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EXPERIMENTAL ARTICLES

Membrane-Bound Forms of Serine Proteases in *Bacillus intermedius*

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Abstract—Proteolytic proteins solubilized from the membrane of *Bacillus intermedius* were studied by electrophoresis. The content of membrane-bound proteinases was lower in cells grown in the presence of glucose. Proteinase enzymograms revealed four molecular forms of subtilisin and four molecular forms of glutamyl endopeptidase. The electrophoretic mobility of one of the molecular forms was similar to those of the mature extracellular proteinases. Chromatography of membrane proteins on a MonoS column yielded four protein fractions that caused hydrolysis of Z-Glu-pNA and four fractions that caused hydrolysis of Z-Ala-Ala-Leu-pNA, which is in agreement with the results of electrophoresis. The molecular forms of proteinases identified in the membrane may reflect various stages of biogenesis of the corresponding extracellular enzymes.

Key words: subtilisin-like thiol-dependent proteinase, glutamyl endopeptidase, membrane, molecular forms, *Bacillus intermedius.*

The secreted proteases of bacilli are synthesized in the cytoplasm as inactive precursors, whose molecules contain an additional polypeptide segment (propeptide) that follows a signal peptide (prepeptide) [1, 2]. Protease maturation and precursor activation occur via multistep processing and cleavage of both signal peptide and propeptide. The propeptide is assumed to maintain the precursor in inactive form during translocation and to direct folding of the protein globule, thus promoting active enzyme formation at the stage of the release from the membrane [1]. The molecular mechanism underlying precursor cleavage, the stages of mature molecule formation, and location of these processes are still not exactly established, although they are of great interest for understanding the biogenesis of secreted proteins.

We have shown previously that *Bacillus intermedius* 3-19 excretes serine proteases into the medium. Among these enzymes, at least 70% are subtilisin-like thiol-dependent proteinases, and about 10% represent glutamyl endopeptidase [3]. Our study of the location of proteolytic enzymes showed that at least 12% of glutamyl endopeptidase (EC 3.4.21.19, 23 kDa) and subtilisin-like thiol-dependent proteinase (EC 3.4.21.66, 32 kDa) remain bound to the membrane [3]. In the presence of inorganic salts of Mg^{2+} and Ca^{2+} (1.0–1.5 M), these proteins can readily be solubilized from the membrane.

In this study we aimed to identify the membrane-associated molecular forms of proteinases from *B. intermedius* using electrophoretic and chromatographic methods. The results obtained provide insight into the mechanism of the mature enzyme formation.

MATERIALS AND METHODS

The subject of this study was the strain of *Bacillus intermedius* 3-19 (Str 500), which is a streptomycin-resistant mutant of the wild-type strain *B. intermedius* 7P from the collection of Kazan State University.

Bacteria were grown in a medium of the following composition (%): peptone, 2; $CaCl_2 \cdot 2H_2O$, 0.01; $MgSO_4 \cdot 7H_2O$, 0.03; NaCl, 0.3; $MnSO_4$, 0.01. Before sterilization, the pH was adjusted to 8.5 with a solution of NaOH. Before inoculation, the following solutions were added to the final concentrations indicated: Na_2HPO_4 (0.01% in the control medium and 0.025% in the experimental medium), glucose (1%), gelatin (1%), and casein (1%). Bacteria were grown in Erlenmeyer flasks at a ratio of medium to flask volumes of 1 : 5 on a rotary shaker (200 rpm) in a Braun temperature-controlled chamber (Germany) at 30°C. Cells of a 48-h culture grown on a medium containing streptomycin sulfate served as the inoculum (1 vol %). The antibiotic was introduced in an amount of 500 μg/ml before inoculation. Bacterial growth was monitored nephelometrically on a KFK-2 photoelectric colorimeter at 590 nm.

The cells were harvested by centrifugation and washed with a 15-fold volume of 10 mM Tris–HCl buffer (pH 7.5) containing 10% sucrose until no pro-

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Specific activity (U/g biomass) serine proteinases of *B. intermedius* in the membrane fraction and culture liquid (CL) during growth on various media

Exogenous factors	Z-Ala-Ala-Leu-pNA		Z-Glu-pNA		Casein	
	CL	Membrane	CL	Membrane	CL	Membrane
Control medium	9800*	370*	500*	70*	1500**	90*
Na_2HPO_4	32000*	400*	1800*	75*	5200*	85*
Gelatin	24000*	380*	1300*	80*	3500**	80*
Casein	21000*	350*	1200*	88*	3320*	83*
Glucose	2500*	88*	160*	18*	400	19**

Notes: * < 10%; ** < 15%.

teolytic activity could be determined in the washing buffer. Then the cells were centrifuged once more.

