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Psychrophilic Trypsin-Type Protease from *Serratia proteamaculans*

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Abstract—A preparative method for purification of a novel protease from the psychrotolerant Gram-negative microorganism *Serratia proteamaculans* (PSP) was developed using affinity chromatography on BPTI-Sepharose. It yielded electrophoretically homogeneous PSP preparation of 60 kD. The PSP properties (temperature and pH stability, high catalytic efficiency) indicate that this enzyme can be defined as a psychrophilic protease. Inhibitory analysis together with substrate specificity indicates that the studied PSP exhibits properties of serine trypsin-like and Zn-dependent protease.

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Psychrophilic and psychrotolerant organisms constantly or part of time, correspondingly, living at low ambient temperatures produce "cold-adapted" enzymes. These organisms are widely distributed in the living world and their enzymes metalloproteinases (e.g. cellulases, collagenases) have industrial application as washing agents, in the process of leather treatment, in food industry, and in molecular biology [1]. Enzymes isolated from these organisms exhibit higher catalytic efficacy but lower thermostability than their mesophilic analogs; higher catalytic activity is usually observed not only at 20-25°C but also at temperatures close to 0°C [2]. Enzymes from psychrophilic organisms now represent attractive objects for intensive studies not only because of their high biotechnological potential. The search for new psychrophilic enzymes and study of their structural–functional features also represent significant theoretical importance for enzymology. For example, such enzymes are good models for studies of protein folding [3] and temperature adaptation [4].

The psychrotolerant Gram-negative microorganism *Serratia proteamaculans* identified during wide screening of natural and museum microorganisms has been used as the research object of this study. Earlier it was demonstrated that the strain *S. proteamaculans* 94 exhibits high collagenolytic activity at low (above 0°C) temperatures; the gene for new thermolysin-like metalloprotease was cloned and its protein product named protealysin was characterized [5]. Medical and biological studies revealed that this strain is not pathogenic for humans and animals and it meets safety criteria required for industrial microorganisms.

In the present study, we have isolated from biomass homogenate of the psychrotolerant bacterium *S. proteamaculans* a proteolytic enzyme (PSP) hydrolyzing a standard trypsin substrate, N_α-benzoyl-DL-arginine *p*-nitroanilide.

Abbreviations: ATEE) N_α-acetyl-L-tyrosine ethyl ester; BAPNA) N_α-benzoyl-DL-arginine *p*-nitroanilide; BPTI) bovine pancreatic trypsin inhibitor; DFP) diisopropylfluorophosphate; DMSO) dimethylsulfoxide; DTDP) 4,4′-dithiodipyridine; PMSF) phenylmethylsulfonyl fluoride; PSP) *Serratia proteamaculans* protease; STI) soybean trypsin inhibitor; TLCK) N_α-*p*-tosyl-L-lysyl-chloromethyl-ketone; Z-Lys-S-Bzl) N_α-benzyloxycarbonyl-L-lysine thiobenzyl ester; buffer A) 0.1 M Tris-HCl, pH 8.0, 50 mM $CaCl_2$; buffer B) 0.1 M Tris-HCl, pH 8.0; buffer C) 0.1 M Tris-HCl, pH 9.0, 50 mM $CaCl_2$, 1 mM $MgCl_2$; buffer D) 10 mM Hepes-KOH, pH 7.5, 1 mM $MgCl_2$.

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MATERIALS AND METHODS

Reagents. The following chemicals were used in this study: N_α-benzoyl-DL-arginine *p*-nitroanilide (BAPNA), N_α-benzyloxycarbonyl-L-lysine thiobenzyl ester (Z-Lys-S-Bzl), egg white ovomucoid, soybean trypsin inhibitor (STI), bovine pancreatic trypsin inhibitor (BPTI), Q-Sepharose, N_α-*p*-tosyl-L-lysyl-chloromethyl-ketone (TLCK), 4,4′-dithiodipyridine (DTDP), *o*-phenanthroline, phenylmethylsulfonyl fluoride (PMSF), iodoacetate, 2-carboxy-2-hydroxy-5′-sulformasyl benzene (zincon), and trifluoroacetic acid (TFA) (HPLC grade) from Sigma (USA); Sepharose 4B and PD-2 columns from Pharmacia (Sweden); acrylamide, ammonium persulfate, N,N′-methylene-*bis*-acrylamide, N,N,N′,N′-tetramethylethylene diamine, Protein Assay kit, and molecular weight protein markers for electrophoresis from Bio-Rad (USA); Tris from Merck (Germany); EDTA, glycerol, glycine, β-mercaptoethanol, SDS, Coomassie R-250, and N_α-acetyl-L-tyrosine ethyl ester (ATEE) from Serva (Germany); N_α-benzyloxycarbonyl-L-alanyl-L-alanyl-L-arginine *p*-nitroanilide (N-Z-Ala-Ala-Arg-*pNA*), N_α-succinyl-glycyl-L-prolyl-L-lysine *p*-nitroanilide (N-Suc-Gly-Pro-Lys-*pNA*), and N_α-succinyl-L-alanyl-L-alanyl-L-prolyl-L-lysine *p*-nitroanilide (N-Suc-Ala-Ala-Pro-Lys-*pNA*) from Bachem (Germany); dimethyl sulfoxide (DMSO) from Fluka (Germany); Hepes from Gerbu (Germany); acetonitrile (HPLC grade) from Kriokhim (Russia); lyophilized bovine trypsin (EC 3.4.23.1) was produced by the Factory for Medical Preparations (St. Petersburg, Russia).

