ISSN 0006-2979, Biochemistry (Moscow), 2009, Vol. 74, No. 10, pp. 1164-1172. © Pleiades Publishing, Ltd., 2009.
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Oligopeptidase B from *Serratia proteamaculans*. I. Determination of Primary Structure, Isolation, and Purification of Wild-Type and Recombinant Enzyme Variants

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Received November 26, 2008
Revision received January 19, 2009

Abstract—A novel trypsin-like protease (PSP) from the psychrotolerant gram-negative microorganism *Serratia proteamaculans* was purified by ion-exchange chromatography on Q-Sepharose and affinity chromatography on immobilized basic pancreatic trypsin inhibitor (BPTI-Sepharose). PSP formed a tight complex with GroEL chaperonin. A method for dissociating the GroEL–PSP complex was developed. Electrophoretically homogeneous PSP had molecular mass of 78 kDa; the *N*-terminal amino acid sequence 1-10 was determined, and mass-spectral analysis of PSP tryptic peptides was carried out. The enzyme was found to be the previously unknown oligopeptidase B (OpdB). The *S. proteamaculans* 94 *OpdB* gene was sequenced and the producer strain *Escherichia coli* BL-21(DE3) pOpdB No. 22 was constructed. The yield of expressed His_6-PSP was 1.5 mg/g biomass.

DOI: 10.1134/S0006297909100137

Key words: oligopeptidase B, trypsin, *Serratia proteamaculans*, GroEL, fusion expression

Oligopeptidase B (OpdB) is a trypsin-like serine proteinase found in ancient unicellular eucaryotes, such as trypanosomes *Trypanosoma cruzi* [1], *Trypanosoma brucei* [2, 3], and *Trypanosoma evansi* [4] and leishmaniae *Leishmania major* [5] and *Leishmania amazonensis* [5]. Genes encoding this enzyme are also found in gram-negative pathogenic bacteria such as *Escherichia coli* [6-9], *Moraxella lacunata* [10], *Salmonella enterica* serovar *typhimurium* [11], mycobacteria *Mycobacterium tuberculosis* and *Mycobacterium leprae* [11], and spirochete *Treponema denticola* [12]. Oligopeptidases B are important virulence factors in trypanosomal infections such as African sleeping sickness and South American Chagas disease.

In *T. cruzi* causing Chagas disease, OpdB provides trypanosomal invasion on blood cells via activation of calcium-signaling factor [1]. In other trypanosomes that are not intracellular parasites (*T. brucei* and *T. evansi*), OpdB is released by dying cells into the mammal host blood circulatory system, where it keeps stability and catalytic activity because it is not inhibited by blood plasma inhibitors such as serpins, cystatins, and α_2-macroglobulin [2]. Parasitic OpdB catalyze abnormal degradation of host peptide hormones such as atrial natriuretic factor, thus being implicated in pathogenesis of African sleeping sickness and other trypanosomiases and leischmaniases [4]. It should be emphasized that genes encoding this enzyme are not found in mammals. Thus, OpdB of protozoan parasites are crucial for virulence and, hence, may serve as therapeutic targets in search for pharmaceuticals against these dangerous infections. Prokaryotic homologs

Abbreviations: BAPNA, N_α-benzoyl-*D*,*L*-arginine *p*-nitroanilide; BPTI, basic bovine pancreatic trypsin inhibitor; buffer A, 0.1 M Tris-HCl, pH 8.0, containing 50 mM $CaCl_2$ and 1 mM $MgCl_2$; buffer B, 10 mM Hepes-KOH, pH 7.5, containing 1 mM $MgCl_2$; buffer C, 20 mM potassium phosphate, pH 7.4, containing 0.5 M NaCl, 10 mM imidazole, 0.1% 2-mercaptoethanol, and 5% glycerol; DMSO, dimethyl sulfoxide; IPTG, isopropyl-β-*D*-thiogalactoside; NMWL, nominal molecular weight limit; OpdB, oligopeptidase B; PSP, proteinase from *Serratia proteamaculans*.

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of this protein are far less studied, but are also supposed to be important targets for antimicrobial chemotherapy [11].

Oligopeptidases B are members of prolyl oligopeptidase family S9 within the clan SC. This group of serine proteinases combines four subgroups differing in substrate specificity but possessing homology of their amino acid sequences, namely prolyl oligopeptidase, dipeptidyl peptidase, acylaminoacyl peptidase, and oligopeptidase B [13]. A characteristic feature of this family is the catalytic triad Ser554, Asp641, and His680 localized in the *C*-terminal catalytic domain that is structurally closer to lipases than to chymotrypsin or trypsin. It is the domain that chiefly determines the likeness between structures of various members of the family. *N*-Terminal domains more considerably differ in amino acid sequence than catalytic *C*-terminal ones – not only between members of various subgroups of this family, but even between OpdB variants from different sources. Nevertheless, the common feature is unusual *N*-terminal domain structure: 7-bladed β-propeller allowing oligopeptides to penetrate to the catalytic triad localized inside the major cavity at the interface of two domains and excluding big globular proteins from it. The maximum size of hydrolyzed peptide substrates is supposed to be no more than 3 kDa [14]. However, this limitation seems not to be absolute because restricted proteolysis of globular proteins, such as histones and aspartokinases I and III, was observed under the action of *S. enterica* OpdB [11] and *E. coli* oligopeptidase B [6], respectively. In this case, the enzyme hydrolyzes proteins devoid of secondary structure. So, it is supposed that oligopeptidases B are specialized enzymes providing protein processing in prokaryotes and lower eucaryotes and hydrolyzing denatured proteins [7, 15].

