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Biochemical Properties of *Bacillus intermedius* Subtilisin-Like Proteinase Secreted by a *Bacillus subtilis* Recombinant Strain in Its Stationary Phase of Growth

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Abstract—Biochemical properties of *Bacillus intermedius* subtilisin-like proteinase (AprBi) secreted by a *B. subtilis* recombinant strain in the early and late stationary phases of growth have been determined. Protein structure was analyzed and its stability estimated. It was noted that the enzyme corresponding to different phases of bacterial growth retains activity in the presence of reducing and oxidizing agents (C_2H_5OH and H_2O_2). Different effects of bivalent metal ions on activity of two proteinase fractions were found. Calcium ions more efficiently activate proteinase secreted in the late stationary phase. Unlike the first enzyme fraction, the second forms catalytically active dimers.

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Key words: subtilisin-like proteinase, AprBi, dimers

Subtilisin-like serine proteinases form the family of subtilases, enzymes that are widespread in nature. The number of members of this family, found both in eukaryotes and prokaryotes, exceeds 200 and steadily increases [1]. Among them there are extracellular and intracellular subtilases [2, 3], but the overwhelming majority of them are secreted proteins synthesized within cells as precursors [4]. Bacterial subtilases exhibit broad substrate specificity, stability at high pH values, and activity over a broad range of temperatures [5]. Tertiary structure was identified for various subtilisin-like enzymes [1] indicative of the existence of calcium-binding sites on the surface of the protein globule [6]. Calcium ions stabilize the enzyme molecule, but unlike calcium ions in metalloproteases they are not involved in catalysis.

The ubiquitous nature of subtilases is associated with their participation in various physiological processes like catabolism of complex protein substrates, formation and germination of bacterial spores and fungal axospores, activation of prehormones, etc. [5, 7, 8]. In recent years the regulatory role of these proteins in cell physiology has become more and more obvious. Thus the regulation of synthesis of these enzymes is intensively studied. Bacterial proteins are the simplest model for such investigations. Bacilli traditionally secrete into the medium different proteinases among which subtilisin-like serine proteases are prevalent.

The *Bacillus intermedius* subtilisin-like proteinase gene was cloned on the multicopy plasmid pCS9. Its expression was studied in the protease-deficient *Bacillus subtilis* strain AJ73 [9]. The base sequence of the gene has been determined (AN AY754946). It has been shown that the enzyme accumulation in culture medium of *B. subtilis* recombinant strain reaches its maximum at the beginning and end of stationary growth phase (28 and 48 h, respectively) and depends on the nutrient medium composition [9, 10]. Change in the mechanism of regulation of gene expression during culture growth has been shown [10, 11]. The *B. intermedius* subtilisin-like proteinase fractions corresponding to different growth phases of the recombinant strain (28 and 48 h) were isolated and partially characterized [12]. It was shown by MALDI-TOF spectrometry that the two protein fractions have identical amino

Abbreviations: AprBi, *Bacillus intermedius* subtilisin-like proteinase; DMFA, dimethylformamide; pNa, *p*-nitroanilide; Z, benzyloxycarbonyl.

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acid sequence, begin with the same amino acid, and their N and C ends coincide. However, it was found that enzyme fractions have different kinetic constants (K_m = 1.85 and 0.86 mM for the first and second fractions, respectively) and differ in substrate specificity.

The aim of this work was comparative analysis of biochemical properties of both fractions of *B. intermedius* subtilisin-like proteinase secreted by *B. subtilis* recombinant strain at different growth phases.

MATERIALS AND METHODS

The *B. subtilis* recombinant strain AJ73 (pCS9) carrying the gene of *B. intermedius* subtilisin-like serine proteinase on the multicopy plasmid pCS9 was used in this work (courtesy of S. V. Kostrov, Institute of Molecular Genetics, Russian Academy of Sciences). The *B. subtilis* recombinant strain was grown for 28 h (first fraction) and 48 h (second fraction) as described earlier [9]. Subtilisin-like proteinase of *B. intermedius* was isolated at 28 and 48 h of growth from 1 liter of *B. subtilis* culture medium by ion-exchange chromatography on CM-cellulose and FPLC on a MonoS column [12].

Protein amount was determined according to Bradford [13]. Specific activity of subtilisin-like proteinases was determined by cleavage of a chromogenic substrate, Z-Ala-Ala-Leu-pNa [14]. The enzyme amount that hydrolyzes under experimental conditions 1 μmol substrate in 1 min was taken as the activity unit. Specific activity is expressed as unit/mg protein.