The peptide H-$(Gly)_5$-Asp-Lys-Phe(NO_2)-Gly was synthesized by I. A. Prudchenko (Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow); the peptides H-Ser-Ile-Pro-Lys-Ser-Leu-Phe-Glu-OH and H-Ser-Asn-Pro-Lys-Ser-Asp-Phe-Glu-NH_2 were kindly presented by T. S. Zamolodchikova (Institute of Bioorganic Chemistry, Russian Academy of Sciences). Other chemicals of especially pure or chemically pure grades were from local suppliers.

Preparation of BPTI-Sepharose. Sepharose 4B was activated by the method of March et al. [6]. BPTI (120 mg) dissolved in 50 ml of 0.2 M bicarbonate buffer, pH 9.5, was condensed with activated Sepharose at 4°C for 20 h under shaking. The resultant sorbent was then incubated for 4 h with 0.1 M ethanolamine at room temperature, sequentially washed with 1 liter of 0.1 M formate buffer, pH 3.0, containing 0.5 M NaCl, 1 liter of 2 M urea with 0.5 M NaCl, 1 liter of 0.1 M bicarbonate buffer, pH 10.0, containing 0.5 M NaCl, and 1 liter of 0.01 M Tris-HCl buffer, pH 8.0, containing 0.5 M NaCl. The capacity of this affinity sorbent was 0.75 mg trypsin per ml.

Electrophoresis in polyacrylamide gel (PAGE). Purity of enzyme preparations was evaluated by SDS-PAGE by the method of Laemmli using a Mini-Protean 3 system. Concentrations of separating and stacking gels were 14 and 4%, respectively.

Gelatinase activity was detected using zymography in 14% polyacrylamide gel in the presence of 0.026% gelatin. Samples were incubated in sample buffer without mercaptoethanol at 4°C for 30 min. Electrophoresis was also carried out at 4°C; gels were washed with 2.5% Triton X-100 and 10 mM Tris-HCl, pH 8.0, incubated in the same buffer at room temperature for 10-12 h, and stained with 0.1% Coomassie R-250. After washing proteases were detected as clear bands on a dark background.

The HPLC procedure was carried out using a Beckman System Gold chromatograph (Beckman, USA).

Absorbance was measured using a Gilford 2400-2 spectrophotometer (Gilford, USA). Hydrolysis of *p*-nitroanilide substrates was registered as the increase in absorbance at 405 nm (at 25°C) occurring in the process of formation of free *p*-nitroaniline ($\Delta\varepsilon_{405}$ = 10,400 $M^{-1}\cdot cm^{-1}$).

Protein concentration was determined by the method of Bradford using a Bio-Rad Protein Assay kit and bovine serum albumin as the standard (BioRad).

Trypsin activity assay using BAPNA as substrate. *Method 1 (standard assay of trypsin activity).* A solution of 1 mM BAPNA (1.425 ml) in 0.1 M Tris-HCl buffer, pH 8.0, containing 50 mM $CaCl_2$ (buffer A) and 10% DMSO was mixed with 75 μl of enzyme preparation in a 1-cm quartz cuvette.

Method 2 (modification for PSP). Buffer A (1.44 ml) was mixed with 30 μl of 10 mM BAPNA in DMSO and 30 μl of the enzyme preparation. The concentration of active PSP was determined by titration with a solution containing a known concentration of BPTI. In kinetic experiments (for determination of hydrolysis constants) the incubation mixture contained from 33 μM to 0.67 mM BAPNA, 2 nM PSP, and 6.7% DMSO in buffer A.

Determination of temperature and pH stability of PSP. Enzyme aliquots were incubated in buffer A with or without 1 mM $MgCl_2$ at various temperatures; in pH stability studies the enzyme was incubated in buffers with various pH values (Tris-HCl or Na-acetate in the absence or in the presence of Mg^{2+}) at 25°C for 1 h and enzyme activity was determined using standard methods (1 or 2) and BAPNA as substrate.

Trypsin activity assay with Z-Lys-S-Bzl as substrate. The enzyme activity was registered by the increase in absorbance at 324 nm and 25°C, which accompanied hydrolysis of the thiobenzyl ester (range of concentrations from 67 μM to 0.13 mM) followed by reaction of the formed SH group with 0.2 mM DTDP in 0.1 M Tris-HCl buffer, pH 8.0 (buffer B) or buffer A using $\Delta\varepsilon_{324}$ = 16,067 $M^{-1}\cdot cm^{-1}$.

Chymotrypsin activity assay. Activity was registered by the decrease in absorbance at 237 nm and 25°C during ATEE hydrolysis [7]; 1 mM ATEE in buffer A was mixed

with 30-100 μl of the enzyme preparation (total volume 1.5 ml).