We found a new trypsin-like proteinase (PSP) in the psychrotolerant Gram-negative microorganism *Serratia proteamaculans* 94. We have also developed a protocol for purification of PSP including ion-exchange chromatography on Q-Sepharose, gel filtration, and affinity chromatography on BPTI-Sepharose with PSP elution in a NaCl gradient [16]. In this study, we have carried out mass spectrometry of tryptic PSP fragments and their analysis using the NCBInr protein database and MASCOT data mining software, which allowed us to identify PSP as a previously unknown oligopeptidase B on the basis of nucleotide sequence from the *S. proteamaculans* 568 genome library. The strain *S. proteamaculans* 94 is not pathogenic for humans and animals and meets the requirements of safety for industrial microorganisms [17].

Unlike other known oligopeptidases B from other sources, PSP is inhibited by basic bovine pancreatic trypsin inhibitor (BPTI), thus allowing development of an effective method for purification of this oligopeptidase by means of affinity chromatography and significant simplification of rather complex and multistage protocols for isolation of other natural oligopeptidases (for instance, from *E. coli* [6] and *T. brucei* [2, 3]). PSP resembles OpdB from other sources in its properties and can serve as a good model in searching for pharmaceuticals against bacterial as well as protozoan pathogens.

MATERIALS AND METHODS

The following chemicals were used: N_α-benzoyl-*D*,*L*-arginine *p*-nitroanilide (BAPNA), basic bovine pancreatic trypsin inhibitor (BPTI), Q-Sepharose, SDS, and isopropyl-β-*D*-thiogalactoside (IPTG) from Sigma (USA); acrylamide, ammonium persulfate, *N*,*N*′-methylene-bis-acrylamide, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine, Protein Assay kit, bovine serum albumin (BSA), and protein standards for electrophoresis from Bio-Rad (USA); Tris and NaCl from Merck (Germany); glycerol from ICN (USA); EDTA, glycine, β-mercaptoethanol, and Coomassie G-250 and R-250 from Serva (Germany); *p*′-guanidinobenzoic acid *p*-nitrophenyl ester and dimethyl sulfoxide (DMSO) from Fluka (Germany); Hepes from Gerbu (Germany); Centricon, Centriplus, and Ultracel ultrafiltration equipment from Millipore (USA); ampicillin from Biokhimak (Russia); bacto tryptone and bacto-yeast extract from Beckon Dickinson (France); lysozyme from Applichem (Germany); Pfu DNA-polymerase from Sileks (Russia); restrictase Sfr2741 and corresponding buffer solution from SibEnzyme (Russia); restrictase NheI, T4 DNA-ligase, and corresponding buffer solutions from Fermentas (Lithuania). Other chemicals of extremely high purity or chemical purity grade were manufactured in Russia.

Determination of PSP activity. The reaction mixture (1.44 ml) containing 0.1 M Tris-HCl, pH 8.0, 50 mM $CaCl_2$, 30 μl of 10-mM BAPNA in DMSO, and 30 μl of enzyme solution was placed into a 1-cm quartz cuvette. Optical density was measured on a Gilford 2400-2 spectrophotometer (USA). Hydrolysis of *p*-nitroanilide-containing substrates was monitored at 25°C from increase in absorbance at 405 nm ($\Delta\varepsilon_{405}$ = 10,400 $M^{-1}\cdot cm^{-1}$) caused by the release of free *p*-nitroaniline. Molarity of enzyme solutions was determined by titration of the active centers with *p*′-guanidinobenzoic acid *p*-nitrophenyl ester [18].

Protein was determined by the method of Bradford using the Bio-Rad Protein Assay with BSA as a standard.

Electrophoresis. Homogeneity of enzyme specimens was determined by SDS-PAGE according to the method of Laemmli using a Mini-Protean 3 electrophoresis cell (Bio-Rad). Concentration of separating gel was 10-12%, and concentration of concentrating gel was 4-5%. Gels were stained with either Coomassie G-250 or silver [19].

Blotting. The protein was transferred onto a Millipore Immobilon-P membrane (pore size 0.45 μm) in a Bio-Rad Mini Trans Blot transfer cell at 200 mA current for 3 h at 4°C. The transfer buffer contained 0.12 M Tris, pH 8.3, 96 mM glycine, and 0.01% SDS. Following the transfer, the membrane was fixed in fixative containing 40% methanol and 10% acetic acid for 10 min, stained with 0.1% Coomassie R-250 in the same solution for 20 min, and washed in the fixative until the background color disappeared.

Preparation of BPTI Sepharose. Sepharose 4B was activated as described by March et al. [20]. The activated Sepharose was added to 50 ml of 0.2 M bicarbonate buffer, pH 9.5, containing 120 mg of BPTI and incubated at 4°C for 20 h with gentle rolling on a shaker. Then the suspension was incubated with 0.1 M ethanolamine for 4 h at room temperature. The prepared sorbent was 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 containing 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, pH 8.0, containing 0.5 M NaCl. The capacity of the prepared affinity sorbent was 15 nmol of trypsin per ml.

