ISSN 0006-2979, Biochemistry (Moscow), 2007, Vol. 72, No. 2, pp. 192-198. © Pleiades Publishing, Ltd., 2007.
Original Russian Text © E. O. Mikhailova, A. M. Mardanova, N. P. Balaban, G. N. Rudenskaya, M. R. Sharipova, 2007, published in Biokhimiya, 2007, Vol. 72, No. 2, pp. 228-235.

Isolation and Characterization of a Subtilisin-Like Proteinase of *Bacillus intermedius* Secreted by the *Bacillus subtilis* Recombinant Strain AJ73 at Different Growth Stages

E. O. Mikhailova[1]*, A. M. Mardanova[1], N. P. Balaban[1], G. N. Rudenskaya[2], and M. R. Sharipova[1]

[1]*Kazan State University, ul. Kremlevskaya 18, 420008 Kazan, Russia; fax: (843) 238-7121; E-mail: katya_o_m@rambler.ru*
[2]*Chair of Natural Compound Chemistry, Faculty of Chemistry, Lomonosov Moscow State University, 119992 Moscow, Russia; fax: (495) 939-3188; E-mail: laboratoriahps@hotmail.com*

Received July 3, 2006
Revision received September 25, 2006

Abstract—Two subtilisin-like serine proteinases of *Bacillus intermedius* secreted by the *Bacillus subtilis* recombinant strain AJ73 (pCS9) on the 28th and 48th h of culture growth (early and late proteinase, respectively) have been isolated by ion-exchange chromatography on CM-cellulose and by FPLC. Molecular weights of both proteinases were determined. The N-terminal sequences of the recombinant protein and mature proteinases of the original strain were compared. Kinetic parameters and substrate specificities of the early and late proteinase were analyzed. Physicochemical properties of the enzymes were studied.

DOI: 10.1134/S0006297907020095

Key words: subtilisin-like proteinases, mass spectra, substrate specificity, Michaelis constant

Subtilisin-like serine proteases (subtilases) are among the most intensively studied enzymes. They are interesting not only in relation to their commercial significance [1], but also because of their wide use in fundamental research. At present, data are available on more than 200 enzymes of this family [2]. Subtilisins have been found in organisms at different evolutionary steps: viruses, archaea, bacteria, fungi, and higher eukaryotes [3]. These enzymes perform various functions. They are involved in embryogenesis of eukaryotes and also in some diseases from Alzheimer's disease and cancer to Ebola fever and AIDS [4, 5]. In the cells of prokaryotes, they are involved in cell differentiation and adaptation to unfavorable conditions of the environment. Despite the great phylogenetic distance between the organisms, the structure and functions of serine proteinase synthesized by them have much in common, and this suggests the evolutionary relationship of these enzymes. The catalytic triad of the active site of this family of enzymes includes Asp-His-Ser. Subtilases are synthesized intracellularly as precursors, preproenzymes, which consist of a signaling peptide responsible for the protein secretion, a propeptide, and the mature enzyme itself [6]. The length of these components varies and is specific for every enzyme. Propeptides are supposed to perform chaperon-like functions during the folding of the mature protein [7] and suppression of proteolytic activities of the enzymes, which can be important for defense of the host bacteria against accidental proteolytic damage [8]. The bacterial subtilisins are strong alkaline enzymes including both thermostable enzymes and those resistant to low temperatures. Molecules of many subtilisins have calcium-binding sites, but calcium ions are not involved in catalysis—they only stabilize the enzyme molecule (as differentiated from calcium ions in metalloproteinases) [9]. It seems rather timely to isolate new bacterial subtilisin-like proteinases, obtain information about the biochemical properties of proteins in living systems (expression level, post-translational modifications), and elucidate the pattern of changes in the protein conformation caused by synthesis and assemblage of proteins during different growth stages of bacteria.

A subtilisin-like proteinase was earlier isolated from the culture fluid of *Bacillus intermedius*, and its physico-

Abbreviations: PMSF) phenylmethylsulfonyl fluoride; DMFA) dimethylformamide; Z) benzyloxycarbonyl; pNa) *p*-nitroanilide.

* To whom correspondence should be addressed.

chemical properties were described [10]. The gene of the protein was cloned into the multicopy plasmid pCS9 [11], and its expression was studied in the recombinant strain of *Bacillus subtilis*. The maximum accumulation of the enzyme of *Bacillus intermedius* in the culture fluid of *Bacillus subtilis* AJ73 was recorded by the 28th and 48th h of culture growth and depended on the nutrient medium composition [12]. The gene sequence has been published in the International GeneBank with the accession code AY754946.