Gel filtration of enzyme solutions was carried out on a Sephadex G-100 column (Pharmacia, Sweden) in 50 mM Tris-HCl buffer, 120 mM KCl, pH 8.5. Lysozyme (14.4 kDa), trypsin inhibitor (21.5 kDa), peroxidase (40 kDa), and BSA (66 kDa) were used as molecular mass markers. The void volume of the column was determined using Dextran blue (Roana, Hungary).

To remove Ca^{2+}, protein solutions were dialyzed against 50 mM Tris-HCl buffer, 1% EDTA, pH 8.5. Protein electrophoresis in native conditions was carried out according to Lebendicker (http://wolfson.huji.ac.il/purification/Protocols/PAGE_Acidic.html).

To study the effect of H_2O_2 on the activity of enzyme fractions, enzyme solutions were preincubated in 0.05 M Tris-HCl buffer containing 10, 20, and 40 mM H_2O_2 for 1 and 24 h at 37°C.

The effect of ethanol on proteinase activity was studied in 0.05 M Tris-HCl buffer containing 5, 10, 25, and 50% ethanol. Activity was determined after preincubation of enzyme solutions with ethanol.

To study the effect of bivalent metal ions on the subtilisin-like proteinase activity, the enzyme was incubated for 30 min at 37°C in 0.05 M Tris-HCl buffer (pH 8.5) containing Mn^{2+}, Cu^{2+}, Mg^{2+}, or Ca^{2+} at final concentrations from 5 to 20 mM. Then activity was determined by the above-described method. Activity of the enzyme incubated without metals was taken as 100%.

The amino acid sequence of the *B. intermedius* subtilisin-like proteinase was analyzed using the ProtParam program (www.expasy.org). The search for homology and alignment of amino acid sequences was carried out using the BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and ClustalW 1.83 (www.ebi.ac.uk/clustalw/) programs, respectively.

Mathematical data processing was carried out using the Microsoft Excel program by calculation of the root-mean-square deviation (σ). The results were considered as confident at $\sigma \leq 15\%$. Student's criterion was used in calculation of the difference confidence taking $p \leq 0.05$ as the reliable level of significance.

RESULTS AND DISCUSSION

As shown by mass spectrometry, the *B. intermedius* subtilisin-like proteinase (AprBi) fractions isolated from culture medium of *B. subtilis* recombinant strain at different phases of growth have an identical amino acid sequence, and the N and C termini of the sequences coincide [12]. The alignment of amino acid sequence of *B. intermedius* subtilisin-like proteinase (accession code Q29ZA8) with sequences of other subtilisin-like proteinases using the BLAST and ClustalW programs has shown 98% identity with subtilisin-like protease BPP-A of *B. pumilus* (Q2HXI3), 77% identity with subtilisin AP01 of *B. subtilis* (Q58GF0), and 76% identity with nattokinase of *B. subtilis.* subsp. *natto* (Q5EFD9) and with subtilisin E of *B. subtilis* 168 (A0FLP4) (Fig. 1). The AprBi sequence does not contain cysteine, and three amino acid residues (D32, H64, S221) form the catalytic triad of the active center, which is characteristic of the subtilisin family [1].

The ratio of amino acids with different properties in microbial subtilisins is shown in the table. It is known that increased number of charged and acidic amino acids in protein structure correlates with resistance of the protein to high temperatures [15]. We showed earlier that both fractions of AprBi proteinase corresponding to different growth phases are not thermostable: temperature optima for the first and second fractions are 37 and 45°C, respectively [12]. Data of amino acid analysis are also indicative of the absence of thermostability in AprBi proteinase. The AprBi primary structure is characterized by low (compared to other subtilisins) content of acidic (5.2%) and charged (10.1%) amino acids. The more stable proteinase K contains 6.2% acidic and 13.7% charged amino acids, which is higher than the similar parameters for another subtilisin-like protein of *Fervidobacterium islandicum* (10.1 and 19.8%, respectively) [16]. The fraction of aromatic amino acids also increases within the range of thermostability growth and is 5.1% for AprBi proteinase, which is half of that for islandisin (9.9%).