Isolation of PSP from *S. proteamaculans* 94. The *S. proteamaculans* 94 cells were suspended in four volumes of 0.1 M Tris-HCl, pH 8.0, containing 50 mM $CaCl_2$, 1 mM $MgCl_2$ (buffer A), and 1 mg/ml of lysozyme. Following incubation at 4°C for 1 h, the cells were disrupted using a UZDN-A ultrasound disintegrator (twice for 1 min). The cell homogenate was centrifuged on a Beckman L8-55 centrifuge with Type 45i rotor at 28,000 rpm for 40 min followed by filtration of the supernatant through a Millex filter unit (pore size 0.45 μm). From 27 g of biomass, 77 ml of supernatant containing 3.46 nmol (0.27 mg) of active PSP was obtained.

The PSP-containing supernatant was applied onto 30 ml of Q-Sepharose (2.8 × 5.0-cm column) equilibrated with buffer A at 4°C and flow rate of 20 ml/h. The sorbent was washed with buffer A to $A_{280} = 0$, and then the PSP was eluted with a linear gradient of 0-0.2 M NaCl (200 + 200 ml) in the same buffer, and 5-ml fractions were collected.

The active fractions were pooled, concentrated to 5.1 ml on an Amicon Ultra-15 centrifugal filter device with Ultracel-50k membrane (nominal molecular weight limit (NMWL) 50 kDa), and dialyzed overnight against 2 liters of 10-mM Hepes-KOH, pH 7.5, containing 1 mM $MgCl_2$ (buffer B). Then methanol was added to final concentration of 20%, and the solution was incubated for two days at 4°C. The precipitate was separated by centrifugation, and the supernatant was filtered on the Amicon Ultra-15 centrifugal filter device with Ultracel-100k membrane (NMWL 100 kDa). The filtrate (11.4 ml) contained 28.2 nmol of PSP. The concentrate (0.5 ml) contained 1.5 nmol of PSP and (from the data of PAGE) a considerable amount of GroEL.

The filtrate obtained at the preceding stage was 5-fold diluted with buffer B, concentrated to 2 ml on a Centricon-50, and applied onto 3 ml of BPTI-Sepharose (1.4 × 2.0-cm column) equilibrated with buffer B at a flow rate of 6 ml/h and 4°C. The sorbent was washed with 50 ml of buffer B at the same rate, PSP was eluted with 0.1 M sodium-acetate buffer, pH 5.0, containing 0.1 M NaCl and 1 mM $MgCl_2$ at a flow rate of 6 ml/h, and 2-ml fractions were collected. To prevent inactivation of PSP in acidic medium, the fractions were collected in tubes containing 1 M Tris-HCl, pH 8.0, 0.3 ml each. Total yield of active PSP was 90%; however, the fractions 2 and 3 containing 10% activity were not homogeneous from the data of PAGE and were not kept. Fractions 4-14 were pooled and concentrated on the Centricon-50.

Sequencing of *S. proteamaculans* 94 *OpdB* gene. The coding fragment of the *S. proteamaculans* 94 oligopeptidase B (OpdB) gene was amplified by PCR. The *S. proteamaculans* 94 cell lysate was used as a template; to prepare it, 10-15 mg of overnight culture cells were suspended in 50 μl of distilled water, heated at 100°C for 10 min, and centrifuged at 8600*g* for 10 min. The supernatant (1-5 μl) was added to the reaction mixture. The PCR primers, OpdB_N (ATGATGACACCCCCTAAGG) and OpdB_C (TTACTCCGCCAGCGCCAGAA), were constructed on the basis of the oligopeptidase B gene sequence from the full-genome *S. proteamaculans* 568 sequence (GenBank ID CP000826). PCR was performed using Pfu DNA-polymerase according to the following protocol: 4 min at 94°C; (1 min at 94°C, 1 min at 58°C, and 4 min at 72°C) – 25 cycles; 1 min at 94°C, 1 min at 58°C, and 8 min at 72°C. PCR products were separated by horizontal electrophoresis in 1.5% agarose gel. The product corresponding to *OpdB* (about 2000 bp) was eluted from the gel using the DNA Extraction Kit (Fermentas) and sequenced using a specific set of primers (CAGGCCGAGAGCGAGC, CTCACGCTGCTCGGCAC, GCCGGTGGCTTGCTG, CGCTGCCACCCATGGC, CATACAGCGTGGTCG, CGAGCGGGTATGGGTG, CGATCGGAGGTGGTC, CACGTGGTGGGTACCG, GTTGGTGATAGTGATCG, and GACAGATCCACTGGCAG).

Construction of producer strain *E. coli* BL-21(DE3) [pOpdB]. Thus prepared PCR product corresponding to the coding fragment of the *OpdB* gene was used as a template for PCR with the primers OpdB_H6 and OpdB_XhoI. The primer OpdB_H6 (AGGTAA**GCTAGC**(CATCAC)$_3$ATGACACCCCCTAAGGCAG) carried the NheI restriction site (printed in bold) on the 5′-end and six histidine codons (underlined). The primer OpdB_XhoI (CCCGCC**CTCGAG**TTACTCCGCCAGCGCCAGAATA) carried the Sfr2741 (XhoI) restriction site (bold). Amplification was carried out using Pfu DNA-polymerase according to the following protocol: 4 min at 94°C; (1 min at 94°C, 1 min at 52°C, and 4 min at 72°C) – 25 cycles; 1 min at 94°C, 1 min at 58°C, and

8 min at 72°C. The PCR product was hydrolyzed with endonucleases NheI and Sfr2741 and ligated according to the manufactorer's protocol with the plasmid pET23b+ (Novagen, USA), which was preliminarily treated with the same endonucleases. The ligation mixture was used for transformation of *E. coli* TG1 cells. The structure of prepared vector named pOpdB was confirmed by sequencing. The pOpdB plasmid was introduced into the strain *E. coli* BL-21(DE3) (Novagen) for expression of the oligopeptidase B gene.