The purpose of this work was to purify and characterize the subtilisin-like serine proteinase of *B. intermedius* secreted at different growth stages by the *B. subtilis* recombinant strain AJ73 transformed by the multicopy plasmid pCS9, which contains the gene of this enzyme.

MATERIALS AND METHODS

The *B. subtilis* AJ73 strain used in this work carries the gene of the subtilisin-like serine proteinase of *B. intermedius* incorporated into the multicopy plasmid pCS9. The *B. subtilis* recombinant strain was cultured for 28 (peak 1) and 48 h (peak 2), as described in [12]. Subtilisin-like proteinases of *B. intermedius* corresponding to these peaks were isolated from 1 liter of the *B. subtilis* culture fluid by ion-exchange chromatography on CM-cellulose and by FPLC on a Mono S column. In the cell-free culture, fluid pH was adjusted to 6.3. The supernatant was diluted tenfold in distilled water and added to CM-cellulose equilibrated with 0.015 M sodium acetate buffer (pH 6.3). The enzyme was absorbed during stirring in the CM-cellulose volume for 60 min. The CM-cellulose was collected, placed onto a column, washed in the same buffer, and the protein was eluted with 0.2 M sodium acetate buffer (pH 6.3). The fractions were used for determination of A_{280} and the specific activity. The protein was determined spectrophotometrically assuming, that the protein concentration of 1 mg/ml corresponded to A_{280} equal to one unit (in a cuvette of 1 cm thickness), and also by Bradford's method [13]. Fractions with high proteolytic activity were combined, diluted 12-fold in water, and placed onto a Mono S column in an FPLC system (Pharmacia, Sweden) equilibrated with 0.015 M sodium acetate buffer (pH 6.3) supplemented with 0.5 mM $CaCl_2$. The protein was eluted using a linear NaCl gradient (0-0.5 M) in the same buffer at the rate of 1 ml/min. The proteolytic activity was determined in the collected fractions.

The purity and molecular weight of the resulting preparations were monitored by SDS-PAGE in 12.5% gel by the Laemmli method [14].

The specific activity of the subtilisin-like proteinases was determined by cleavage of the chromogenic substrate Z-Ala-Ala-Leu-pNa (where pNA is *p*-nitroanilide and Z is benzyloxycarbonyl) as described in [15]. The enzyme quantity hydrolyzing under the experiment conditions 1 μmol substrate per min was taken as the activity unit. The specific activity was expressed in units per mg protein.

The purified protein fractions corresponding to different growth stages of the culture (28 and 48 h) were analyzed by mass spectrometry. Solutions of the *B. intermedius* proteinase were treated with trypsin according to the method described at the site (http://www.biochem.unitzh.ch/services/protocol/ingeldigestion.pdf). The resulting peptides with different molecular weights were identified using a Vision 2000 TOF mass spectrometer (ThermoBioanalysis, Great Britain). The findings were processed using the Peptide Mass Fingerprint and Peptide Mass programs.

The substrate specificity of the resulting fractions of the enzymes was studied using synthetic *p*-nitroanilide substrates Glp-Ala-Ala-Leu-pNa, Glp-Phe-Ala-Ala-pNa, Z-Ala-Ala-Val-pNa, Glp-Phe-Gly-pNa, Z-Ala-Ala-Leu-pNa, and Z-Glu-pNa dissolved in 20% dimethylformamide (DMFA) at the final concentration of 2 mg/ml.

The proteinase specificity was determined by their action on the B-chain of oxidized sheep insulin as described in [16].

The K_m value for the protein fractions of *B. intermedius* secreted by *B. subtilis* was determined by hydrolysis of the specific chromogenic substrate Z-Ala-Ala-Leu-pNa using a double-beam Lambda 35 spectrophotometer (Perkin Elmer, USA). The calculations were performed using a plot in Lineweaver–Burk coordinates using the Excel program. The catalytic constant k_{cat} was calculated by the formula:

$$k_{cat} = V_{max}/[E],$$

where [E] is the enzyme concentration.

Effects of various inhibitors on the activities of the subtilisin-like proteinases were studied using ovomucoid, the sea anemone inhibitor, trypsin inhibitor, and *o*-phenanthroline at the enzyme/inhibitor ratio 1 : 10, and also phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), and EDTA at the ratio of 1 : 1000. The protein solution was incubated with the inhibitor for 1 h at 37°C in Tris-HCl buffer (pH 8.5), and the proteolytic activity was determined with respect to the chromogenic substrate Z-Ala-Ala-Leu-pNa as described above.