Cell protoplasts of *B. intermedius* 3-19 and their lysates were obtained as described earlier [3]. To solubilize enzymes from protoplasts, the latter were treated with inorganic salt solutions (1 M $CaCl_2$, 1 M $MgCl_2$) in a 1 mM Tris–HCl buffer, pH 8.0, in the presence of 20% sucrose. After incubation for 20 min at room temperature, the mixture was centrifuged at 10000 *g* for 30 min. The proteins solubilized from membranes were desalted by gel-filtration through a PD-10 column with G-25 (Pharmacia, Sweden). Desalted fractions were passed through a membrane filter with pore diameter of 0.22 μm. Then, protein chromatography was performed on a column with MonoS HR 5/5 using an FPLC system (Pharmacia, Sweden) and a linear NaCl gradient from 0 to 1.5 M in a 0.015 M Na-acetate buffer (pH 6.3) containing 0.5 mM $CaCl_2$.

To obtain membrane preparations, protoplasts were centrifuged at 1000 *g*, and their lysis was induced by osmotic shock (treatment with a 5 mM Tris–HCl buffer, pH 7.8, at 4°C). The mixture was incubated with DNase (1 mg/ml) (Serva, Germany) for 30 min at 30°C and centrifuged at 3000 *g* for 1 h.

The protein concentration was measured on a spectrophotometer (assuming that A_{280} = 1 in a 1-cm-thick cuvette corresponds to a protein concentration of 1 mg/ml) and by the Bradford method [4] (the standard curve was obtained using bovine serum albumin).

Caseinolytic activity was measured by Kaverzneva's method [5] using as the substrate 2% casein solution (Serva, Germany) in 0.1 M Tris–HCl buffer, pH 8.5. One unit of caseinolytic activity was defined as the amount of enzyme that catalyzed a release of 1 μmol of tyrosine in 1 min.

The activity of the thiol-dependent serine proteinase was determined from the cleavage of the chromogenic peptide substrate Z-Ala-Ala-Leu-pNA by the method described in [6]. One unit of enzymatic activity was defined as the amount of enzyme that hydrolyzed 1 μmol of substrate in 1 min. The result obtained was multiplied by the coefficient 1000.

The activity of glutamyl endopeptidase was measured by the method described in [6]. *para*-Nitroanilide of carbobenzoxy-L-glutamic acid (Z-Glu-pNA) served as the substrate. One unit of activity was defined as the amount of enzyme that hydrolyzed 1 [mu]mol of substrate in 1 min. The result obtained was multiplied by the coefficient 1000.

The proteins of the cell envelope and cytoplasm were studied by electrophoresis in 12.5% polyacrylamide gel in the presence of sodium dodecyl sulfate and β-mercaptoethanol (the Laemmli method [7]). To obtain proteinase enzymograms, electrophoresis of the cell fractions in 12.5% polyacrylamide gel was conducted in the absence of sodium dodecyl sulfate and β-mercaptoethanol in the Laemmli buffer system [7]. After electrophoresis, the gel was fragmented to elute

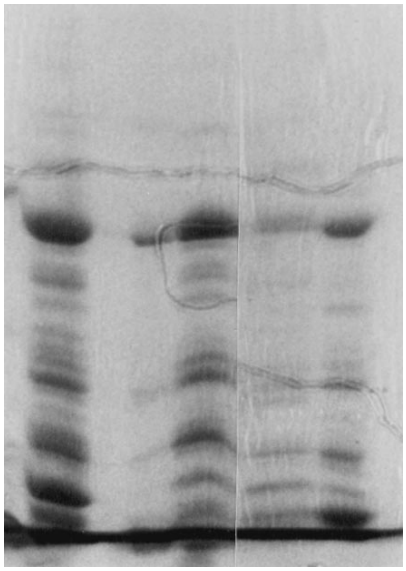

Fig. 1. Electrophoresis in 12.5% SDS-PAAG of the fraction of proteins solubilized with a 1 M solution of $CaCl_2$ from membranes of *B. intermedius* cells grown on media supplemented with (*1*) 1% gelatin, (*3*) 0.025% inorganic phosphate, (*2*, *4*, *5*) 1% glucose.