Isolation and purification of His_6-PSP. The producer strain *E. coli* BL-21(DE3) pOpdB No. 22 was inoculated into 5 ml of LB medium containing 50 μg/ml of ampicillin and grown overnight. On the next day, 5 ml of the overnight culture was re-inoculated into 500 ml of LB medium containing 50 μg/ml of ampicillin and grown at 37°C with continuous agitation for 3 h until $A_{550} \approx 0.6$. The cells were induced by addition of IPTG to the final concentration of 1 mM followed by incubation at 25°C with continuous agitation for 3 h.

The biomass was separated by centrifugation at 3000 rpm for 20 min. The supernatant was discarded, and the pellets were pooled, suspended in a small volume of buffer A, and recentrifuged at 3000 rpm for 20 min. The biomass was either immediately used for isolation of total protein or stored at −20°C.

Cells (3.5 g) were suspended in four volumes of 20 mM potassium-phosphate buffer, pH 7.4, containing 0.5 M NaCl, 10 mM imidazole, 0.1% β-mercaptoethanol, and 5% glycerol (buffer C). Then lysozyme was added to the final concentration of 0.1 mg/ml followed by incubation on an ice bath for 1 h. The suspension was ultrasonicated (twice for 1 min; 15-ml portions) on the UZDN-A ultrasound disintegrator.

The homogenate was centrifuged on an Eppendorf centrifuge at 14,000 rpm for 15 min. The supernatant was collected, its PSP activity was determined according to the standard protocol, and the pellet was discarded.

The supernatant was filtered through Millex filter units with pore size of 0.45 μm. The clarified specimen was applied onto a 1.7 × 4.5-cm column with 10 ml of Ni-NTA-agarose equilibrated with buffer C. The column was washed with the same buffer to $A_{280} = 0$, protein was eluted with a linear 10-250 mM imidazole gradient in buffer C at a flow rate of 30 ml/h, and 3.5-ml fractions were collected.

The active fractions were pooled, dialyzed against the buffer A, and concentrated to 8.5 ml on the Amicon Ultra-15 centrifugal filter device with Ultracel-50k membrane (NMWL 50 kDa). The filtered solution was applied onto a 2.8 × 5.0-cm column with 30 ml of Q-Sepharose equilibrated with buffer A at a flow rate of 20 ml/h at 4°C. The sorbent was washed with buffer A to $A_{280} = 0$, His_6-PSP was eluted with a linear 0-0.1 M NaCl gradient (100 + 100 ml) in the same buffer, and 2-ml fractions were collected.

RESULTS AND DISCUSSION

The PSP specimen we prepared earlier [16] contained (from the data of electrophoresis under reducing conditions) a major protein band with $M_r = 60$ kDa. However, the *N*-terminal amino acid sequence of this protein (AAKDVE(K)FGNDAEVY(E)V) appeared to be virtually identical to sequence 2-16 of Cpn60 family chaperonins, such as *E. coli* GroEL, with molecular mass of 60 kDa (MAAKDVKFGNDARVKM).

Mass-spectral analysis (MALDI-TOF, Ultraflex-TOF/TOF; Bruker, Germany) of tryptic fragments of the 60-kDa protein band from the PSP specimen using the Swiss-Prot database and the MASCOT data mining software (http://www.matrixscience.com) allowed identification of this polypeptide as a subunit of a previously unknown *S. proteamaculans* GroEL-type chaperonin. The amino acid sequence of this protein has 50.4% homology with those of several chaperonins with molecular masses of 60 kDa (Cpn60, a GroEL fragment): O66206, O66212, O66222, O66202, Q6D9J0, O66218P0A1D3, P0A1D4, and Q5PL62. The GroEL molecule is composed of 14 subunits of 60 kDa each.

To reveal true molecular mass of the studied proteinase under non-denaturing conditions, we used ultrafiltration of PSP specimens obtained from different purification stages followed by determination of enzymatic activity (using BAPNA as substrate), as well as electrophoretic mobility of the concentrates and filtrates. Virtually all PSP activity remained in a concentrate when specimens were filtered through a membrane with NMWL of 50 kDa; however, a significant portion of activity (10-30%, depending on the purification stage) passed a membrane with NMWL of 100 kDa and was found in the filtrate. When the PSP specimen after ion-exchange chromatography was filtered through a Centricon-100 membrane, the major portion of activity was determined in the concentrate, whereas after affinity chromatography the portion of PSP activity in the filtrate significantly increased. The protein band corresponding to molecular mass of 60 kDa was abundantly found in PAGE under reducing conditions of the prepared concentrates, whereas filtrates through the Centricon-100 showed only small amounts of such protein. On the whole, these data suggest molecular mass of PSP ranging within 50-100 kDa, but this protein is in a tight complex with high molecular weight chaperone. This complex gradually, but not completely, dissociates in the course of purification, particularly at the stage of affinity chromatography. Attempts to use detergents such as Triton X-100 and octyl glucoside for dissociation of this complex were ineffectual.