Determination of initial rates of hydrolysis of peptide substrates by HPLC. A peptide substrate (0.1-0.2 mM) in buffer A was incubated with the enzyme (trypsin or PSP) at 25°C. At selected time intervals, aliquots of 20 μl were taken and analyzed using a Luna C18 column (250 × 4 mm) (Phenomenex, USA). The chromatographic procedure was run in 0.1% TFA using an acetonitrile gradient 0-60% at the elution rate 0.3 ml/min. The initial reaction rate was calculated by the ratio of peak area of substrate and of products (representing not more than 20% of substrate conversion). Difference in molecular optical absorbance at 222 nm was corrected after total hydrolysis.

RESULTS AND DISCUSSION

Purification of trypsin-like enzyme from *S. proteamaculans*. *Biomass preparation.* Under sterile conditions, a suspension of daily grown culture of *S. proteamaculans* (1 ml) was added to conical flasks containing 100 ml of the cultivation liquid (peptone-rich medium containing technical collagen). Biomass was grown under constant moderate stirring at 4°C for 5 days. A portion of the cultivation liquid without cell suspension was used as control. Biomass was separated from the cultivation liquid by centrifugation at 800*g* for 20 min. The sediment was suspended in five volumes of 0.1 M Tris-HCl buffer containing 50 mM $CaCl_2$, pH 9.0, and centrifuged again for removal of cultivation liquid.

Isolation of total protein preparation from S. proteamaculans. All procedures were carried out at 4°C. The biomass (freshly isolated or thawed) was suspended in five volumes of 0.1 M Tris-HCl buffer containing 50 mM $CaCl_2$, 1 mM $MgCl_2$, pH 9.0 (buffer C). Cells were disrupted using an ultrasound disintegrator (10 times for 6 sec). The suspension was centrifuged at 1000*g* for 20 min. We found that use of the buffer with pH 8.0 for cell disruption yielded two times higher protein content than the use of corresponding buffer with pH 9.0; however, activity of the enzyme of interest changed insignificantly. So in subsequent experiments we used buffer C, giving higher specific activity of this enzyme at the first purification stage.

Thus, BAPNA-hydrolyzing activity was detected in homogenate of *S. proteamaculans* biomass. We did not detect such activity in the cultivation liquid.

Development of the method for purification of a new trypsin-like protease required investigation of its pH and temperature stability. It is known that thermolability, a characteristic property of psychrophilic enzymes, reflects their structural features. The enzyme was stable during long term storage of homogenate at 4°C. Incubation at 20-25°C for 1 h did not influence the enzyme activity, but incubation for 12 h caused its total inactivation. These changes were more pronounced at 37°C: 50% inactivation was observed during incubation for 1 h. As a rule, pH optimum of psychrophilic enzyme stability is at alkaline pH, whereas in acidic media such enzymes are less stable [8-11]. Homogenate enzyme activity was nearly the same at pH 8.0, 9.0, and 10.0. Enzyme inactivation was noted at pH < 8.0. Total inactivation was observed during incubation of the enzyme preparation at pH 4.0 for 1 h.

Ion-exchange chromatography on Q-Sepharose. After cell disruption, supernatant (containing about 13.6 nmol of the enzyme of interest) was decanted and immediately applied onto a column packed with Q-Sepharose (sorbent volume of 10.5 ml) with the rate 20 ml/h. After sample application, the column was washed with buffer C. The enzyme was eluted by a gradient of ionic strength in buffer C (0-0.6 M NaCl; 100 + 100 ml). The volume of collected fractions was 6 ml. Protein elution was monitored by absorbance at 280 nm, and enzyme activity was assayed with BAPNA as substrate. Active enzyme was desorbed at ionic strength of 0.10-0.15 M NaCl (Fig. 1a). The enzyme activity yield was 86.7%.

Attempts at concentrating and dialysis of PSP preparations using Centricon-50 ultrafiltration device were not successful because of total inactivation of the enzyme. The purified preparation was stored at −20°C.

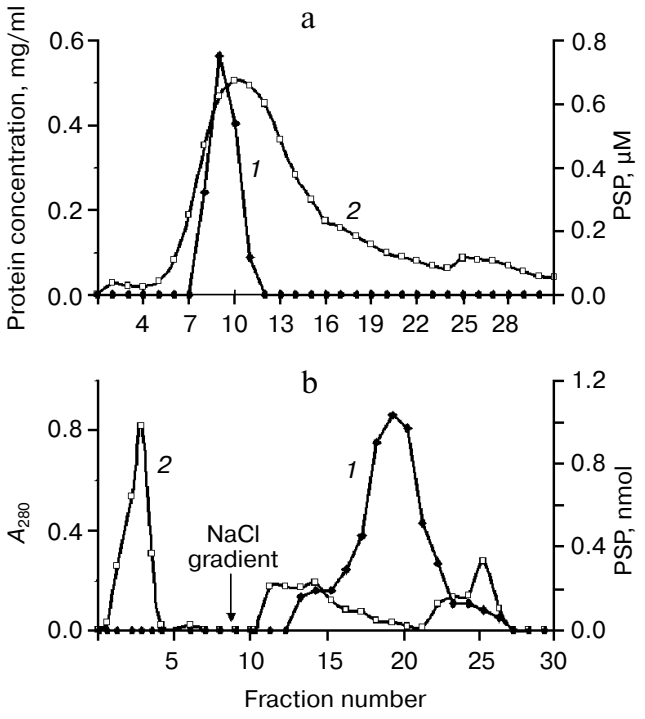

Fig. 1. a) Ion-exchange chromatography of total protein preparation from *S. proteamaculans* on Q-Sepharose: *1*) concentration of active PSP; *2*) protein concentration. b) Affinity chromatography of PSP preparation (partially purified on Q-Sepharose) on BPTI-Sepharose: *1*) active enzyme; *2*) A_{280}.