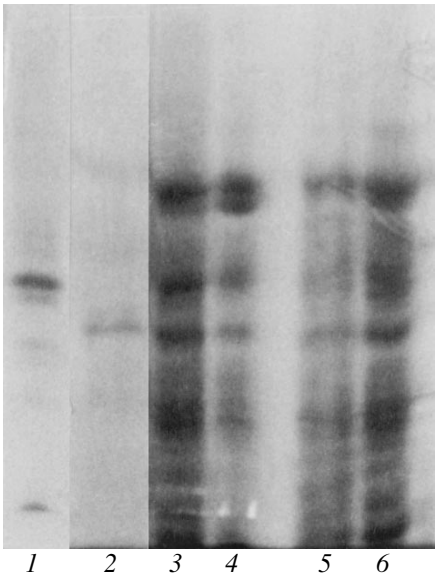

Fig. 2. Electrophoresis in 12.5% SDS-PAAG of the fraction of proteins solubilized from membranes of *B. intermedius* and of the homogeneous extracellular proteinases. Lane *1*, extracellular thiol-dependent proteinase; lane *2*, extracellular glutamyl endopeptidase; lanes *3* and *4*, proteins solubilized from membranes of cells grown on media supplemented with 0.025% inorganic phosphate; lanes *5* and *6*, proteins solubilized from membranes of cells grown on media supplemented with 1% gelatin. The membrane proteins were solubilized with solutions of $CaCl_2$ and $MgCl_2$ (1 M).

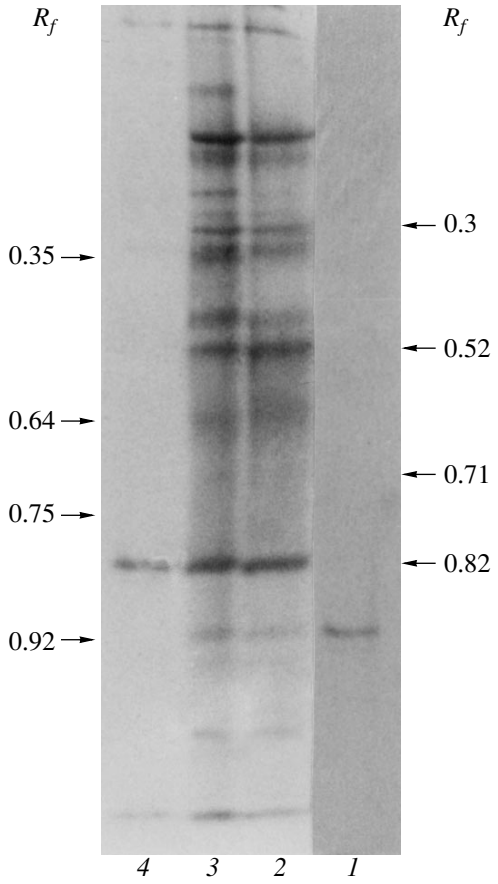

Fig. 3. Electrophoresis in 12.5% SDS-PAAG of (*2*, *3*) the fraction of proteins solubilized from membranes of *B. intermedius* cells and of homogeneous extracellular proteinases: (*1*) extracellular thiol-dependent proteinase and (*4*) extracellular glutamyl endopeptidase. Arrows on the left indicate bands that display hydrolytic activity with respect to Z-Ala-Ala-Leu-pNA; arrows on the right indicate bands that display hydrolytic activity with respect to Z-Glu-pNA.

the enzymes with a 0.25 M Tris–HCl buffer, pH 8.5, and determine their activity from the cleavage of casein and specific substrates.

Homogeneous enzymes (subtilisin-like thiol-dependent proteinase and glutamyl endopeptidase) were isolated from the culture liquid by the methods described earlier [8, 9].

Statistical treatment of the results was performed using Microsoft Excel software. Mean square deviations σ were calculated; the results were considered significant at $\sigma \le 15\%$. The significance of differences was estimated using Student's test, taking $P \le 0.05$ as the significance level.