Preparation of homogeneous PSP specimens was also complicated by the presence of several protein bands in the desired interval of 50-100 kDa in addition to the already identified 60-kDa GroEL subunit – not only

after ion-exchange, but also after affinity chromatography. It is likely that high molecular weight chaperonin GroEL carries a set of *S. proteamaculans* proteins, including PSP proteinase that, being associated in the complex, is accessible both to substrates, such as BAPNA, and to inhibitor BPTI, even immobilized (BPTI-Sepharose). This complex behaves virtually like a single molecule during sorption–desorption on the affinity sorbent, preventing preparation of a homogeneous PSP specimen.

Detailed examination of electrophoregrams and activity has shown that a minor protein band with molecular mass of 78 kDa is present in both filtrates and concentrates, which intensity is in a good conformity with proteinase activity. So, we have supposed that this minor band with molecular mass of 78 kDa corresponds to PSP in prepared specimens.

Firm intersubunit contacts do exist in the GroEL molecule: the interaction force between 14- and 60-kDa subunits is comparable with that between antigen and antibody [21]. The GroEL complexes with protein substrates, that is, the proteins localized in the internal cavity of GroEL-GroES chaperone and undergoing folding, are also rather tight: their dissociation constants are 0.01-10 nM; such complexes do not dissociate in ion-exchange chromatography and gel filtration [22]. The PSP specimens we have prepared also contain the tight complex of the proteinase with chaperonin, which hardly dissociates in the course of affinity chromatography. At the same time, most protein substrates of GroEL are rather small (less than 35-40 kDa), which allows them to tuck into the central cavity of the GroEL ring [23]. Nonetheless, many proteins with higher molecular masses (60-120 kDa) do not undergo processing but can form stable complexes with GroEL [23]. Polypeptides bound in the internal cavity of the chaperonin are released from the complex with GroEL in the presence of ATP and magnesium ions [22, 23]. However, such treatment did not result in dissociation of the PSP–GroEL complex, which might be indicative of another complex type. Only one alternative method for dissociation of GroEL complexes with protein ligands is described in the literature: incubation in buffer containing 20% methanol [23]. We have found that incubation in 20% methanol (10-20 h, 4°C) really results in partial decomposition of the high molecular weight PSP–GroEL complex with precipitation of denatured chaperonin and a small (no more than 10%) loss of PSP activi-

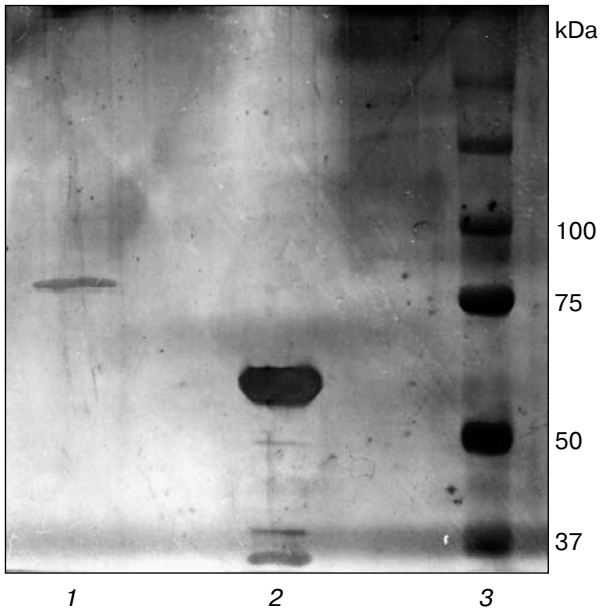

Fig. 1. SDS-PAGE (reducing conditions, staining with silver) of PSP specimens purified by chromatography on Q-Sepharose and ultrafiltration through a Centriplus-100 filter. Lanes: *1*) filtrate (after affinity chromatography on BPTI-Sepharose); *2*) concentrate; *3*) protein standards.

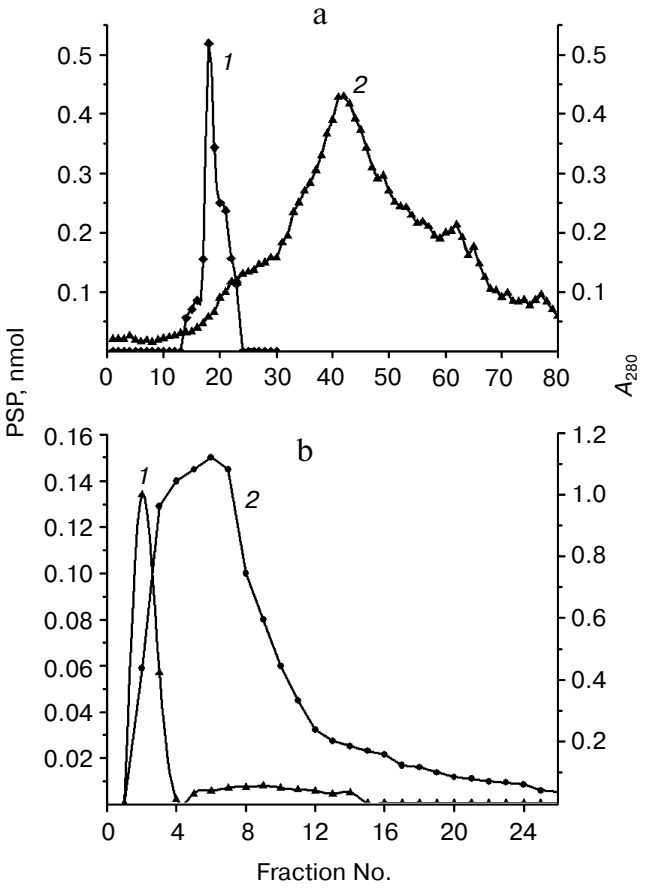

Fig. 2. a) Ion-exchange chromatography of total protein from *S. proteamaculans* on Q-Sepharose. b) Affinity chromatography of a PSP specimen partially purified by ion-exchange chromatography and ultrafiltration in 20% methanol (filtrate after Ultracel-100k). *1*) Enzyme activity; *2*) absorbance of fractions, A_{280}.

ty. This allowed development of a protocol for preparation of homogeneous PSP.