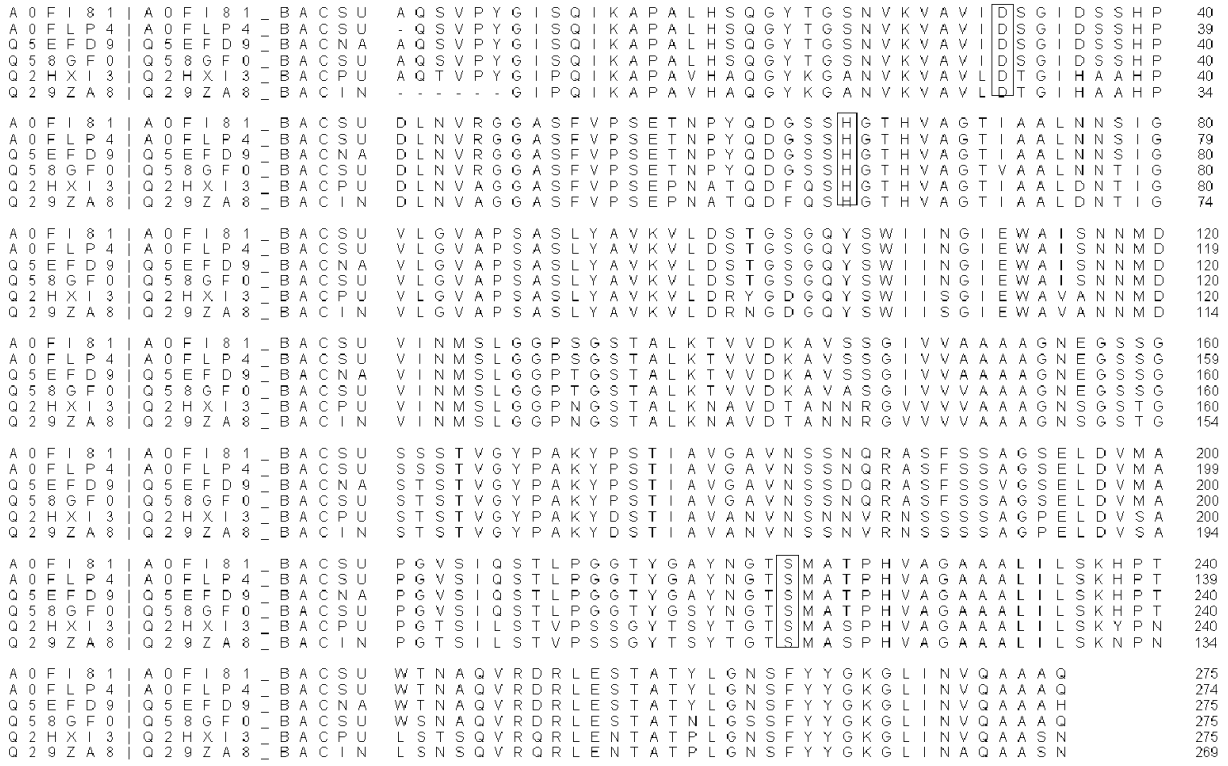

Fig. 1. Comparison of primary structure of *B. intermedius* subtilisin-like proteinase secreted by the *B. subtilis* recombinant strain with other subtilisins. Codes of access to protein sequences are shown to the right. Amino acid residues of the active center are in the frames.

Amino acid analysis of microbial subtilisins

Protease	T_{opt}, °C	pH optimum	Amino acids, %								
			charged				acidic	aromatic	basic	polar	hydrophobic
			total number	– Asp+ Glu	+ Lys+ Arg	Arg					
Proteinase AprBi	37 (1st fraction) 45 (2nd fraction)	9.5-11	10.1	14	13	5	5.2	5.1	4.9	31.2	36.3
Protease BPP-A	55	11-13	12	14	13	5	5.1	6.2	4.7	31.3	42.9
Protease YaB	–	10.5-12	8.2	8	13	9	3.3	4.8	4.9	26.5	37.7
Subtilisin Carlsberg	60	8.5-10.5	11.7	13	13	4	5.1	6.6	4.7	28.1	37.2
E	55	8.5-10.5	10.9	14	8	4	5.1	6.6	4.4	31.6	35.3
BPN'	60	8.5-10.5	12.4	15	13	2	5.5	5.8	4.7	28.4	36.7
Proteinase K	75	7.5-8.0	13.7	18	20	12	6.2	9	7.2	33.4	33.2
Islandisin	80	8.0	19.8	71	61	25	10.1	9.9	8.7	25.6	36.6

Note: Data for subtilisin BPN′ (*B. amyloliquefaciens*), subtilisin Carlsberg (*B. licheniformis*), subtilisin-like protease BPP-A (*B. pumilus*), subtilisin E (*B. subtilis*), proteinase YaB (*Bacillus* sp.), proteinase K (*Tritirachium album*), and islandisin (*Fervidobacterium islandicum*) were obtained from the Uniprot Knowledgebase database (www.cn.expasy.org/sprot).