RESULTS AND DISCUSSION

B. intermedius was grown under conditions favorable for active synthesis and secretion of proteinases (on media containing inorganic phosphate (Pi), gelatin, and casein), as well as under conditions leading to a sharp decrease in the synthesis of extracellular proteinases (in the presence of glucose in the medium). The proteolytic enzyme activities, namely the activities of glutamyl endopeptidase and subtilisin-like thiol-dependent proteinase, were determined in the culture liquid and in the membrane protein fraction of *B. intermedius*. It can be seen from the table that in the fraction of membrane proteins, the specific activity (U/g of biomass) of glutamyl endopeptidase and thiol-dependent proteinase, determined from the cleavage of specific substrates, remained at the level of the control variant. In contrast, in the culture liquid, the activity increased two- and fourfold on the media containing Pi and gelatin, respectively. On glucose-containing medium, both extracellular and membrane-bound enzymatic activities were four times lower than in the control.

The stable content of membrane-bound proteinases in the presence of various exogenous factors (casein, gelatin, and Pi) that induce unequal increases in specific activities of extracellular enzymes may be explained by a low number of the membrane sites involved in protein translocation. According to the published data, the potential number of these sites in *B. subtilis* is estimated as 2.5×10^4 per cell [10]. In *E. coli*, the maximum number of the translocation sites estimated at complete membrane saturation during secretion of the hybrid protein malE/lacZ was 2.0×10^4 [11].

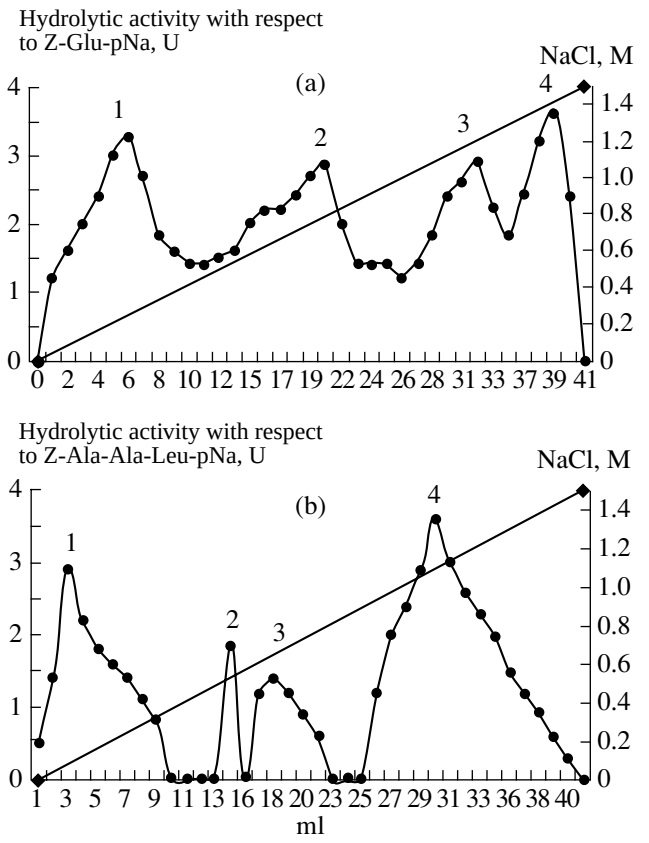

Fig. 4. Chromatography on the MonoS column in NaCl gradient (0.0–1.5 M) of proteinases solubilized from membranes with $CaCl_2$. (a) Hydrolytic activity with respect to Z-Glu-pNA; (b) hydrolytic activity with respect to Z-Ala-Ala-Leu-pNA.

Electrophoretic analysis of proteins from the membrane fraction of *B. intermedius* was conducted in polyacrylamide gel in the presence of sodium dodecyl sulfate. Cells grown on media with gelatin, Pi, and glucose were fractionated, and the fraction of proteins solubilized from membrane by a solution of $CaCl_2$ (1 M) was analyzed by electrophoresis. The sample loads were equal in terms protein concentration (0.25 A_{280} units per 1 ml of solution). The results obtained are shown in Fig. 1. The membrane extracts yielded protein bands whose intensity decreased when the bacteria were grown on glucose-containing medium. The inhibitory effect of glucose on the formation of proteins solubilizable from the membrane by a solution of $CaCl_2$ (1 M) testifies to the presence of secreted proteinases in this cell fraction. This conclusion is in agreement with the data showing that in *B. intermedius*, extracellular proteolytic activity correlates with the activity of proteinases bound with the cytoplasmic membrane [3].

