leu-pNA. The pH dependence of the proteinase activity was investigated using the substrate diluted in 0.05 M Tris-HCl buffer in the range of pH 7.2-9.5. To study the effect of pH on the protein stability, the enzymes were incubated for 2 h at room temperature in 0.05 M Tris-HCl buffer within the range of pH 7.2-9.5 in the presence and in the absence of 0.5 mM $CaCl_2$, and then the activity was determined as described above. The activity of the enzyme in the presence of Ca^{2+} without preincubation was taken as 100%. To determine the optimal temperature of the reaction, the reaction mixture was incubated at 22-65°C in the presence and in the absence of 0.5 mM $CaCl_2$. To investigate the temperature stability, the enzymes were heated during 1 h at 22, 37, 45, 50, and 55°C in the presence and in the absence of 0.5 mM $CaCl_2$, and then the activity was

determined as described above. The activity of the enzyme without heating was taken as 100%.

Synthesis of peptide bonds in the presence of the proteinases. Enzymatic synthesis of the peptide Z-Ala-Ala-Leu-pNA was performed as follows: the reaction mixture contained 31 mg (0.1 mM) of Z-Ala-Ala-OCH_3 (Sigma), 25 mg (0.1 mM) of Leu-pNA (Sigma), 0.1 ml of dimethylformamide, 0.01 ml of ethyl acetate, and 0.08 ml of an enzyme solution (2 μg protein). The solution was incubated at room temperature for 2 h under constant stirring. The resulting precipitate was dissolved in 2 ml of ethyl acetate and washed with 3% $NaHCO_3$ (3 times with 1 ml), water (2 times with 1 ml), 0.5 M HCl (3 times with 1 ml), and again with water (2 times with 1 ml). The obtained solution of Z-Ala-Ala-Leu-pNA in ethyl acetate was evaporated. The purity of the peptide was tested using thin-layer chromatography in the systems benzene–acetone–acetic acid (100 : 28 : 4) and chloroform–ethanol (4 : 1). The chromatograms were assayed for admixtures using the qualitative reactions for free groups of *p*-nitroanilide (pNA) (treatment with ninhydrin).

Statistical analysis of the results was made using Microsoft Excel. The root-mean-square deviations (σ) were calculated. The results were considered to be significant when $\sigma \leq 15\%$.

RESULTS AND DISCUSSION

The dynamics of the accumulation of the proteolytic activity in the culture liquid of *B. amyloliquefaciens* was investigated during the bacterial growth including the stationary phase. The proteolytic activity appears in the culture liquid on the growth deceleration phase, reaching its maximal value in the stationary phase (28th and 46th hour of growth for proteinases 1 and 2, respectively) (Fig. 1).

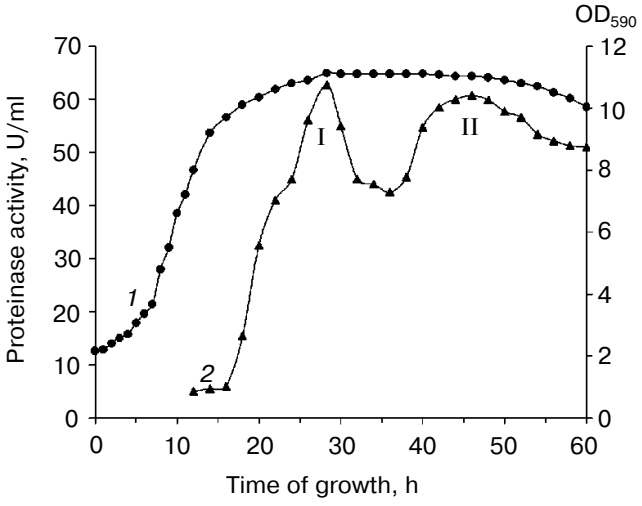

Fig. 1. Dynamics of the culture growth (*1*) and biosynthesis of subtilisin-like proteinases (*2*) secreted by the bacteria *Bacillus amyloliquefaciens* H2: I) proteinase 1; II) proteinase 2.