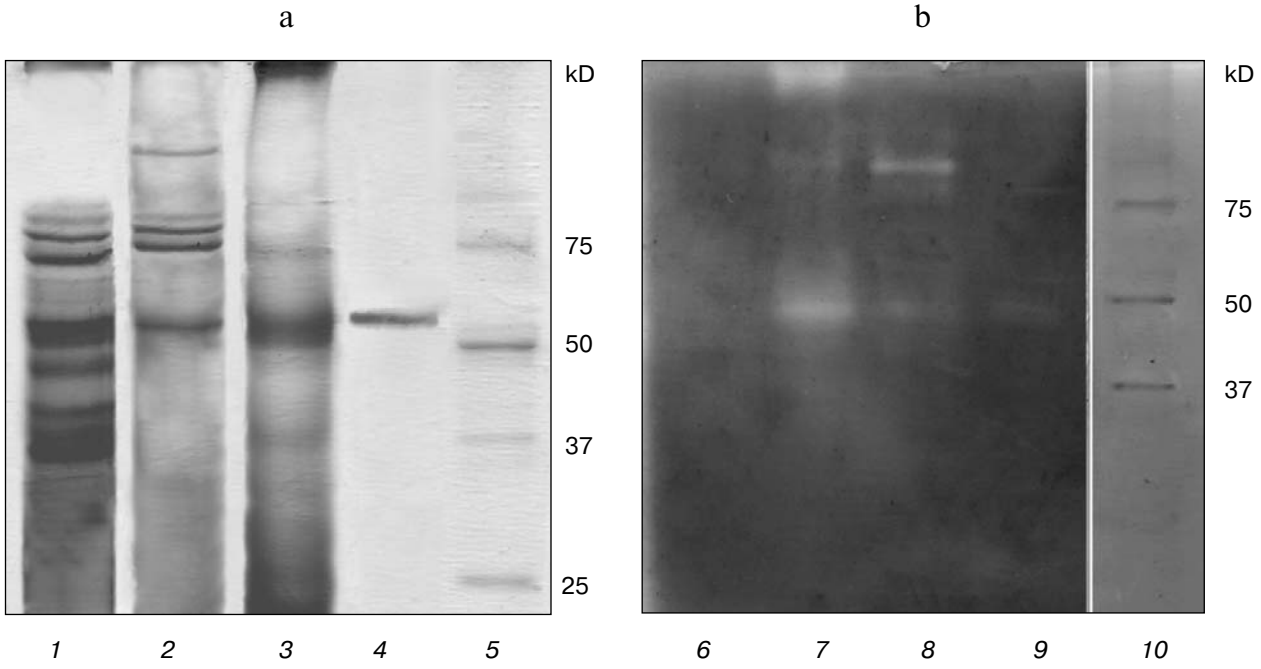

Fig. 2. a) Elution profile of PSP protein during enzyme purification (14% SDS-PAGE): *1*) total protein preparation after cell disruption; *2*) ion-exchange chromatography on Q-Sepharose; *3*) affinity chromatography on BPTI-Sepharose with 0.5 M NaCl elution; *4*) affinity chromatography on BPTI-Sepharose, elution with NaCl gradient of 0-0.5 M (Fig. 1b, fraction No. 20); *5*) protein markers. b) The zymogram of enzyme fractions obtained during affinity chromatography (Fig. 1b): *6*) fraction No. 20 (homogenous PSP); *7*) fraction No. 3 (washing with buffer A before the use of NaCl gradient); *8*) fraction No. 14; *9*) fraction No. 17; *10*) protein markers.

Electrophoresis in polyacrylamide gel revealed that the resulting preparations were not homogenous (Fig. 2). Determination of molecular mass of PSP was complicated by the fact that comparison of active and inactive fractions revealed at least three protein bands (60, 92, and 174 kD), which were not found in the inactive fractions (Fig. 2a).

Trypsin-like enzymes exhibit calcium-dependence and so all purification procedures employed 50 mM $CaCl_2$. However, certain evidence also exists in the literature that in the case of metal-dependent enzymes 1 mM magnesium ions prevent complex formation between Tris-HCl and metal ions (possibly zinc) required for catalytic activity [12, 13], and this increases the stability of such enzymes.

Pilot experiments on isolation of the trypsin-like protease from *S. proteamaculans* were carried out in the absence of magnesium ions in the buffer (both on the stage of cell disruption and ion-exchange chromatography on Q-Sepharose). This was accompanied by significant loss of the active protein during all stages of isolation and purification (yield of active PSP was 37%). The use of 1 mM $MgCl_2$ increased yield of the active PSP preparation after Q-Sepharose up to 87%.