To determine the temperature optimum, the enzyme activities with respect to Z-Ala-Ala-Leu-pNa were measured in the temperature range from 0 to 65°C. To assess the thermostability, the enzyme solutions in 0.05 M Tris-HCl buffer (pH 8.5) were incubated in the temperature range from 0 to 70°C for 30 min, then Z-Ala-Ala-Leu-pNa solution in 20% DMFA was added, and the activity was determined at 37°C, as described above.

To find the pH optimum, the enzyme activities with respect to Z-Ala-Ala-Leu-pNa were determined in 0.1 M

Table 1. Purification of the subtilisin-like proteinase of *B. intermedius* secreted by the recombinant strain *B. subtilis* AJ73 at different stages of the culture growth

Stage of purification	Volume, ml	Protein, A_{280}	Total activity, U	Specific activity, U/mg	Degree of purification	Yield, %
Culture fluid						
early proteinase	920	25 875	1811.25	0.07	1	100
late proteinase	1000	38 500	2310	0.06	1	100
Chromatography on CM-cellulose						
early proteinase	23	41.4	510.6	12.33	176.1	28.2
late proteinase	41	96	893.1	9.3	155	38.7
Chromatography on a Mono S column (FPLC)						
early proteinase	15	5.2	206.2	39.65	566.4	11.4
late proteinase	28	15	456.2	30.41	506.8	19.8
Rechromatography in the FPLC system						
early proteinase	10	3.5	174.3	49.8	711.4	9.6
late proteinase	17	8.5	379.8	44.68	744.7	16.4

universal buffer and 0.05 M Tris-HCl buffer. The 0.1 M universal buffer contained 0.04 M solutions of CH_3COOH, H_3PO_4, and H_3BO_3, and pH was adjusted with 0.2 M NaOH. To determine the pH stability, the protein solutions were incubated in the universal buffer for 1 h at 25°C, then solution of Z-Ala-Ala-Leu-pNa in 20% DMFA was added, and the activity was determined at 37°C as described above.

The results were processed using the Microsoft Excel program by calculating the mean square deviation (σ). The results were considered significant at $\sigma \leq 15\%$. The significance of the resulting differences was calculated using Student's test, assuming $P \leq 0.05$ as the reliable significance level.

RESULTS AND DISCUSSION

The accumulation of subtilisin-like proteinases of *B. intermedius* in the culture fluid of *B. subtilis* was earlier shown to reach the maximum by the 28th (early proteinase) and 48th h (late proteinase) of the growth, which corresponded to the early and late stationary phases of the bacterial growth [12]. By ion-exchange chromatography on CM-cellulose and FPLC on a Mono S with a subsequent rechromatography, two fractions of a subtilisin-like proteinase were isolated, with the purification degree of 711.4 and 745 for the early and late enzyme, respectively. The enzyme yield by activity was 9.48 and 16.67%, respectively (Table 1).

The protein fractions corresponding to different stages of the culture growth were analyzed by mass spectrometry (MALDI-TOF). This method is based on the protein cleavage by trypsin followed by obtaining of peptides with different molecular weights, which are identified using a mass spectrometer. This establishes the primary sequence and the molecular weight of the mature protein. The results obtained by MALDI-TOF were processed using the Peptide Mass Fingerprint and Peptide Mass programs. Using the Peptide Mass program the cleavage was theoretically determined of the native strain protein with the amino acid sequence found earlier [11]. The data processing with the Peptide Mass Fingerprint program allowed us to establish the primary sequence of both recombinant proteins. The results indicated that both fractions of the recombinant strain proteinase corresponding to the growth at different stages had identical amino acid sequences, began from the same amino acid, and their N- and C-terminal sequences were coincident. The molecular weight of both protein fractions was

Table 2. Substrate specificity of subtilisin-like proteinases of *B. intermedius* secreted by the *B. subtilis* recombinant strain

Substrate	Early proteinase, U/mg	Late proteinase, U/mg
Glp-Ala-Ala-Leu-pNa	115.6	138.7
Glp-Ala-Ala-Phe-pNa	1.0	1.6
Z-Ala-Ala-Val-pNa	0	0
Glp-Phe-Gly-pNa	0	0
Z-Ala-Ala-Leu-pNa	115.0	121.7
Z-Glu-pNa	0	0