The tight high molecular weight complex of *S. proteamaculans* GroEL chaperonin with PSP was isolated by affinity chromatography on Q-Sepharose. Following treatment with methanol, the PSP proteinase was separated from the high molecular weight fraction by ultrafiltration through a Centriplus-100 membrane. No less than 50-60% of the PSP activity was in the filtrate, whereas the concentrate contained the high molecular weight GroEL chaperonin dissociating into 60-kDa subunits in SDS-electrophoresis (Fig. 1, lane *2*). Active filtrates were concentrated by filtration through a Centricon-50 membrane and subjected to final purification by affinity chromatography on BPTI-Sepharose. Thus prepared PSP specimens were homogeneous from the data of SDS-PAGE and had molecular mass of 78 kDa (Fig. 1, lane *1*).

Mass-spectral analysis (MALDI-TOF, Ultraflex-TOF/TOF; Bruker) of PSP tryptic peptides using the protein database NCBInr and the MASCOT data mining software (http://www.matrixscience.com) identified PSP as a previously unknown oligopeptidase B on the basis of the nucleotide sequence from the *S. proteamaculans* 568 genome library: YP_001478168.1 GI:157370179, NC_009832.1 [24].

The *N*-terminal amino acid sequence 1-10 of homogeneous PSP specimen containing a single 78-kDa protein band in SDS-PAGE was determined as MMTPP-KAEKR. This sequence also corresponds to the sequence of fragment 1-10 of the *S. proteamaculans* 568 oligopeptidase B gene [24].

It is worth noting that no data have been reported in the literature on complexes of oligopeptidases B with chaperones; however, the molecular mass of natural oligopeptidase B from *E. coli* was initially determined (by electrophoresis) as 58 kDa [6]. Following cloning and expression of the corresponding gene, this molecular mass was shown to be ~80 kDa, which led to discussion

Table 1. Purification of PSP from *S. proteamaculans* 94

Purification stage	Protein, mg	PSP, nmol/mg	Yield, %
Cell-free extract	920	0.0038	100
Ion-exchange chromatography on Q-Sepharose	5.25	0.37	55.8
Ultrafiltration in 20% methanol; filtrate	1.48	0.82	35.0
Affinity chromatography on BPTI-Sepharose	0.07	12.6	25.4

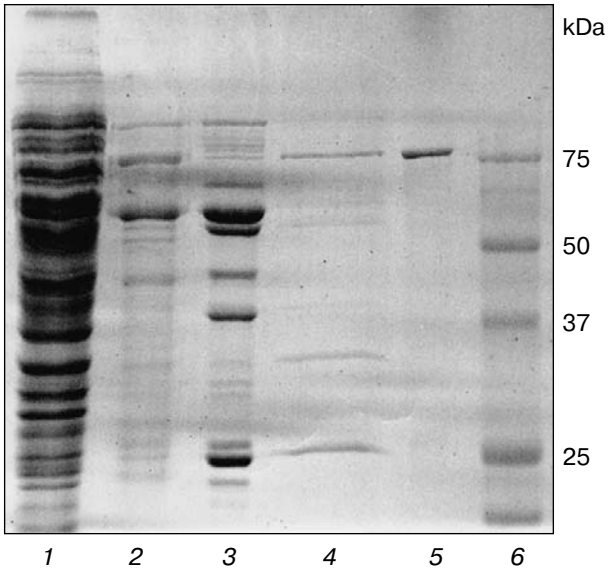

Fig. 3. Protein patterns at different stages of *S. proteamaculans* PSP purification (SDS-PAGE in 10% gel). Lanes: *1*) total protein after cell disruption; *2*) ion-exchange chromatography on Q-Sepharose; *3*) incubation with 20% methanol followed by ultrafiltration (Ultracel-100k), concentrate; *4*) incubation with 20% methanol followed by ultrafiltration (Ultracel-100k), filtrate; *5*) affinity chromatography of filtrate after Ultracel-100k on BPTI-Sepharose; *6*) protein standards.

on the causes of this discrepancy [8]. Earlier, we also erroneously determined the molecular mass of PSP as 60 kDa [16] because the major electrophoretic band of partially purified enzyme specimens is the 60-kDa subunit of the GroEL chaperonin, whereas PSP proteinase itself gives a minor band of 78 kDa.

On the basis of these experiments, we developed a protocol for preparation of oligopeptidase B from *S. proteamaculans* 94 (Fig. 2 and Table 1). Note that incubation of the GroEL–PSP complex with 20% methanol denatures GroEL and possibly other proteins to form a precipitate. The PSP activity remains virtually unaltered.

Thus a homogeneous specimen of the desired proteinase was prepared (Fig. 3). The PSP concentration in this specimen was 12.6 nmol/mg protein (Table 1).