The PSP preparation isolated in the presence of magnesium ions exhibited higher thermostability and resistance to shift of pH values into the acidic region; nevertheless, total enzyme inactivation was observed during incubation for 1 h at both 42°C (pH 8.0) and 25°C (pH 3.5). Higher concentration of Mg^{2+} (10 mM) ions caused inhibition of enzyme activity by 12.6%.

Enzyme instability at acidic pH significantly complicated its subsequent purification (after ion-exchange chromatography). Indeed, use of standard methods of affinity chromatography of trypsin-like enzymes using immobilized protein trypsin inhibitors including soybean trypsin inhibitor (STI) or bovine basic pancreatic trypsin inhibitor (aprotinin) (BPTI) requires acidic pH (<3.0) of the eluting solution for protein desorption from the affinity sorbent. Thus, development of the method for affinity chromatography PSP required additional detailed study of interaction between putative trypsin inhibitor ligands with this enzyme partially purified by ion-exchange chromatography on Q-Sepharose.

Experiments revealed that ovomucoid concentrations up to 0.2 mM did not influence enzyme activity, and STI caused 31% inhibition at the concentration of 35 μM. BPTI caused rapid and potent inhibition of this enzyme activity but its effect depended on the presence of calcium ions in the medium. For trypsin-like enzymes, such dependence was demonstrated for the first time. We found that inhibitor concentrations required for 50% inhibition of enzymatic activity differed by one order of magnitude in the presence and in the absence of calcium ions using both BAPNA and Z-Lys-S-Bzl as substrates

Table 1. Effect of BPTI on the activity of PSP (7.2 nM); [BAPNA] = 0.2 mM

Buffer	$[I]_{50}$, M
A	$8.0 \cdot 10^{-6}$
B	$1.4 \cdot 10^{-7}$
B*	$1.8 \cdot 10^{-7}$
0.01 M Hepes, pH 7.2	$4.0 \cdot 10^{-8}$
Buffer B, 0.5 M NaCl**	$\geqslant 2.0 \cdot 10^{-5}$

* Substrate Z-Lys-S-Bzl.
** [PSP] = 50 nM.

(Table 1). Enzyme inhibition was also influenced by the nature and concentration of the buffer and pH values. The most potent inhibition was found in 0.01 M Hepes-KOH buffer, pH 7.2 (Table 1). Ionic strength (NaCl) also influenced PSP inhibition by BPTI (Table 1). At 10 μM BPTI total inhibition of 0.2 mM BAPNA hydrolysis catalyzed by 45 nM PSP was observed in buffer B; in the presence of 0.1 and 0.5 M NaCl the inhibitory effect was reduced to 84 and 21%, respectively. (In the presence of 0.5 M NaCl, 20 μM BPTI caused 45% inhibition.)

Development of affinity chromatography method for isolation of homogenous PSP preparation. Data of Table 1 on inhibition of the trypsin-like enzyme from *S. proteamaculans* by BPTI were used for the development of the affinity chromatography method for subsequent purification of this enzyme on immobilized BPTI. We selected 10 mM Hepes-KOH buffer, pH 7.5, containing 1 mM $MgCl_2$ (buffer D) for adsorption of PSP preparation on the affinity sorbent. For desorption of the active enzyme from the immobilized inhibitor we proposed to use elution with the same buffer containing $CaCl_2$ and/or NaCl. This precludes the need for acidic buffers with pH of 2.0-3.0 for elution and allows homogenous enzyme preparation to be obtained.

Enzyme preparation obtained after ion-exchange chromatography on Q-Sepharose contained 0.15 M NaCl. Usually protein solutions applied onto an affinity sorbent contain 0.5 M NaCl. This prevents nonspecific protein sorption. In our case, use of such NaCl concentration was not applicable due to negative effect of rather high ionic strength on PSP binding to BPTI (Table 1). Moreover, a pilot affinity chromatography experiment revealed lack of enzyme binding to the affinity sorbent even in the presence of 0.15 M NaCl. Since dialysis of enzyme preparation obtained after ion-exchange chromatography (Centricon-50 and -30) resulted in its total inactivation, we used gel filtration on a PD-2 column for PSP transfer into buffer D. The yield of enzyme preparation was 90%.

Pilot (analytical) chromatography of the desalinated PSP preparation on BPTI-Sepharose demonstrated binding of the active enzyme to the immobilized inhibitor (10 nmol/ml sorbent); however, it was not eluted with 50 mM $CaCl_2$. Washing of the affinity sorbent with buffer D containing 0.5 M NaCl was accompanied by quantitative desorption of the active enzyme. Thus, 0.5 M NaCl in the buffer with alkaline pH (7.5) can be used for elution of PSP from immobilized BPTI.

However, the resulting preparation was not electrophoretically homogenous (Fig. 2a). Gelatin zymography also revealed gelatinase activity of this preparation (data not shown). The presence of impurities may be attributed to the use of solutions with low ionic strength for PSP application onto BPTI-Sepharose, promoting nonspecific sorption of contaminant proteins. Pulsed elution with NaCl caused contamination of the PSP preparation. So, in subsequent experiments we used NaCl gradient elution for isolation of homogenous PSP preparation.