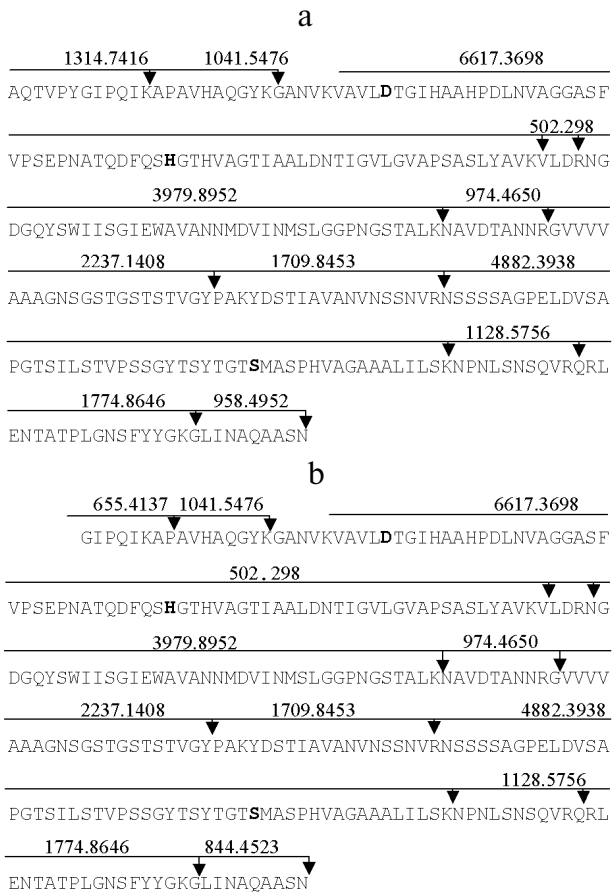

Fig. 1. Comparison of amino acid sequences of subtilisin-like proteinases of *B. intermedius* secreted by the native strain of *B. intermedius* 3-19 (a) and the *B. subtilis* recombinant strain AJ73 (b).

27 kD. However, the comparison of the sequences of these proteins revealed that the N-terminal sequence of the enzymes from the *B. subtilis* recombinant strain was six amino acid residues shorter than the N-terminal sequence of the mature enzymes from the *B. intermedius* native strain (Fig. 1). Data on the molecular weight of the proteins were confirmed electrophoretically (Fig. 2) and found to be close to values for other bacterial subtilisins, such as subtilisin NAT from *B. subtilis var. natto* (27.5 kD), subtilisin DFE (28 kD) from *B. amyloliquefaciens* DC-4, subtilisin Carlsberg from *B. licheniformis* (27.3 kD), and subtilisin BPN′ from *B. amyloliquefaciens* (27.5 kD) [17-19]. With the Peptide Mass program, the theoretical isoelectric point (p*I*) of the subtilisin-like proteinases of *B. intermedius* secreted by the *B. subtilis* recombinant strain was found to be 6.64. This value is slightly lower than the p*I* values of other serine proteinases, which are usually in the neutral or alkaline areas (7.8 for BPN′, 9.4 for Carlsberg), although there are some exceptions. Thus, p*I* of protease HS from the *Bacillus* sp. strain GX6638 is 4.2 [20].

The substrate specificity of the resulting protein fractions was studied using different synthetic peptides. Subtilisin-like proteinases of *B. intermedius* secreted by the *B. subtilis* recombinant strain failed to hydrolyze substrates specific for chymotrypsin (Glp-Phe-Gly-pNa) and Glu-Asp-specific proteases (Z-Glu-pNa) (Table 2). Both proteinases were the most active with the substrates specific just for subtilisins (Glp-Ala-Ala-Leu-pNa and Z-Ala-Ala-Leu-pNa), and this suggested that these enzymes really are subtilisin-like proteinases (Table 2). Most bacterial subtilisins preferentially hydrolyze the bonds between aromatic (Phe) and aliphatic (Leu) amino acids (subtilisin BPN′, savinase, esperase), but there are also subtilisin-like proteases more effective relative to basic amino acid residues (e.g., SAM-P45 secreted by *Streptomyces albogriseolus* [21, 22]). The enzymes under study also more effectively hydrolyzed *p*-nitroanilides containing hydrophobic amino acids leucine and phenylalanine.