A high number of hydrophobic and charged amino acids on the surface of the subtilisin protein molecule is an adaptation mechanism of enzyme stabilization at high pH values [17]. Besides, the distribution of charged residues in the polypeptide chain contributes significantly to its conformation. The fraction of hydrophobic amino acids within subtilisin-like proteinase of *B. intermedius* is 36.3% and does not exceed that for other subtilisins (table). The number of positively charged amino acids (Arg + Lys) in the AprBi structure correlates with that in other highly alkaline subtilisins, and the number of arginines, supposed to be responsible for maximal contribution to the molecule stabilization in alkaline medium, is even higher than in classical subtilisins such as BPN′, E, and Carlsberg [18]. The pH optimum of AprBi proteinase activity in a universal buffer is 11.0 and in Tris-HCl buffer it is 9.5 [12]. Data of amino acid analysis also characterize AprBi proteinase as an alkaline enzyme. Thus, analysis of the ratio of different amino acids in the AprBi proteinase primary structure has shown that the enzyme is stable in alkaline medium and relatively non-thermostable.

The weak point of all subtilisins is inactivation due to oxidation of methionine located immediately after serine forming the catalytic triad [19]. At the present time subtilisin-like proteases E-1, KP-9860, LP-Ya, and other bacillar (*Bacillus* sp.) subtilisins resistant to H_2O_2 are known [20]. We found that the first and second fractions of subtilisin-like proteinase of *B. intermedius* retained activity in the presence of 10 mM H_2O_2 at the control level, and the activity of both fractions decreased only slightly in the presence of 20 and 40 mM H_2O_2 (Fig. 2a). Preliminary incubation of both fractions of subtilisin-like proteinase of *B. intermedius* with 10 and 20 mM H_2O_2 for 1 h also had little effect on their activity, but in the case of 40 mM H_2O_2 concentration activity of the early proteinase decreased by 40% (Fig. 2b). Preincubation of the proteins for 24 h with 10 mM H_2O_2 decreased activity by 80% and with 20 and 40 mM H_2O_2 by more than 90% (Fig. 2c). These data do not contradict the results obtained for other proteases, such as NH1 protease that is active in the presence of 1-5% H_2O_2. Alkaline protease of *B. clausii* I-52 retained its activity in the presence of 10% H_2O_2 [21, 22].

Figure 3 shows the effect of ethanol on the activity of the subtilisin-like proteinase of *B. intermedius*. Activity of the first proteinase fraction decreased only slightly (0-30%) in the presence of 5-10% ethanol without preincubation; the effect of ethanol at these concentrations on the activity of the second fraction was more pronounced (20-50%). Ethanol at high concentrations (25, 50%) inhibited the activity of both proteinase fractions by 80 and 95%, respectively (Fig. 3a). Similar results were also obtained for other subtilisins, the activity of which did not decrease in the presence of ethanol at low concentrations [16]. Preincubation of enzyme solutions with 5% ethanol for 1 h resulted in the loss of activity of both proteinase fractions by more than 60%, whereas 50% ethanol completely inhibited the enzyme activity (Fig. 3b). Thus, preincubation with ethanol resulted in the loss of activity of both enzyme fractions, which is indicative of the instability of the proteinase in the presence of this substance.