We applied 6.11 nmol of PSP preparation in 4 ml of buffer D onto 2 ml of BPTI-Sepharose at a flow rate of 6 ml/h. The sorbent was washed with the same buffer until $A_{280} = 0$ (14 ml). PSP activity was not detected in the washing buffer. The enzyme was eluted by a 0-0.5 M NaCl gradient (25 + 25 ml) in buffer D at the same flow rate (collecting fractions of 2 ml each) (Fig. 1b). Electrophoretically homogenous PSP preparation was obtained at the ionic strength of 0.17-0.23 M NaCl (Fig. 2a) with 87% yield. According to electrophoresis, the molecular mass of the PSP was 60 kD. Before and after use of NaCl gradient (at 0.05-0.1 M NaCl) contaminant proteins were washed out of the affinity sorbent; they exhibited poor BAPNA activity but possessed gelatinase activity. According to zymography data, molecular masses of these proteins were 170-180, 90, and 50 kD, and the protease of 50 kD was homogenous (Fig. 2b). Homogenous PSP preparation was free of gelatinase activity.

Preincubation of 15 μl PSP (0.3 μM in buffer A) with 20 μl of 2% SDS for 20 min at 4°C resulted in total enzyme inactivation. Thus, during determination of gelatinase activity in PSP preparations where protein sample is incubated in a lysing buffer containing 2% SDS one should take into consideration possible enzyme inactivation.

We found abnormally high inhibitory effect of DMSO on PSP activity ($[I]_{50}$ = 7%). So we modified the method of enzyme activity assay employing method 2 (0.2 mM BAPNA, 2% DMSO) instead of method 1 (1 mM BAPNA, 10% DMSO).

Effect of temperature and pH on enzyme stability and catalytic activity. The isolated enzyme was more thermolabile than bovine trypsin; incubation of the purified enzyme for 1 h at 42°C caused its total inactivation. Mesophilic trypsin is inactivated at 60°C, whereas psychrophilic trypsin from *Paranotothenia magellanica* Forster fish living at low temperature is inactivated at 52°C [10] (Fig. 3a).

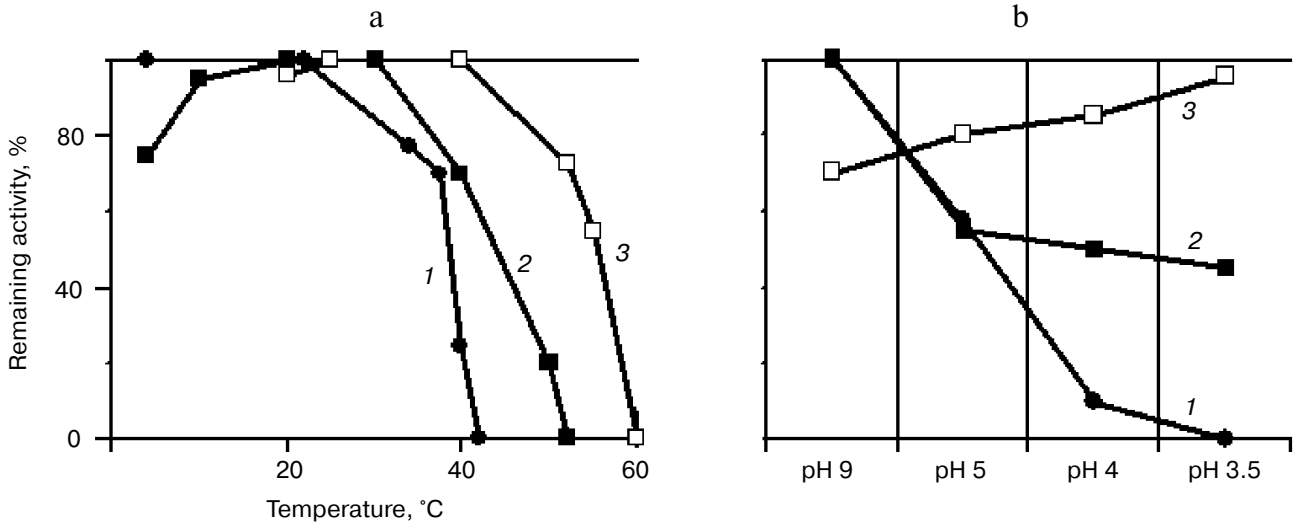

Fig. 3. Comparison of temperature and pH stability of PSP, psychrophilic trypsin from fishes living at low temperature, and mesophilic bovine trypsin. a) Temperature resistance: *1*) PSP; *2*) *P. magellanica* trypsin [10]; *3*) bovine trypsin [10]. b) pH tolerance: *1*) PSP; *2*) *G. ogac* trypsin [11]; *3*) bovine trypsin [11].

In the pH range 3.5-9.0, changes in stability of the studied enzyme were closer to the corresponding dependence for psychrophilic trypsins from fishes living at low temperature than to the pH stability of mesophilic bovine trypsin. It should be noted that mesophilic trypsin is more stable at acidic pH than in alkaline pH. For psychrophilic trypsins from fishes *P. magellanica* [10] and *Gadus ogac* [11] there was reversed dependence: stability at pH > 8.0 and rapid inactivation at acidic pH. In the case of protease from *S. proteamaculans* (PSP) the enzyme inactivation was especially high at pH < 3.0 (Fig. 3b).