Michaelis constants of the proteins of *B. intermedius* were determined by hydrolysis of a specific chromogenic substrate. From the plot in Lineweaver–Burk coordinates, K_m values were found to be 1.85 and 0.86 mM for the early and late proteinase, respectively. The K_m values

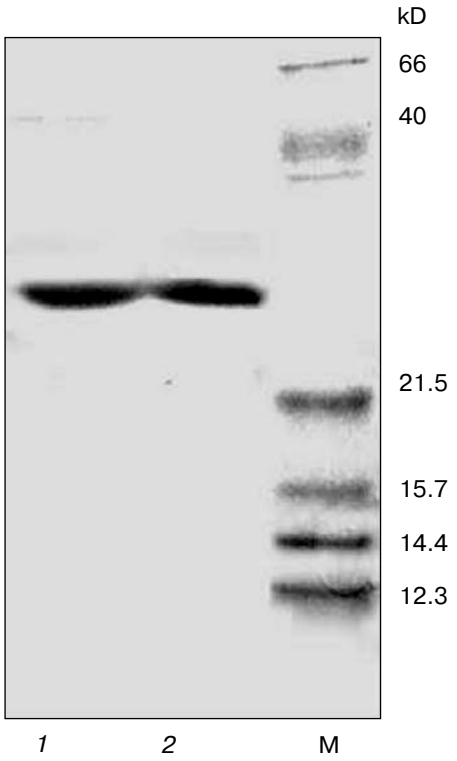

Fig. 2. SDS-PAGE of serine proteinases of *B. intermedius* secreted by *B. subtilis* AJ73. M, markers: RNase (Binase) (12.3 kD), lysozyme (14.4 kD), RNase A (15.7 kD), trypsin inhibitor (21.5 kD), peroxidase (40 kD), BSA (66 kD); *1*) the early subtilisin-like proteinase; *2*) the late subtilisin-like proteinase.

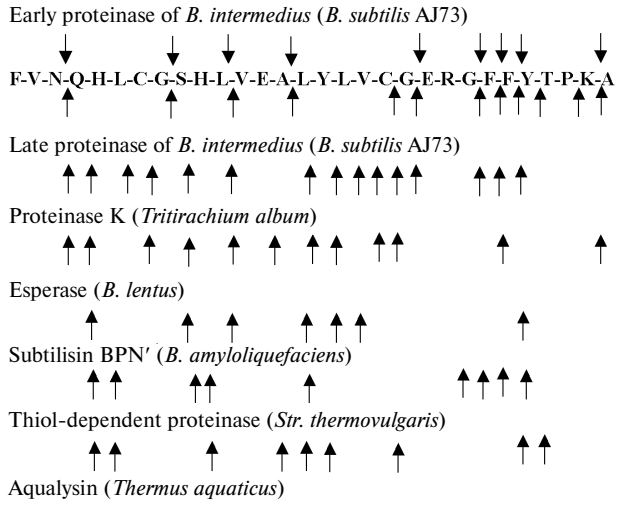

Fig. 3. Hydrolysis of the insulin B-chain by the subtilisin-like proteinase of *B. intermedius* 3-19 secreted by the *B. subtilis* recombinant strain at different growth stages.

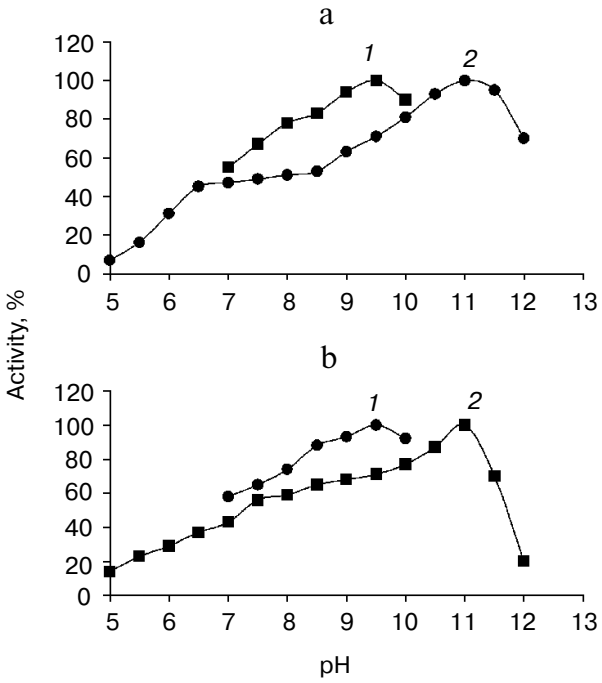

Fig. 4. The pH optimum (a) and pH stability (b) of the subtilisin-like proteinase of *B. intermedius* secreted by the *B. subtilis* recombinant strain AJ73 at different growth stages in Tris-HCl buffer (*1*) and in universal buffer (*2*).