Amino acid sequences of OpdB encoded by corresponding genes of gram-negative bacteria are very similar: 89% homology was found between the enzymes from *E. coli and S. enterica*. High level of homology was found between the latter and corresponding homologs from some γ-proteobacteria, such as *Yersinia pestis* (63%) and *Shewanella putrefaciens* (44%), and even eukaryotic homologs such as oligopeptidases B from *T. brucei* (33%) and *L. major* (33%) [11].

Using point mutations of oligopeptidase B from *S. enterica*, a pair of glutamic acid residues, Glu576 and Glu578, was found to determine the trypsin-like P1-specificity of the oligopeptidase B [11]. A second pair,

Asp460 and Asp462, can determine P2-specificity to basic residues and thus be responsible for preferable hydrolysis of a polypeptide chain after a pair of basic residues, which is characteristic of this enzyme [11].

The enzyme has no inactive precursors. It was found by amino acid sequencing that the *N*-terminal residue of the active enzyme is Met encoded by initiating codon ATG [8]. Moreover, unlike trypsin, the free *N*-terminus of oligopeptidase B is not necessary for its enzymatic activity. This allows the use of *N*-terminal hexahistidine sequence in oligopeptidase B gene constructs expressed in *E. coli*, which considerably facilitates rather complex and multistage purification of this enzyme in native state. It was shown that the removal of the hexahistidine sequence had no effect on the enzymatic features of the recombinant oligopeptidase B [4, 9, 11].

PSP resembles OpdB variants from other sources and can serve as a good model in search for pharmaceuticals against both bacterial and protozoan pathogens. However, purification of the natural enzyme from *S. proteamaculans* is rather complex because it includes decomposition of its complex with chaperone (Fig. 3 and Table 1), and the yield is low: 0.26 mg from 100 g of initial biomass. So, we have sequenced the *S. proteamaculans* 94 *OpdB* gene using primers designed on the basis of the *S. proteamaculans* 568 genome library [24]. The coding fragment of *S. proteamaculans* 94 oligopeptidase B gene was sequenced in both chains (GenBank ID EU751293). The *Serratia proteamaculans* 94 oligopeptidase B producer strain *E. coli* BL-21(DE3) [pOpdB] has been constructed on the basis of the commercial expression system comprising the strain *E. coli* BL-21(DE3) and the vector pET23b+. Results concerning His_6-PSP are shown in the Fig. 4 and Table 2. The yield of expressed His_6-PSP was 150 mg from 100 g of biomass.

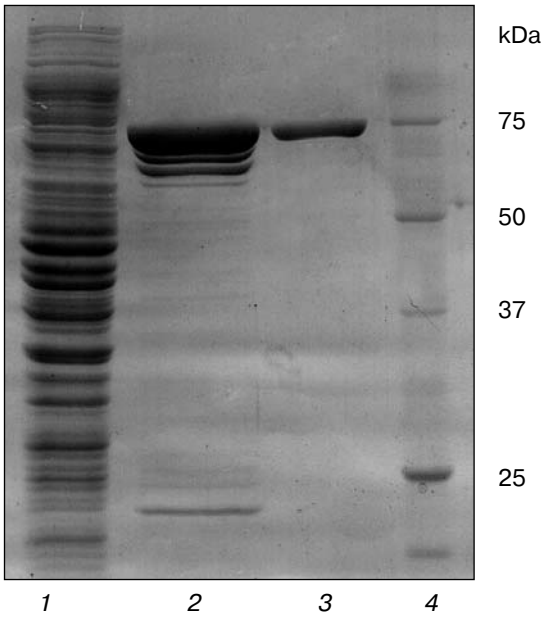

Fig. 4. Protein patterns at different stages of His_6-PSP purification (SDS-PAGE in 10% gel). Lanes: *1*) total protein after cell disruption; *2*) chromatography on Ni-NTA-agarose; *3*) ion-exchange chromatography on Q-Sepharose; *4*) protein standards.

Table 2. Purification of His_6-PSP

Purification stage	Protein, mg	PSP, nmol/mg	Yield, %
Cell-free extract	2616	0.062	100
Affinity chromatography on Ni-NTA-agarose	13.9	4.9	41.7
Ion-exchange chromatography on Q-Sepharose	5.1	12.1	37.7

The amino acid sequence of *S. proteamaculans* 94 OpdB (Fig. 5) has 11 substitutions (Thr34, Ala52, Thr113, Gln220, Thr260, Glu299, Asp396, Ser439, Ala441, Glu547, and Gln585) in comparison with the corresponding enzyme from *S. proteamaculans* 568 (Ala34, Val52, Ile113, Lys220, Lys260, Asp299, Gly396, Lys439, Thr441, Arg547, and Glu585) [24].

The amino acid sequence of oligopeptidase B from *S. proteamaculans* (PSP) closely resembles those of oligopeptidases B from *E. coli* and *S. enterica* (68% homology; for *C*-terminal catalytic domain – 85% homology). An interesting feature of the primary structure of *S. proteamaculans* oligopeptidase B is the absence of one residue of the Asp/Glu pair controlling P2-specificity of the enzyme: Asp462 is substituted by Ala. In trypanosomal and some bacterial oligopeptidases B, this pair Asp/Glu 460 and 462 is obligate, but the second residue is substituted by an uncharged one in corresponding enzymes from *L. major* and *L. amazonensis* [5]. There is no data on specificity of enzymes from leishmaniae. The most homologous to the PSP amino acid sequence is that of OpdB (deduced from the gene sequence) from *Y. pestis* biovar *Microtus str.* 91001 (causative agent of plague), in which the negatively charged residue 462 is also absent. In this case the degree of identity is 87%, and it is 90% for the more conservative *C*-terminal catalytic domain.