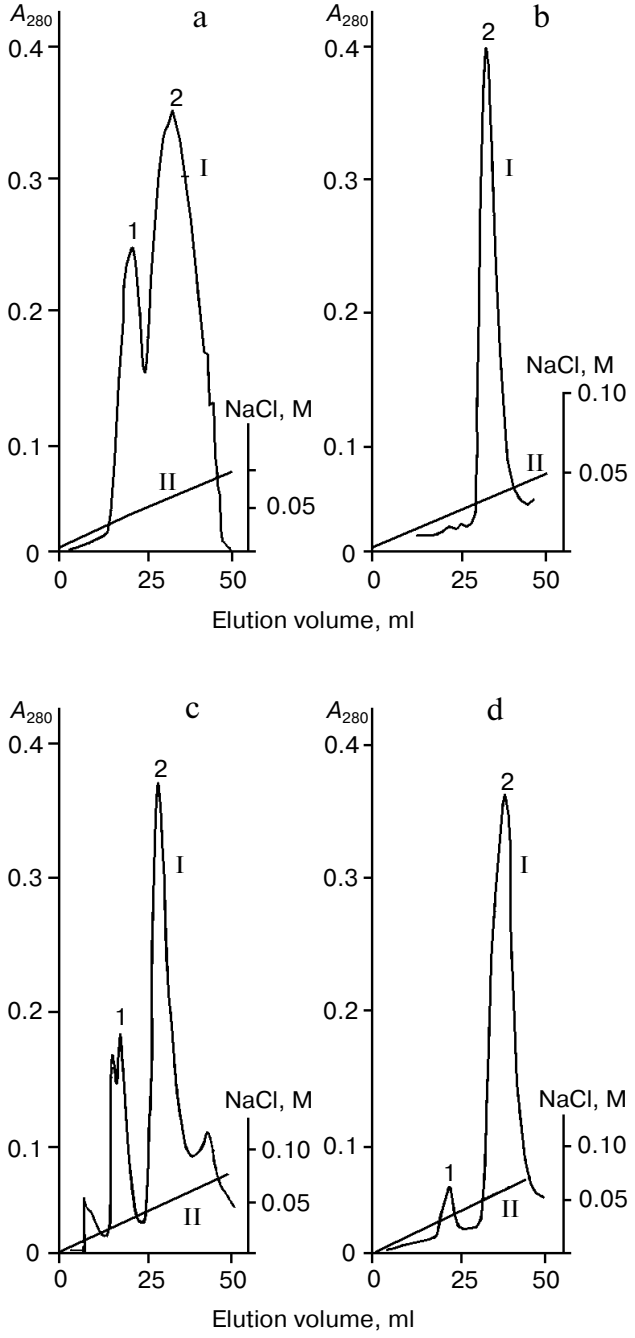

Fig. 2. Chromatographic profiles of the serine proteinases from *B. amyloliquefaciens* isolated in the early (a, b) and late (c, d) stationary growth phases. Chromatography (a, c) and re-chromatography (b, d) of the proteinases on a Mono S column are shown: I) A_{280}; II) 0-0.5 M NaCl gradient.

Proteolytic enzymes of the early and late stationary growth phases were isolated from the culture liquid of *B. amyloliquefaciens* using ion-exchange chromatography on CM-cellulose and HPLC on a Mono S column. Figure 2 presents chromatographic profiles of the pro-