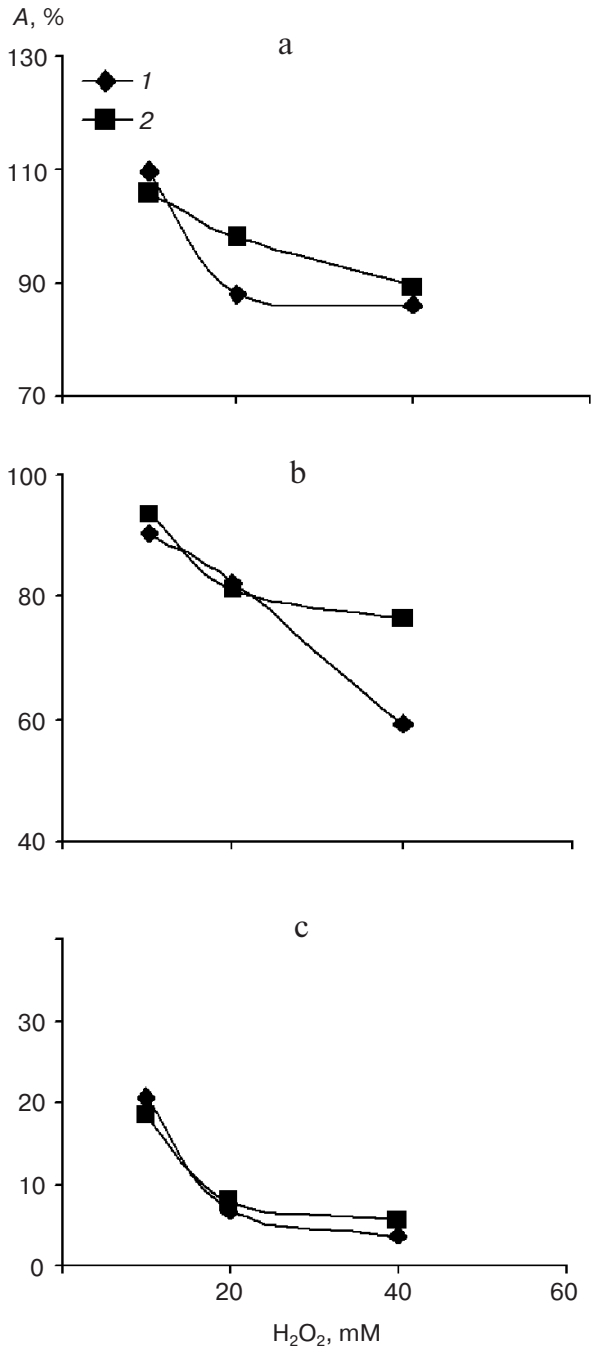

Fig. 2. Effect H_2O_2 on activity of *B. intermedius* subtilisin-like proteinase fractions secreted by the *B. subtilis* recombinant strain at different growth phases: a) without preincubation; b) preincubation for 1 h; c) preincubation for 24 h; *1*) first fraction; *2*) second fraction.

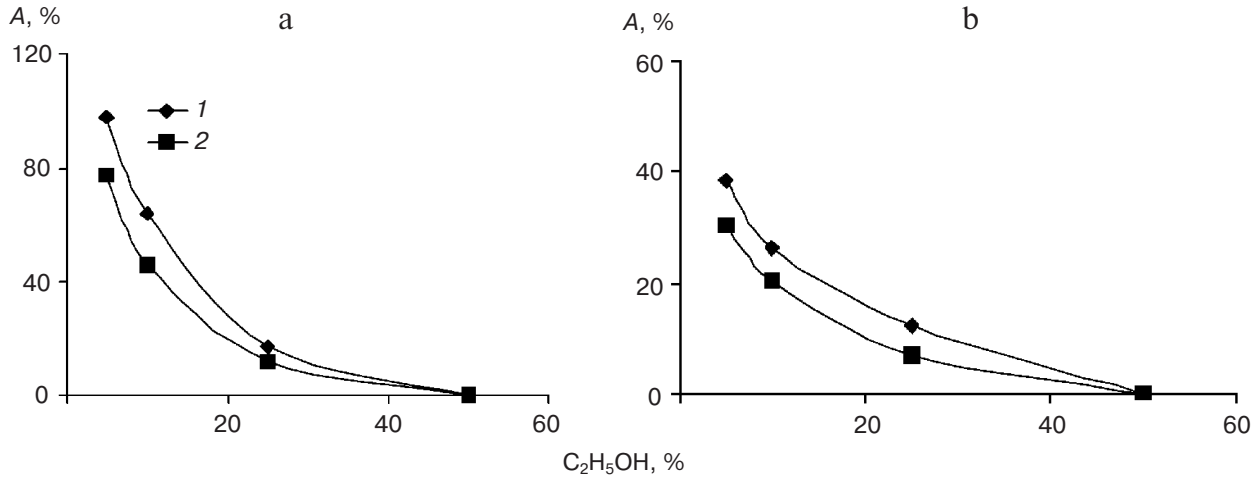

Fig. 3. Effect of ethanol on activity of *B. intermedius* subtilisin-like proteinases secreted by the *B. subtilis* recombinant strain: a) without preincubation; b) preincubation for 1 h; *1*) first fraction; *2*) second fraction.