One of the trypanosomal OpdB enzymes, namely that from *T. brucei*, is both a serine and SH-dependent proteinase: its activity is controlled by Cys256 residue of *N*-terminal domain, as well as by Cys559 and Cys597 drawn together with Ser of the active center [25]. In contrast, inhibitory analysis of PSP has shown that the enzyme is not SH-dependent [16], and it has been found later that, unlike corresponding enzymes from other bacteria, the amino acid sequence of OpdB from *S. proteamaculans* (Fig. 5) deduced from its gene sequence has no cysteine residues at all.

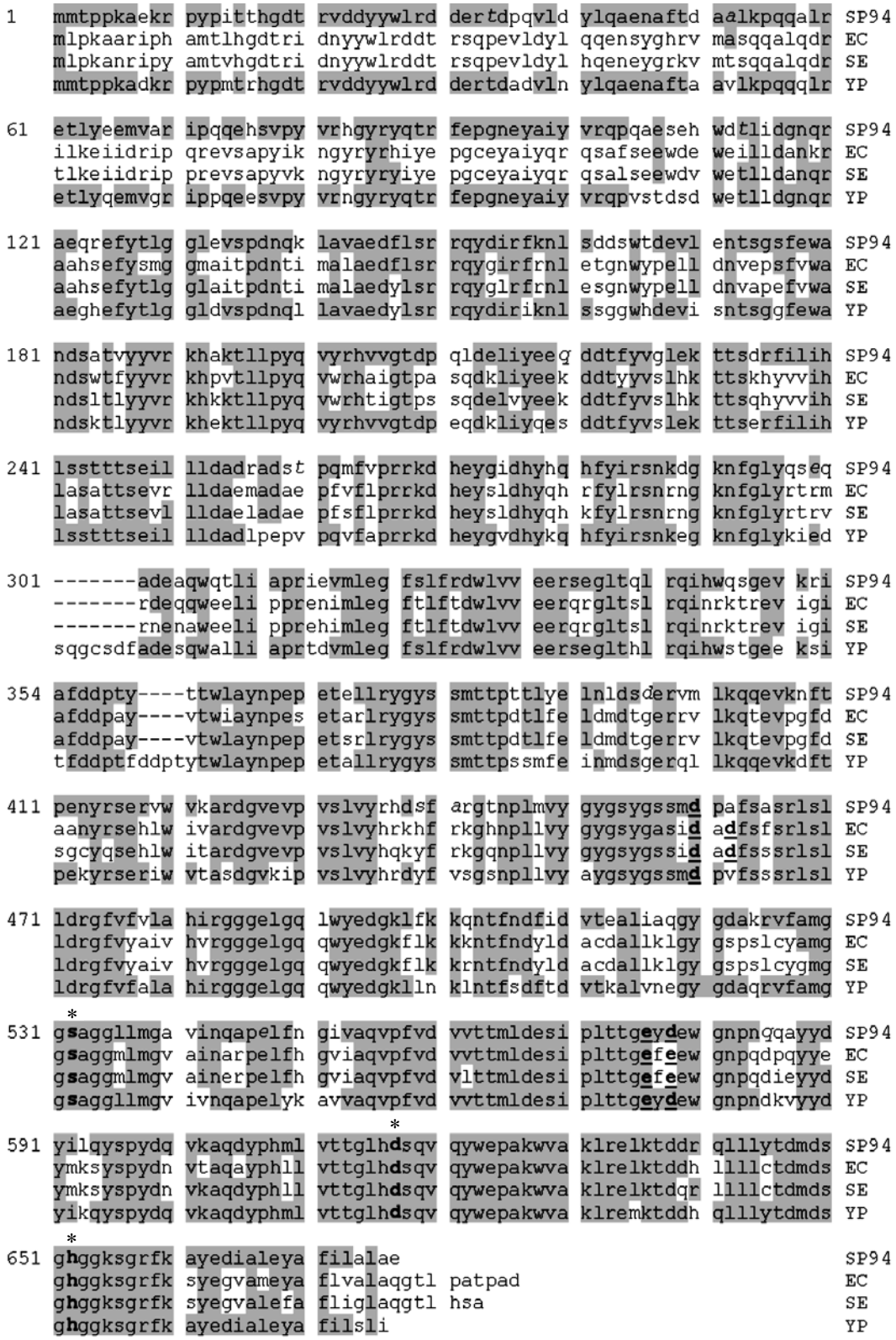

Fig. 5. Comparison of amino acid sequences of OpdB variants from *S. proteamaculans* 94 (SP94), *E. coli* (EC), *S. enterica* (SE), and *Y. pestis* (YP). The fragments coinciding for OpdB from *S. proteamaculans* and at least one enzyme of those from other sources are indicated with gray. Eleven amino acid residues of *S. proteamaculans* 94 OpdB differing from corresponding residues of OpdB from *S. proteamaculans* 568 are italicized. The Asp/Glu residues of substrate-binding centers S1 and S2 are underlined. The asterisk indicates the amino acid residues (Ser, His, and Asp) of the catalytic triad.

This study was supported by the Russian Foundation for Basic Research (grant 07-04-00569) and the Program "Combinative Approaches to Development of Novel Inhibitors of Key Proteinases of Neurodegenerative and Infectious Disease Progression" GK No. 02.512.11.2208.

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