Table 1. Isolation of proteinases from *B. amyloliquefaciens*

Purification stage	Volume, ml	Protein, mg	Specific activity, U/mg	Yield, %
Culture liquid				
Proteinase 1	1875	35 100	0.07	100
Proteinase 2	1900	38 000	0.039	100
CM-cellulose chromatography				
Proteinase 1	28.5	68.4	11.7	32
Proteinase 2	33	57.8	10.3	40.2
Mono S chromatography				
Proteinase 1	17.5	19.6	12.8	10.1
Proteinase 2	15.5	11.2	17.4	13.2
Mono S re-chromatography				
Proteinase 1	8.5	7.8	19.9	6.2
Proteinase 2	3.7	2	34	4.6

teinases of *B. amyloliquefaciens* isolated in different growth phases of the bacteria. The proteins eluted from the column at NaCl concentration of 0.55 mM (Figs. 2a and 3a) were identified with the use of the specific chromogenic substrate Z-Ala-Ala-Leu-pNA as subtilisin-like proteinases. Re-chromatography yielded homogeneous proteinases 1 and 2 of *B. amyloliquefaciens* (Figs. 2b and 2d). The results of three-step purification of the enzymes are presented in Table 1. Proteinase 1 was isolated with the purification degree of 284 and the yield of 6.2%, and for proteinase 2 these parameters were 872 and 4.6%, respectively.

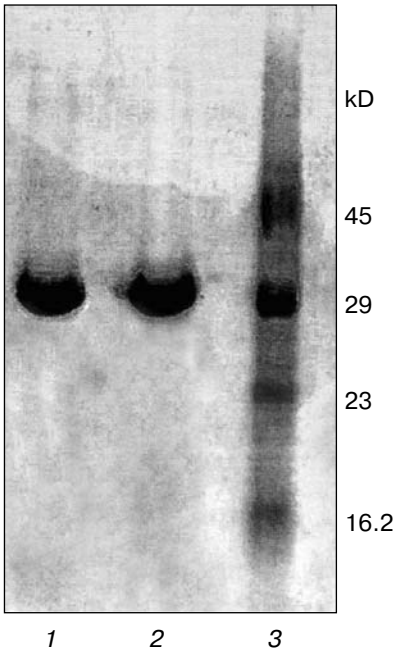

Fig. 3. SDS-PAGE of proteinases from *B. amyloliquefaciens*: *1*) proteinase 1; *2*) proteinase 2; *3*) protein standards: hen egg ovalbumin (45 kD), carboanhydrase from bovine erythrocytes (29 kD), soybean trypsin inhibitor (23 kD), and α-lactalbumin (16.2 kD).

Purity of the isolated proteins and their molecular weights were determined in 12.5% polyacrylamide gel under denaturing conditions (Fig. 3). As seen from the figure, each of protein fractions contains one polypeptide of 29 kD. As known from literature data, molecular weights of the subtilisin-like proteinases isolated from *Bacillus* vary in the range 27-32 kD [1]. For example, the classic subtilisins BPN′ and Carlsberg have molecular weights of 27.5 and 27.3 kD, respectively [1, 5]. The molecular weights of the subtilisin-like proteinases from *B. intermedius* were found to be 32.5 and 28 kD for the early and late stationary growth phase, respectively [10, 12].

The effect of proteolytic enzyme inhibitors on the activity of proteinases 1 and 2 of *B. amyloliquefaciens* was investigated. Specific inhibitors of serine proteinases DFP and PMSF completely inhibited the activity of the purified enzymes of *B. amyloliquefaciens*. The specific inhibitor of metalloproteinases (*o*-phenanthroline), compounds reacting with thiol groups ($HgCl_2$ and *p*-chloromercuribenzoate), and natural protein inhibitors did not affect the activity of the proteinases. This suggests the absence of cysteine residues in the proteinases, which is characteristic for classic bacterial subtilisins. Thus, considering the effects of the inhibitors, the isolated proteinases of *B. amyloliquefaciens* can be assigned to the family of the subtilisin-like serine proteinases [2].