The nature of temperature dependence of PSP activity (Fig. 4) is typical for psychrophilic enzymes [2, 14]: apparent maximal activity at 37°C (due to enzyme inactivation at moderate temperatures) and relatively high activity (>20% of maximal) at 4°C. Thus, this protease exhibits properties typical for enzymes from psychrophilic organisms.

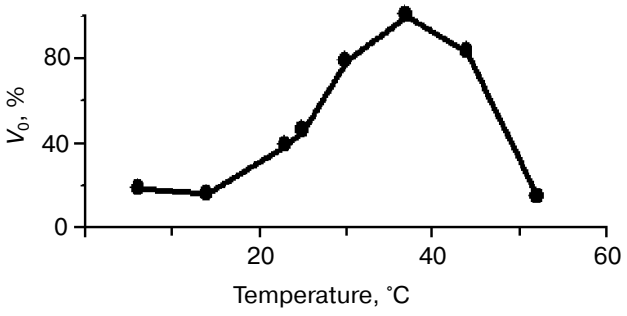

Fig. 4. Temperature dependence of PSP activity; [BAPNA] = 0.2 mM (method 2).

Substrate-inhibitory analysis. Search for a new trypsin-like protease from *S. proteamaculans* and also assay of catalytic activity during partial purification of this enzyme were carried out using the standard trypsin substrate (BAPNA). Indeed, we found BAPNA hydrolyzing activity in the homogenate of *S. proteamaculans* biomass. However, this activity might be attributed to various types of collagenases, such metal-dependent enzymes as serralysins and astacins, and also cysteine proteases. Consequently, it was necessary to analyze activity of this new protease from *S. proteamaculans* with respect to other trypsin substrates. Characterization of substrate specificity of this enzyme also required analysis for possible chymotrypsin activity.

Substrate specificity. Experiments revealed that both the homogenate of *S. proteamaculans* biomass and purified enzyme preparation hydrolyzing BAPNA were completely inactive with respect to the chymotrypsin substrate ATEE. This suggests lack of chymotrypsin-like activity of this enzyme.

Kinetic studies of BAPNA hydrolysis by this enzyme revealed the following constants: $K_m = 0.1$ mM and $k_{cat} = 90$ min^{-1}. This K_m value is close to that for trypsins from *Streptomycetes* [15, 16] and significantly lower than corresponding values for mammalian trypsin (4 mM) [17]. Such low K_m values are typical for psychrophilic trypsins [8-11].

The isolated enzyme effectively hydrolyzed the other classic trypsin peptide substrate, Z-Lys-S-Bzl, and also elongated *p*-nitroanilides—Z-Ala-Ala-Arg-*pNA* and Suc-Gly-Pro-Lys-*pNA*. However, even during prolonged incubation PSP did not hydrolyze Suc-Ala-Ala-Pro-Lys-*pNA*, the latter being effectively hydrolyzed by trypsin ($k_{cat}/K_m = 2.88 \cdot 10^7$ M^{-1}·min^{-1} [18]). Some other known

substrates of trypsin and trypsin-like proteases were also tested: H-$(Gly)_5$-Asp-Lys-Phe(NO_2)-Gly, H-Ser-Ile-Pro-Lys-Ser-Leu-Phe-Glu-OH, and H-Ser-Asn-Pro-Lys-Ser-Asp-Phe-Glu-NH_2. Using HPLC, we found that incubation of these peptides with PSP resulted in formation of the same products as in the case of trypsin, enteropeptidase [19], and duodenase [20]. This suggests that hydrolysis of the polypeptide chain occurs after a lysine residue. Effectiveness of hydrolysis of these substrates was much lower than that of arginine-containing substrates (Z-Ala-Ala-Arg-*pNA* and even BAPNA). Trypsin also preferred substrates containing an arginine residue at P1 position over substrates containing a lysine residue in it. However, in the case of PSP such preference was much more pronounced, especially with BAPNA. At 25°C, effectiveness of its hydrolysis by PSP (k_{cat}/K_m = $9{\cdot}10^5$ $M^{-1}{\cdot}min^{-1}$) was 20 times higher than the effectiveness of hydrolysis of this substrate by trypsin (k_{cat}/K_m = $4{\cdot}10^4$ $M^{-1}{\cdot}min^{-1}$) [17]. We found only one lysine substrate, Z-Lys-S-Bzl, which was effectively hydrolyzed by PSP. It is possible that PSP is a highly specific enzyme hydrolyzing polypeptide chains after arginine residues.

We also investigated the effect of calcium ions on the activity of PSP. Interestingly, they caused opposite effects on hydrolysis of different substrates. For example, 50 mM $CaCl_2$ slightly increased enzyme activity (by 28%) with BAPNA as substrate, but decreased it with Z-Lys-S-Bzl as substrate by 26%. Such phenomenon was not found with trypsin. We have already mentioned abnormal enzyme behavior towards BPTI in the presence and in the absence of calcium ions.

Effects of inhibitors and some other reagents on activity of PSP. Since BAPNA is a substrate not only for serine trypsin-like proteases but also for metallo- and cysteine proteases, its further typing required inhibitory analysis using group-specific inhibitors. We found that specific cysteine protease inhibitors, iodoacetamide and DTDP, did not influence enzyme activity. Mercaptoethanol concentrations up to 0.1% (14.3 mM) are known to activate thiol enzymes. However, mercaptoethanol inhibited PSP activity (Table 2). This indicates that PSP does not belong to the group of cysteine proteases.