obtained for both proteins were comparable with the values for other subtilisins: the K_m for the early proteinase was virtually the same as the K_m values for subtilisin BPN′ (1.86 mM) and proteinase from *Thermoactinomyces vulgaris* (1.9 mM), whereas the K_m value for the late proteinase was slightly higher than that for *Streptomyces thermovulgaris* (0.4 mM). These findings revealed that the late proteinase had the higher affinity for the substrate Z-Ala-Ala-Leu-pNa than the early proteinase and the higher efficiency than subtilisin BPN′ and proteinase from *T. vulgaris*. The early enzyme hydrolyzed the substrate similarly to subtilisin BPN′. The k_{cat} value (5574 sec^{-1}) of the late proteinase was higher than the k_{cat} value (4748 sec^{-1}) of the early proteinase. Thus, the late proteinase catalyzed the cleavage more effectively than the early protein. These data confirmed the results obtained by hydrolysis of the insulin B-chain. The late enzyme hydrolyzed the insulin B-chain more effectively than the early protein.

The substrate specificity of the proteinase under study was similar to the specificity of other subtilisins, and the hydrolysis depth was comparable with the effects of proteinase K and esperase [22]. Both fractions of the enzyme actively hydrolyzed the bonds formed by hydroxyl groups of hydrophobic amino acids (Leu11–Val12, Phe25–Tyr26, etc.) and also of hydrophilic amino acids including acidic and basic ones (Gly8–Ser9, Gln4–His5, etc.) (Fig. 3). However, the proteolytic activities of the enzymes were different: the late proteinase hydrolyzed two bonds more than the early proteinase did.

Table 3 presents the effects of a number of inhibitors on the subtilisin-like proteinases of *B. intermedius* secreted by the *B. subtilis* recombinant strain. The data for both fractions were similar. The late and early enzymes were not inhibited by nonspecific natural inhibitors, such as ovomucoid, sea anemone inhibitor, and trypsin inhibitor. DTT also did not affect the activities of the early and late

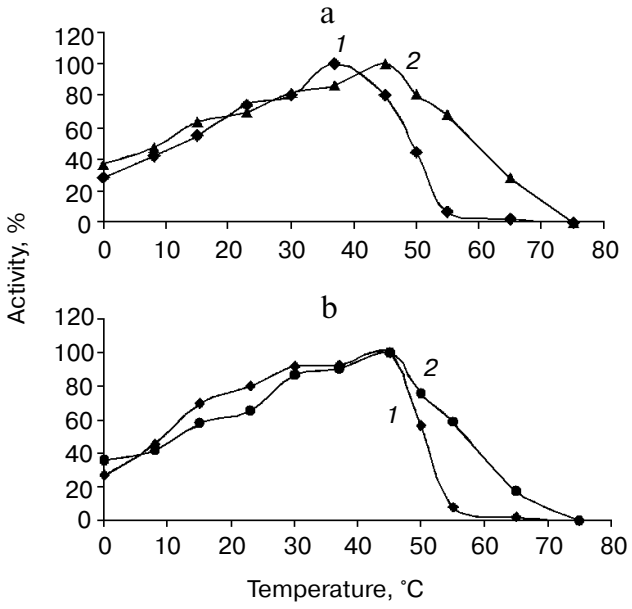

Fig. 5. Temperature optimum of the early (a) and late (b) subtilisin-like proteinase of *B. intermedius* in the absence of Ca^{2+} (*1*) and in the presence of Ca^{2+} (*2*).

proteinases. Both protein fractions were completely inhibited by the specific inhibitor of serine proteinases, PMSF, and were not inhibited by specific inhibitors of metalloproteinases, EDTA and *o*-phenanthroline, and this confirmed their belonging to the serine proteinase family.

The literature data suggest that many bacterial subtilisin-like proteinases retain activity at high pH values. Such proteinases include subtilisin Sendai from *Bacillus* sp. G-825-6, which retains stability even at pH 12.0, AprP from *Bacillus pumilus* TYO-67 (pH 9.0), etc. This stability is usually due to the more rigid and hydrophobic surface area of the protein globule of subtilisins [23, 24]. Our findings are consistent with the data for other subtilisins: the pH-optimum of both fractions under study of the subtilisin-like proteinases of *B. intermedius* secreted by the *B. subtilis* recombinant strain at different growth stages was 11 in the universal buffer and 9.5 in Tris-HCl buffer. The enzymes retained stability in the pH range from 7.5 to 11.5 in the universal buffer and from 7 to 10 in Tris-HCl buffer (Fig. 4).