The substrate specificity of the subtilisin-like proteinases 1 and 2 was determined by the hydrolysis of chro-

Table 2. Substrate specificity of the subtilisin-like proteinases from *B. amyloliquefaciens*

Substrate	Specific activity, U/mg	
	proteinase 1	proteinase 1
Z-D-Ala-Leu-pNA	0.8	1
Glp-Ala-Phe-pNA	0.06	0.02
Glp-Ala-Ala-pNA	31	36
Pyr-Phe-Ala-pNA	0.1	0.2
Z-Ala-Ala-Leu-pNA	11	19
Z-Gly-Ala-Phe-pNA	0	0
Z-Ala-Ala-Phe-pNA	0	0
Ovalbumin	0.01	0.01
Globin	0.01	0.01
Azocasein	1.9	2
Azoalbumin	0.4	0.3

mogenic synthetic di- and tripeptides, as well as some natural substrates (Table 2). Both proteinases exhibited their maximal activity towards the synthetic dipeptide Glp-Ala-Ala-pNA and tripeptide Z-Ala-Ala-Leu-pNA containing the residues Ala and Leu in P1 position, respectively. The Ala residue presumably plays a determinative role in the reaction of hydrolysis. The subtilisin-like proteinases 1 and 2 hydrolyze the substrates containing Ala in P1 position (Glp-Ala-Ala-pNA) 3- and 2-fold, respectively, faster than the substrate containing Leu in P1 position (Z-Ala-Ala-Leu-pNA). The peptide containing Phe in the P1 position is the worst substrate for these enzymes (Glp-Ala-Phe-pNA). Among natural substrates such as azocasein, azoalbumin, globin, and ovalbumin, azocasein is the best substrate for the subtilisin-like proteinases of *B. amyloliquefaciens*.

The substrate specificity of the subtilisin-like proteinases isolated in different growth phases of *B. amyloliquefaciens* was also estimated by hydrolysis of the B-chain of oxidized insulin (Fig. 4). Unlike the subtilisin-like proteinase 1, proteinase 2 additionally hydrolyzes in this molecule the bonds F1-V2, Y26-T27, and T27-P28. The investigated proteinases exhibit wide substrate specificity, since the hydrolysis yields many peptide fragments. Both proteinases hydrolyze bonds formed by the carboxyl groups of the hydrophobic amino acids glycine, leucine, and phenylalanine (G8-S9, G20-E21, L11-V12, L17-V18, F24-F25, etc.) as well as the hydrophilic amino acids serine, glutamine, and tyrosine (Q4-H5, Y16-L17, etc.). The character of the hydrolysis of the insulin B-chain by the investigated proteinases from *B. amyloliquefaciens* differs from that of the subtilisin-like proteinase 1 from *B. intermedius* that hydrolyzes more bonds in the insulin molecule [11]. However, compared with other known proteinases like subtilisin BPN′, proteinase K, and thermitase, the subtilisin-like proteinases of *B. amyloliquefaciens* appeared to be similar to the subtilisin-like proteinase 2 of *B. intermedius* and proteinase K of the fungus *Tritirachium album* in terms of substrate specificity (Fig. 4) [15-18].

Investigation of enzymatic properties of the proteinases demonstrated that the maximal activity of the enzymes towards the synthetic chromogenic substrate Z-Ala-Ala-pNA was observed at pH 8.5 for proteinase 1 and at pH 9.0 for proteinase 2. Both enzymes are stable in the wide range of pH from 7.2 to 9.5, the activity increasing in the presence of 0.5 mM Ca^{2+} by 20-25%. Optimal pH values for the activity of the proteinases of *B. amyloliquefaciens* are the same as for the proteinases of *B. intermedius* [19]. Interestingly, for the classic subtilisins BPN′ and Carlsberg, the pH optimum is shifted to the alkaline region, to 10.5, but BPN′ is stable within the pH range of 7-8 [5]. The optimal temperature for the subtilisin-like proteinases 1 and 2 of *B. amyloliquefaciens* is 37°C, this being significantly lower than for the subtilisins BPN′ and Carlsberg (60°C) and for the subtilisin-like proteinases 1 and 2 of *B. intermedius* (50 and 55°C) [20, 21].