Irreversible group-specific inhibitors of serine proteases. Interaction of diisopropylfluorophosphate (DFP) with serine proteases results in phosphorylation of a catalytically active serine residue. DFP inhibited PSP activity assayed with BAPNA and Z-Lys-S-Bzl (Table 2). Total PSP inactivation by 0.13 mM DFP occurred within 15-20 min. (Such time dependence is also typical for trypsins).

An active site serine residue selectively reacts with PMSF. Our experiments also revealed potent inhibition of PSP by this reagent (Table 2).

Chloromethylketones, which are amino acid derivatives, interact with $N\varepsilon^2$ histidine atom of serine proteases. Certain evidence also exists that they can alkylate other residues (e.g., methionine). N_α-*p*-tosyl-L-lysyl-chloromethyl-ketone (TLCK) is a specific trypsin inhibitor. It acts rather slowly, and so we preincubated PSP with 3 mM TLCK in buffer A at 25°C. After 5 min, remaining enzyme activity was 12.5% of control values. Total PSP inactivation was observed after 30 min (Table 2).

Table 2. Effect of inhibitors and some other reagents on activity of PSP (7.2 nM) with 0.2 mM BAPNA

Reagent	Reagent concentration, M	Remaining activity, %
DTDP*	$2.0 \cdot 10^{-4}$	100
Iodoacetamide	$1.0 \cdot 10^{-3}$	100
Mercaptoethanol	$1.6 \cdot 10^{-2}$	50
DFP	$1.3 \cdot 10^{-4}$	0
DFP**	$1.3 \cdot 10^{-4}$	0
PMSF	$2.3 \cdot 10^{-3}$	19.4
PMSF**	$1.8 \cdot 10^{-3}$	28.6
TLCK***	$3.0 \cdot 10^{-3}$	0
Zincon	$5.0 \cdot 10^{-4}$	56.2
EDTA***	$5.0 \cdot 10^{-3}$	61.5
o-Phenanthroline****	$5.0 \cdot 10^{-3}$	0
$Zn(AcO)_2$*	$1.0 \cdot 10^{-3}$	0
$CdCl_2$, $CuCl_2$*	$1.0 \cdot 10^{-3}$	0
$MgCl_2$*	$1.0 \cdot 10^{-3}$	100
	$1.0 \cdot 10^{-2}$	87

* Buffer A, buffer B.
** Substrate Z-Lys-S-Bzl, buffer A.
*** Preincubation for 30 min.
**** Buffer B.

Chelating agents and bivalent metal ions. In the very beginning of this work (based on literature data), we suggested that trypsin-like activity detected in homogenate of *S. proteamaculans* biomass might be attributed to a zinc-dependent enzyme of serralysin family. Indeed, such proteases were found in various Gram-negative bacteria (*Serratia* sp. E-15 [21], *Serratia marcescens* [22], *Pseudomonas aeruginosa* [23], etc.). Serralysins hydrolyze peptide bonds after Arg residues, particularly, in *p*-nitroanilides; their activity is sensitive to inhibition by such chelating agents as *o*-phenanthroline and EDTA. Effects of these chelating agents on PSP supported our hypothesis. The presence of 5 mM *o*-phenanthroline caused nearly immediate enzyme inactivation. (*o*-Phenanthroline is a highly reactive reagent employed for removal of zinc from zinc-containing enzymes.) EDTA (5 mM) also caused marked inhibition of PSP activity (Table 2).

It is known that Zn^{2+}-containing enzymes can be inhibited by such bivalent ions as Cd^{2+} and Cu^{2+} [24], as well as by Zn^{2+} [25]. Indeed, we found that in the micromolar concentration these cations caused inactivation of PSP (Table 2). Enzyme stabilization by low concentrations of Mg^{2+} typical for Zn^{2+}-containing enzymes [12, 13] was also demonstrated for PSP. Finally, PSP was markedly inactivated by zincon, a diagnostic reagent for zinc proteases (Table 2).

It remains unclear how to interpret all these data. According to inhibitory analysis using group-specific inhibitors, PSP can be referred to the group of serine proteases, and substrate specificity of PSP well corresponds to that of trypsin. It is possible that metal (Zn) is essential for stabilization of the enzyme structure, but we cannot exclude its putative role in catalysis.

Although vertebrate trypsins belong to the groups of the best studied representatives of serine proteases, trypsins from invertebrate organisms have been poorly investigated and they are referred to one common group [26]. Trypsins from psychrophilic microorganisms are not known. Recently, the psychrophilic bacterium *Colwellia* sp. NJ341 secreting serine protease exhibiting proteolytic activity with respect to casein was found in samples of Antarctic sea ice [14]. This enzyme exhibited similar behavior in response to effects of temperature and pH on activity and stability, sensitivity to PMSF inhibition as PSP; molecular mass of 60 kD, and some other properties are also similar in these enzymes. However, there is no information available on primary structure and proteolytic (trypsin or chymotrypsin) specificity of this enzyme.

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