The stability of bacterial proteinases at extreme temperatures correlates with the elevated number of hydrogen and ionic bonds, electrostatic interactions, and Van der Waals forces inside the protein molecule [25]. Disulfide bridges formed by cysteine residues also significantly contribute to stabilization of the protein globule of some subtilases. The temperature optima of the two subtilisin-like proteinases of *B. intermedius* secreted by the *B. subtilis* recombinant strain were 37 and 45°C. The enzymes retained stability in the temperature range from 0 to 50°C (Fig. 5). Instability of the subtilisin-like proteinases of *B. intermedius* at the higher temperature could be explained by the absence of disulfide bonds responsible for the stability of thermophilic proteinases, such as proteinase K and aqualysin possessing two such structures, and a psychrophilic *Vibrio* proteinase with four disulfide bridges [26].

Table 3. Inhibitory analysis of subtilisin-like proteinases of *B. intermedius* secreted by *B. subtilis* recombinant strain AJ73

Inhibitor	Molar ratio enzyme/ inhibitor	Early proteinase activity, %	Late proteinase activity, %
PMSF	1 : 1000	0	0
DTT	1 : 1000	100	100
EDTA	1 : 1000	99.5	99.8
o-Phenanthroline	1 : 10	100	100
Ovomucoid	1 : 10	100	100
Trypsin inhibitor	1 : 10	100	100
Sea anemone inhibitor	1 : 10	100	100

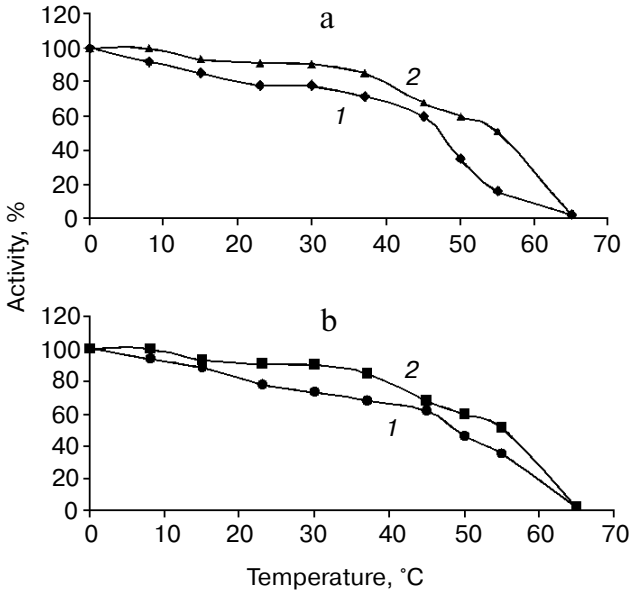

Fig. 6. Thermostability of the early (a) and late (b) subtilisin-like proteinases of *B. intermedius* in the absence of Ca^{2+} (*1*) and in the presence of Ca^{2+} (*2*).

Calcium ions are known to stabilize the structure of the subtilisin molecule. In the literature, two and three calcium-binding sites are reported, respectively, for subtilisin BNP′ and the Carlsberg subtilisin [27]. At the final concentration of 5 mM, calcium ions did not considerably influence the activity of the late proteinase of *B. intermedius* but slightly stabilized the early proteinase, for which the range of the temperature stability increased to 55-60°C (Fig. 6).

Thus, both fractions of the subtilisin-like proteinase of *B. intermedius* secreted by the *B. subtilis* recombinant strain and corresponding to different stages of the culture growth were identical in molecular weight and similar in physicochemical properties. These findings indicated that the enzymes studied belonged to the family of bacterial subtilisin-like proteinases. However, the early and late proteinases had different catalytic characteristics and slightly different substrate specificities that seemed to be due to different conformations of the protein globules associated with changes in the folding mechanisms during the different growth stages of the bacillus.

The authors are grateful to S. V. Kostrov who presented the *B. subtilis* recombinant strain AJ73 carrying the plasmid pCS9 with the gene of the subtilisin-like proteinase from *B. intermedius*.

This work was supported by the Russian Foundation for Basic Research (project No. 05-04-48182-a).