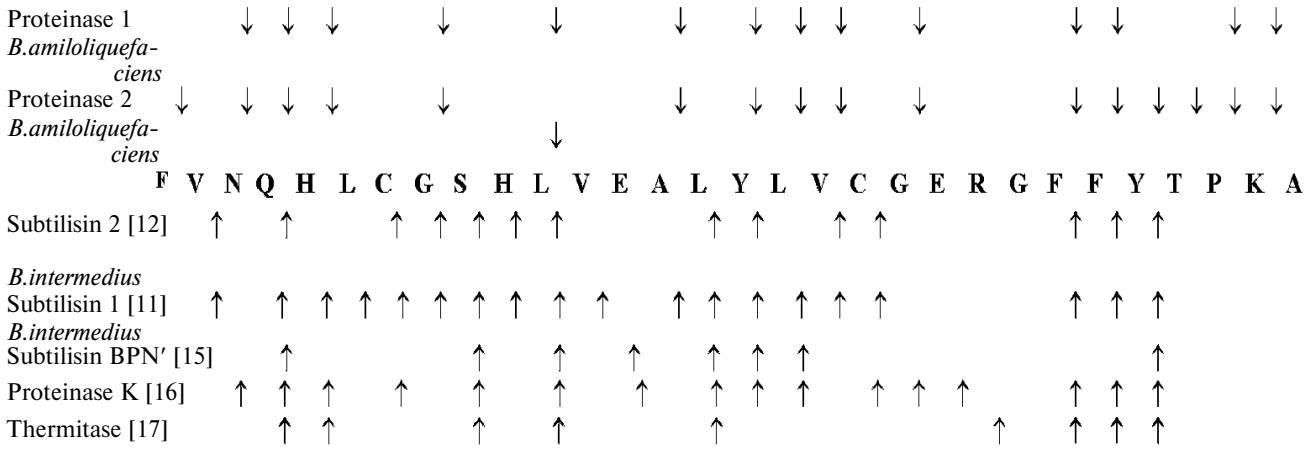

Fig. 4. Hydrolysis of B-chain of insulin by the subtilisin-like proteinases of microorganisms.

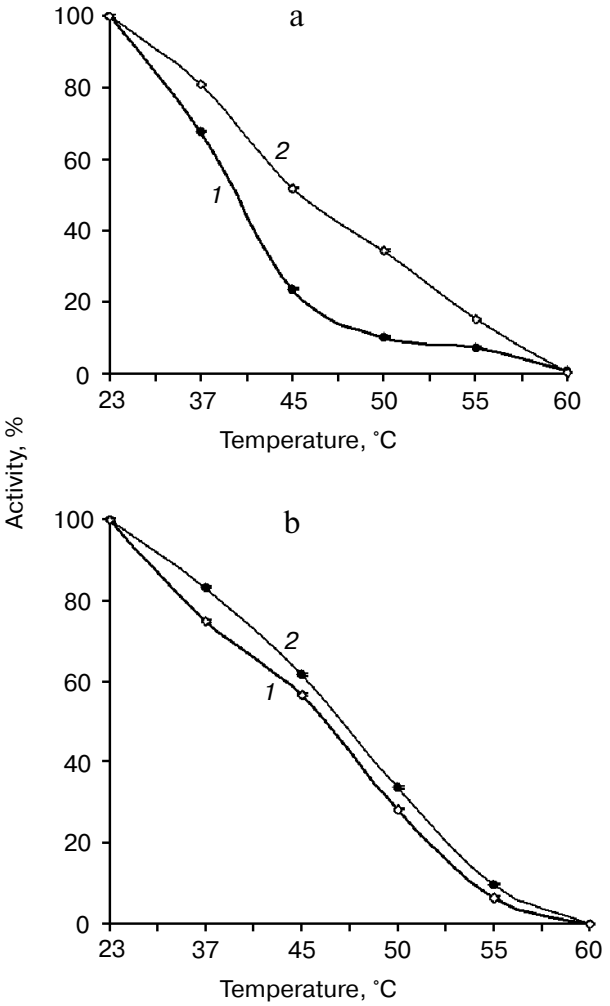

Fig. 5. Thermostability of subtilisin-like proteinases 1 (a) and 2 (b) of *B. amyloliquefaciens* in the presence (*1*) and in the absence (*2*) of Ca^{2+}.