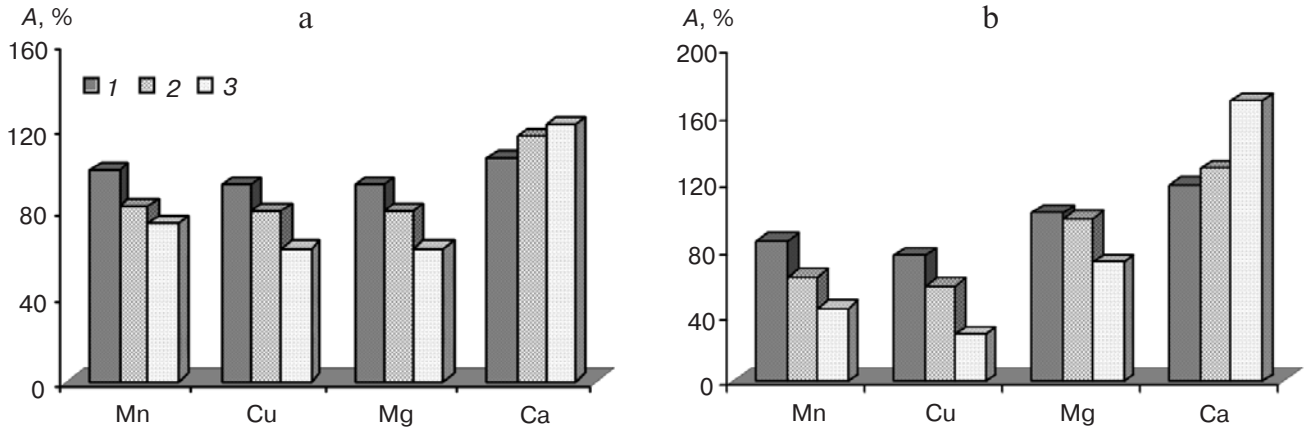

Fig. 4. Effect of metal ions on activity of *B. intermedius* subtilisin-like proteinase secreted by the *B. subtilis* recombinant strain. Activity in the absence of metal ions is taken as 100%. a) First fraction; b) second fraction; *1-3*) 5, 10, and 20 mM, respectively.

The effect of different metal ions on the activity of the enzyme fractions corresponding to different phases of bacterial growth was studied. Tertiary structure of subtilisin-like proteases is characterized by formation of calcium-binding sites contributing to the protein globule conformation and stability [1]. Figure 4 shows the effect of bivalent metal ions on activity of the first and second fractions of AprBi proteinase. Manganese ions at 10 and 20 mM concentrations were more efficient against activity of the second AprBi fraction (40 and 55%, respectively), whereas activity of the first fraction decreased only by 20 and 25%. A low concentration of Mn^{2+} (5 mM) had no effect on the activity of the first fraction and only slightly decreased that of the second fraction. Maximal inhibitory effect of Cu^{2+} on the second enzyme fraction (up to 75%) was noted. Activity of many serine proteinases is inhibited in the presence of Cu^{2+} [16, 23, 24]. However, an activating effect of this metal ion on some serine proteases was noted [25]. The effect of Mg^{2+} was more pronounced with the first AprBi fraction: at 5 mM concentration it decreased by 10% the activity of the first AprBi fraction (Fig. 4a), whereas activity of the second fraction did not change. Manganese ions at 10 and 20 mM concentrations inhibited activity of the first fraction by 20 and 40% and activity of the second fraction by 5 and 30%, respectively. The inhibition of proteolytic activity by Mg^{2+} was also shown for other subtilisins [14], as well as activation of subtilisin-like protease SAM-P20 by Mg^{2+} at low concentrations [26].