The results of SDS-electrophoresis of proteins solubilized from the membrane and of homogeneous preparations of subtilisin-like thiol-dependent proteinase and glutamyl endopeptidase, obtained from the culture liquid of *B. intermedius* 3-19, are shown in Fig. 2. The culture was grown on media containing Pi (0.025%) and gelatin (1%). It can be seen that the electrophoretic patterns of the membrane extracts contained protein bands with a mobility (R_f) similar to that of the mature extracellular enzymes.

Proteinase enzymograms obtained using the oligopeptide substrates Z-Ala-Ala-Leu-pNA and Z-Glu-pNA revealed four molecular forms of subtilisin (R_f 0.35, 0.64, 0.75, and 0.92) and four molecular forms of glutamyl endopeptidase (R_f 0.3, 0.52, 0.71, and 0.82) (Fig. 3). One of the molecular forms of each enzyme solubilized from the membrane had an electrophoretic mobility similar to that of the mature proteins: $R_f = 0.82$ and $R_f = 0.92$ for glytamyl endopeptidase and subtilisin, respectively. The lower R_f values were characteristic of the remaining three molecular forms of both enzymes.

The molecular forms of the membrane proteinases revealed in this study may correspond to various stages in biogenesis of the extracellular enzymes. According to published data, the precursors of secreted proteinases of bacilli, except for the signal one, contain a propeptide sequence [1, 2]. Maturation of extracellular proteins occurs via multistep processing, which yields intermediate forms differing in length. There are data suggesting that the formation of extracellular serine proteinases in bacilli occurs via autoprocessing [12], and, in the course of secretion, the active form of these proteins first appears in the membrane.

The proteins solubilized from the membrane were desalted on a PD-10 column, which was followed by chromatography on a MonoS column in the FPLC system with a sodium chloride gradient from 0 to 1.5 M. This procedure yielded four protein fractions active in the hydrolysis of Z-Glu-pNA and four protein fractions active in the hydrolysis of Z-Ala-Ala-Leu-pNA (Fig. 4). Four peaks of proteins with a glutamyl endopeptidase activity were obtained at the NaCl concentrations of 0.18, 0.74, 1.18, and 1.42 M. Four peaks of proteins with the activity of thiol-dependent proteinase were obtained at the NaCl concentrations of 0.08, 0.48, 0.62, and 1.06 M. All fractions retained their activity in the presence of EDTA (2 mM) and *o*-phenanthroline (2 mM); in the presence of PMSF (2 mM), inhibition of the activity toward Z-Ala-Ala-Leu-pNA was observed. Thus, chromatography of proteins solubilized from the membrane confirmed the electrophoresis data that suggested the presence of several molecular forms of extracellular proteinases in the membranes of *B. intermedius.*

Three cell-associated precursors of metalloprotease were identified in *Pseudomonas aeruginosa* [13]. The authors determined the cleavage sites involved in the proteolytic processing of this enzyme. Site-directed mutagenesis of the corresponding amino acids, as well as His-223 substitution in the active center of the protein, resulted in accumulation of inactive proelastase in the membrane [14]. PrsA lipoprotein (33 kDa) bound to the outer surface of the cytoplasmic membrane was found and characterized in bacilli [15]. This protein is

involved in the activation of the *B. subtilis* subtilisin [15]. Increasing the content of protein PrsA in cells by the introduction of a multicopy plasmid led to a two- to sixfold higher level of secretion of α-amylase and protease. PrsA is 30% homologous to PrtM protein from *Lactococcus lactis*, which is involved in folding the secreted serine protease [16]. The authors concluded that the PrsA lipoprotein of bacilli is an extracellular molecular chaperone located on the membrane outer surface. Interaction with this protein occurs on the outer surface of the membrane and is a stage of enzyme maturation.

Thus, during the release of serine proteinases from *B. intermedius* cells into the culture liquid, membrane-bound forms of the corresponding enzymes are formed: forms with R_f 0.35, 0.64, 0.75, and 0.92 in the case of subtilisin and forms with R_f 0.3, 0.52, 0.71, and 0.82 in the case of glutamyl endopeptidase. These forms are, presumably, the precursors of mature extracellular proteins.

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