Stabilization of the structure of bacterial proteinases is provided by hydrophobic and ionic interaction, as well as hydrogen bonds and disulfide bridges [22]. Analysis of the known tertiary structures of subtilisins revealed two or three Ca^{2+}-binding sites involved in the stabilization of the proteinase molecules [1]. The investigated enzymes of *B. amyloliquefaciens* do not exhibit high thermostability (Fig. 5). After heating the enzymes for 1 h at 37°C, the activity of proteinases 1 and 2 decreased by 34 and 20%, respectively. In the presence of Ca^{2+}, the thermostability of both proteins increases, this presumably being due to the stabilization of the protein structure. Heating of proteinase 1 for 1 h at 45°C in the absence of Ca^{2+} significantly (by 75%) decreases the enzyme activity, while in the presence of Ca^{2+} the activity decreases only by 50%. Proteinase 2 is more resistant to high temperatures. Heating of proteinase 2 for 1 h at 45°C in the presence or in the absence of Ca^{2+} decreases the enzyme activity by 35%. A further increase in the temperature (above 50°C) results in the complete inactivation of the enzyme. Subtilisin-like proteinases of *B. intermedius* are less sensitive to higher temperature: a significant decrease in the activity of proteinases 1 and 2 was observed after heating of the enzymes at 50 and 55°C, respectively. Ca^{2+} was shown to increase the thermostability of these enzymes [10, 12].

The subtilisin-like proteinases of *B. amyloliquefaciens* were shown to catalyze synthesis of the tripeptide Z-Ala-Ala-Leu-pNA from carbobenzoxy-alanyl-alanine methyl ester (Z-Ala-Ala-OCH_3) and *p*-nitroanilide-leucine (Leu-pNA). The product of the reaction, tripeptide Z-Ala-Ala-Leu-pNA, was less soluble in the reaction mixture and precipitated. This resulted in a shift of the equilibrium in the direction of the synthesis. Using the subtilisin-like proteinases 1 and 2 of *B. amyloliquefaciens*, 24.2 and 21.5 mg of the tripeptide Z-Ala-Ala-Leu-pNa were obtained, the yield of the product being 46 and 41%, respectively. There is information concerning the catalysis of the analogous reactions by serine proteinases of *B. subtilis* [23] and *Asp. oryzae* [24]. Using the subtilisin-like proteinase of *B. licheniformes*, tri- and tetrapeptides were synthesized containing not only hydrophobic, but also oppositely charged amino acid residues [25]. Thus, the subtilisin-like proteinases of *B. amyloliquefaciens* can be used as catalysts of peptide bond formation. The enzymatic synthesis of peptides is a promising direction of modern organic chemistry.

Thus, the subtilisin-like proteinases secreted by the culture of *B. amyloliquefaciens* in different growth phases were isolated and purified. The enzymes of different growth phases do not exhibit significant differences in enzymatic properties and substrate specificity. Previously, it was shown that the subtilisin-like proteinases of *B. intermedius* secreted in the different growth phases are products of the same gene, whose expression is regulated by different mechanisms depending on the growth phase of the bacteria [19, 26]. It can be assumed that the enzymes of *B. amyloliquefaciens* secreted in the early and late phases of the culture growth, as the subtilisin-like proteinases of *B. intermedius*, are products of the same gene. The enzymes secreted in the early and late stationary growth phases of *B. amyloliquefaciens* can be the isoforms of the same protein that insignificantly differ in their properties, this being due to the changes in the regulatory mechanisms of biosynthesis of the corresponding enzyme in the late stationary phase during the period of the cell differentiation of bacteria.

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