Activation by Ca^{2+} of various subtilisins is known, but there are also exceptions (such as subtilisin Carlsberg whose activity does not increase in the presence of calcium ions) [6]. Calcium ions activated both AprBi fractions but to different extent. The highest activation effect was observed for the second protein fraction, whose activity

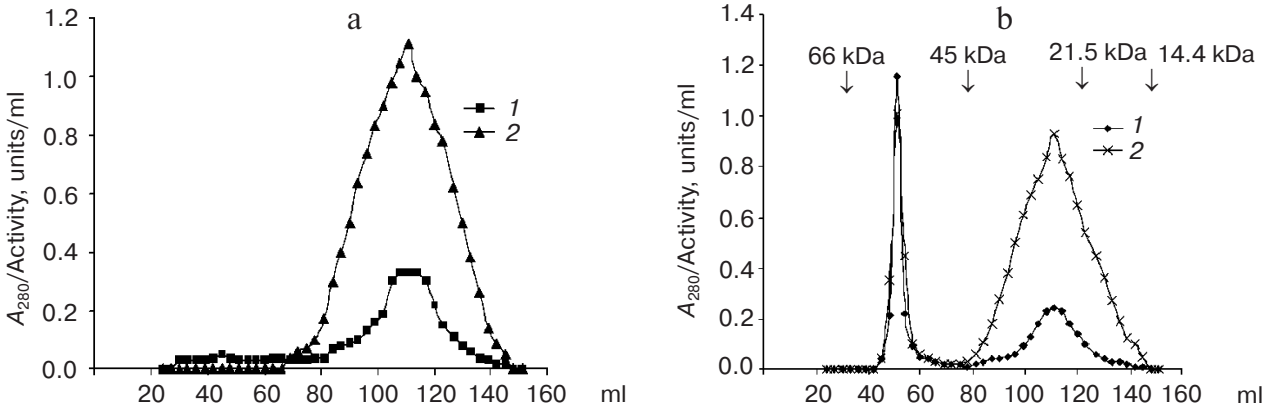

Fig. 5. Gel filtration of *B. intermedius* subtilisin-like proteinase fractions on Sephadex G-100: a) first fraction; b) second fraction; *1*) protein amount; *2*) activity. Marker proteins: lysozyme (14.4 kDa), trypsin inhibitor (21.5 kDa), egg albumin (45 kDa), and BSA (66 kDa).

increased for 17 and 27% at Ca^{2+} concentrations 5 and 10 mM and by 70% at 20 mM Ca^{2+} concentration. Activity of the first fraction increased by 15 and 21% at the Ca^{2+} concentrations 10 and 20 mM, respectively. We suppose that stabilization of the second fraction more than the first in the presence of Ca^{2+} is caused by features of the protein globule. This may be indicative of changes in bacterial physiology and the necessity for increase in the resistance of the protein to unfavorable environmental factors during the late stationary phase of growth. Secretion of the first fraction corresponds to early stages of bacterial culture sporulation, when the requirement for calcium is not as high as for the late stationary phase, when calcium ions are extremely necessary for the full-value sporulation in which subtilisin-like proteinases are involved [27]. In the case of calcium deficiency, the sporulation process is inhibited by 98% and total level of proteolysis by 60% [28]. In addition, the level of proteolysis at late stages of sporulation is for 4-5 times higher than that at early stages of this process [29]. Thus, the second AprBi fraction should be more dependent on calcium ions, which is confirmed by our studies.

Serine proteases are represented both by monomeric and dimeric proteins. Dimeric forms are described for extracellular serine protease of *Leishmania amazonensis* [30], thermostable extracellular proteases of archaeons [31] and cyanobacteria [32]. Dimer formation has been shown for many intracellular subtilisins [2, 3, 33-35]. It was shown that all of them require calcium to maintain activity and stability. We have studied the possibility of dimer formation for AprBi proteinase. Gel filtration of the first AprBi proteinase fraction through Sephadex G-100 revealed the existence of a single protein peak corresponding to the peak of activity (Fig. 5a). The molecular mass of this protein, determined using a set of marker proteins, was 27 kDa. Gel filtration of the second proteinase fraction revealed two protein peaks corresponding to peaks of activity (Fig. 5b). The molecular mass values were 54 and 27 kDa, which suggests formation of proteinase dimer secreted during the late stationary growth phase. Thus, the found conformational changes might be due to AprBi dimer formation at the late phases of bacillar growth. Other examples of the existence of dimers are known for intracellular subtilisins of *B. subtilis*, *B. licheniformis*, and *B. amyloliquefaciens* [2, 3, 33-36].