REFERENCES

1. Ferrari, E., Jarnagin, A. S., and Schmidt, B. F. (1993) in *Bacillus subtilis and Other Gram-Positive Bacteria* (Sonenshein, A. L., Hoch, J. A., and Losick, R., eds.) American Society for Microbiology, Washington, DC, pp. 917-937.
2. Siezen, R. J., and Leunissen, J. A. (1997) *Protein Sci.*, **6**, 501-523.
3. Rawlings, N. D., and Barrett, A. J. (1994) *Meth. Enzymol.*, **244**, 19-61.
4. Henrich, S., Cameron, A., Bourenkov, G. P., Kiefersauer, R., Huber, R., Lindberg, I., Bode, W., and Than, M. E. (2003) *Nature Struct. Biol.*, **10**, 520-526.
5. Thomas, G. (2002) *Nature Rev. Mol. Cell Biol.*, **3**, 753-766.
6. Wells, J. A., Ferrari, E., Henner, D. J., Estel, D. A., and Chen, E. Y. (1983) *Nucleic Acids*, **11**, 7911-7925.
7. Eder, J., Rheinnecker, M., and Fersht, A. R. (1993) *Mol. Biol.*, **233**, 293-304.
8. Huang, H.-W., Chen, W.-Ch., Wu, Ch.-Y., Yu, H.-Ch., Lin, W.-Y., Chen, Sh.-T., and Wang, K.-T. (1997) *Protein Eng.*, **10**, 1227-1233.
9. Bryan, P. N. (2000) *Biochim. Biophys. Acta*, **1543**, 203-222.
10. Balaban, N. P., Mardanova, A. M., Sharipova, M. R., Gabdrakhmanova, L. A., Sokolova, E. A., Rudenskaya, G. N., and Leshchinskaya, I. B. (2004) *Biochemistry (Moscow)*, **69**, 420-426.
11. Sharipova, M., Balaban, N., Kayumov, A., Kirillova, Y., Mardanova, A., Leschinskaya, I., Rudenskaya, G., Akimkina, T., Safina, D., Demidyuk, I., and Kostrov, S. (2007) *Microbiol. Res.*, in press.
12. Kirillova, Yu. M., Mikhailova, E. O., Balaban, N. P., Mardanova, A. M., Kayumov, A. R., Rudenskaya, G. N., Kostrov, S. V., and Sharipova, M. R. (2006) *Mikrobiologiya*, **75**, 1-178.
13. Bradford, M. M. (1976) *Analyt. Biochem.*, **8**, 248-254.
14. Laemmli, U. K. (1970) *Nature*, **227**, 680-685.
15. Lyublinskaya, L. A., Khaidu, I., and Balandina, G. N. (1987) *Bioorg. Khim.*, **13**, 748-753.
16. Itskovich, E. L., Balaban, N. P., Maradanova, A. M., Shakirov, E. V., Sharipova, M. P., Leshchinskaya, I. B., Ksenofontov, A. L., and Rudenskaya, G. N. (1997) *Biochemistry (Moscow)*, **62**, 49-53.
17. Nakamura, T., Yamagata, Y., and Ichishima, E. (1992) *Biosci. Biotechnol. Biochem.*, **56**, 1869-1871.
18. Peng, Y., Huang, Q., Zhang, R. H., and Zhang, Y. Z. (2003) *Comp. Biochem. Physiol. B Biochem. Mol. Biol.*, **134**, 45-52.
19. Markland, F. S., and Smith, E. L. (1971) in *The Enzymes*, Vol. 3 (Boyer, P. D., ed.) Academic Press, Inc., New York, pp. 561-608.
20. Durham, D. D., Stewart, D., and Stellawg, E. J. (1987) *J. Bacteriol.*, **169**, 2762-2768.
21. Suzuki, M., Taguchi, S., Yamada, S., Kojima, S., Miura, K. I., and Momose, H. (1997) *J. Bacteriol.*, **179**, 430-438.
22. Georgieva, D. N., Stoeva, S., Voelter, W., Genov, N., and Betzel, C. (2001) *Arch. Biochem. Biophys.*, **387**, 197-201.
23. Aoyama, M., Yasuda, M., Nakaci, K., Kobamoto, N., Oku, H., and Kato, F. (2003) *Appl. Microb. Biotechnol.*, **53**, 390-395.
24. Maeda, H., Mizutani, O., Yamagata, Y., Ichishima, E., and Nakajima, T. (2001) *J. Biochem.*, **129**, 675-682.
25. Vielle, C., and Zeikus, G. (2001) *Microbiol. Mol. Biol. Rev.*, **65**, 1-43.
26. Kristjansson, M. M., Magnusson, O. T., Gudmundsson, H. M., Alfredson, G. A., and Matzusawa, H. (1999) *Eur. J. Biochem.*, **260**, 752-760.
27. Bryan, P., Wang, L., Hoskins, J., Ruvinov, S., Strausberg, S., Alexander, P., Almong, O., Gililand, G., and Gallagher, T. (1992) *Biochemistry*, **31**, 4937-4945.