To understand the mechanism of dimer formation, both fractions were subjected to electrophoresis of under native conditions. Electrophoresis under non-denaturing conditions did not reveal dimers of the second AprBi proteinase fraction (Fig. 6). We supposed that dimer formation by the second fraction is due to electrostatic interaction between monomers that can be mediated by calcium ions.

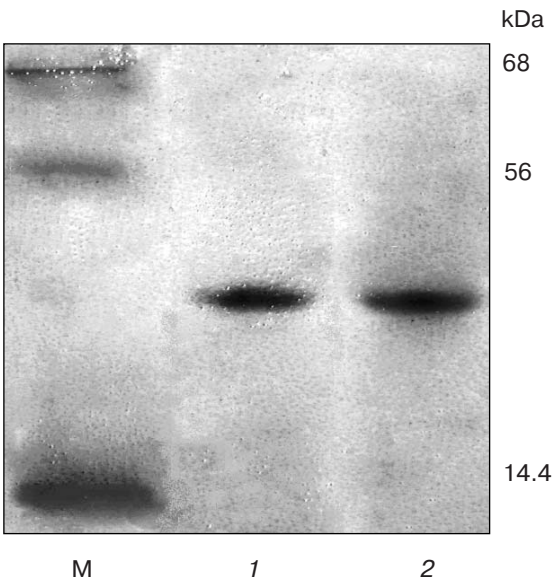

Fig. 6. Electrophoresis of *B. intermedius* subtilisin-like proteinase fractions under native conditions. Lanes: *1*) first fraction; *2*) second fraction; *M*, marker proteins: lysozyme (14.4 kDa), phosphatase (56 kDa), and hemoglobin (68 kDa).

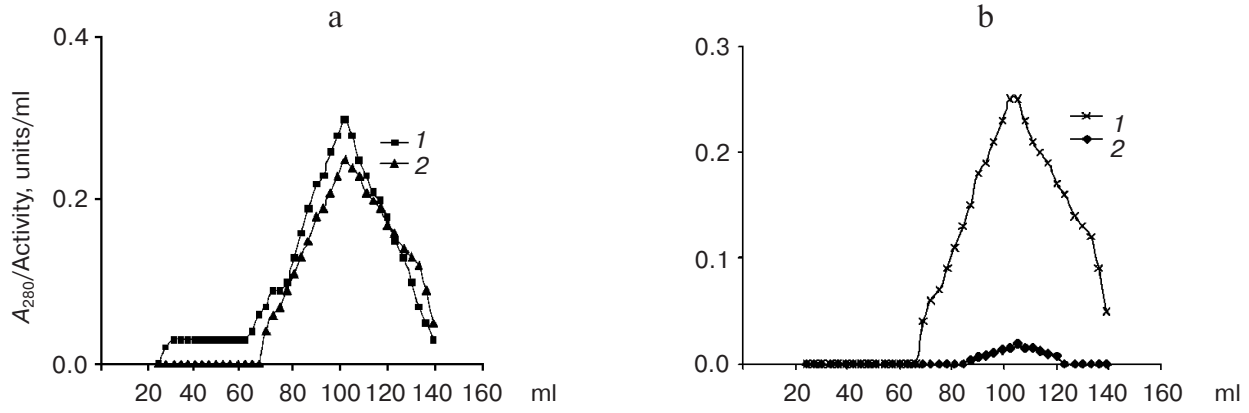

Fig. 7. Gel filtration of *B. intermedius* subtilisin-like proteinase fractions on Sephadex G-100 after dialysis against Tris-HCl buffer, 1% EDTA: a) first fraction; b) second fraction; *1*) protein amount; *2*) activity.

To remove calcium ions from the AprBi proteinase fractions, each of their solutions was dialyzed against Tris-HCl buffer, 1% EDTA, pH 8.5. Subsequent gel filtration showed that the second fraction of AprBi proteinase, devoid of calcium ions, not only does not form dimers, but completely looses its catalytic activity (Fig. 7b). Activity of the first fraction decreased after a similar procedure by 70% compared to its activity in the presence of calcium ions (Figs. 5a and 7a). This means that dimer formation by the second fraction of AprBi proteinase may be due to bonding of two protein globules via calcium ions in calcium-binding sites.

So, we suppose that the difference between the AprBi proteinase catalytic and enzymatic properties, corresponding to different stages of bacillar growth, is caused by dimer formation for the second proteinase fraction. In the physiological aspect, dimer formation can be considered as a characteristic enzyme peculiarity that protects the enzyme against autolysis.

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