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Oligopeptidase B from *Serratia proteamaculans*. II. Enzymatic Characteristics: Substrate Analysis, Influence of Calcium Ions, pH and Temperature Dependences

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Abstract—Enzymatic properties of a novel oligopeptidase B from psychrotolerant gram-negative microorganism *Serratia proteamaculans* (PSP) were studied. The substrate specificity of PSP was analyzed using *p*-nitroanilide substrates, and the influence of calcium ions on the enzyme activity was studied. Hydrolysis of oligopeptides by PSP was studied using melittin as the substrate. Optimal conditions for the PSP activity (pH and temperature) have been established. It was found that PSP shares some properties with oligopeptidases B from other sources containing two Asp/Glu residues in the S2 site, but it differs significantly in some characteristics. The S2 site of PSP contains only one Asp460 residue. The secondary specificity of PSP has a number of specific features: an unusual substrate inhibition by peptides with hydrophobic residues at the P2 position, as well as the drastic influence of calcium ions on substrate characteristics of the enzyme. It is assumed that the PSP molecule contains a large hydrophobic substrate-binding site, and significant conformational rearrangements of the enzyme active site are induced by Ca^{2+} binding and by the formation of the enzyme–substrate complex. The temperature characteristics of PSP (high activity at low temperature as well as low apparent temperature optimum (25°C)) confirm that PSP is a psychrophilic enzyme.

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Key words: oligopeptidase B, *Serratia proteamaculans*, psychrophilic enzymes, substrate analysis, melittin, pH dependence

Oligopeptidase B (OpdB) is a trypsin-like serine peptidase that is present in ancient unicellular eukaryotes: trypanosomes *Ttypanosoma cruzi* [1], *T. brucei* [2, 3], and *T. evansi* [4], as well as in the leishmania *Leishmania major* [5] and *L. amazonensis* [5]. The genes encoding this enzyme are found in the gram-negative pathogenic bacteria *E. coli* [6-9], *Moraxella lacunata* [10], and *Salmonella enterica* serovar *typhimurium* [11], in the mycobacteria *M. tuberculosis* and *M. leprae* [11], as well as in the spirochete *Treponema denticola* [12]. During trypanosome infections (Chagas diseases, African sleeping sickness), OpdB is an important factor of the virulence [1-4]. In mammals, no genes encoding for this enzyme have been revealed. This suggests that OpdB of protozoan parasites is a target for therapeutic preparations for curing of these dangerous diseases. Homologous oligopeptidases of prokaryotes are less studied. However, it is suggested that the bacterial OpdB also can be important targets for antimicrobial pharmacotherapy [11].

Oligopeptidases B belong to the family of serine prolyl oligopeptidases (clan SC, family S9) [13]. Like trypsin, OpdB hydrolyzes peptide bonds formed by Arg and Lys residues, preferring Arg to Lys. The residues in the P2 position of the substrate that are most preferable for the hydrolysis are also Arg or Lys [14]. It is suggested

Abbreviations: BAPNA, N_α-benzoyl-DL-arginine-*p*-nitroanilide; buffer A, 0.1 M Tris-HCl, pH 8.0; buffer B, 0.1 M Tris-HCl, pH 8.0, 50 mM $CaCl_2$; Bz, benzoyl; DMSO, dimethyl sulfoxide; OpdB, oligopeptidase B; *p*-NA, *p*-nitroanilide; PSP, oligopeptidase B from *Serratia proteamaculans*; Suc, succinyl; Z, benzyloxycarbonyl; Z-Lys-S-Bzl, N_α-benzyloxycarbonyl-L-lysine thiobenzyl ester.

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that the length of the peptide substrates of OpdB does not exceed 3 kDa [14]. However, such a limitation is unlikely to be absolute [6, 11]. It is supposed that OpdB are specific enzymes for processing of proteins in prokaryotes and lower eukaryotes that also are capable of hydrolyzing denatured proteins [7, 15].

In the psychrotolerant gram-negative microorganism *Serratia proteamaculans* 94, we found a new trypsin-like proteinase called PSP [16]. The isolated preparation of PSP with molecular weight of 78 kDa was homogeneous by the data of SDS-PAGE and was identified as a previously unknown OpdB. The gene of the enzyme was sequenced, and the enzyme was produced in *E. coli* BL-21 (DE3) pOpdB cells. A method for isolation of the recombinant enzyme (His_6-PSP) was developed [17].

In the present work, we investigated the substrate specificity of PSP and the effect of calcium ions, pH, and temperature on the enzyme activity.

MATERIALS AND METHODS

The following chemicals were used in this study: N_α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), N_α-benzyloxycarbonyl-L-lysine thiobenzyl ester chlorohydrate (Z-Lys-S-Bzl), 4,4′-dithiodipyridine (DTDP), trifluoroacetic acid for HPLC (TFA), and melittin from Sigma (USA); Protein Assay kit (BioRad, USA); Tris, NaCl, and glycerol (ICN, USA); *p*′-guanidinobenzoic acid *p*-nitrophenyl ester and dimethyl sulfoxide (DMSO) (Fluka, Germany); Hepes (Gerbu, Germany); acetonitrile for HPLC (Kriokhim, Russia); *p*-nitroanilide substrates (*p*-NA): Z-Phe-Arg-pNA (Z = benzyloxycarbonyl), Bz-Pro-Phe-Arg-pNA (Bz = benzoyl), Z-Ala-Ala-Arg-pNA, Suc-Gly-Pro-Lys-pNA, and Suc-Ala-Ala-Pro-Lys-pNA (Suc = succinyl) (Bachem, Switzerland). Other chemicals were of domestic production.

The substrates Ac-Leu-Lys-Arg-pNA and Ac-Leu-Leu-Arg-pNA were synthesized in the Laboratory of Proteolytic Enzyme Chemistry (Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry) using standard methods of peptide synthesis. Homogeneity of each peptide was tested using HPLC. The identity of the compounds was confirmed by mass-spectrometric analysis (Ultraflex TOF/TOF; Bruker, Germany).

The natural oligopeptidase B from *S. proteamaculans* (PSP) and the corresponding recombinant enzyme (His_6-PSP) were obtained using methods developed in our laboratory [17]. Molar concentration of enzyme solutions was determined by the titration of the active sites with *p*′-guanidinobenzoic acid *p*-nitrophenyl ester [18].

Absorption was measured using a Gilford 2400-2 spectrophotometer (USA). Hydrolysis of *p*-nitroanilide substrates was monitored by an increase in the absorption at 405 nm (25°C) due to the formation of free *p*-nitroaniline ($\Delta\varepsilon_{405}$ = 10,400 $M^{-1}\cdot cm^{-1}$).

Protein concentration was determined by the Bradford method using a BioRad Protein Assay kit and bovine serum γ-globulin as the standard.

Incubation mixtures were analyzed by HPLC (Luna C8 column, 2 × 250 mm; Phenomenex, USA) using a Beckman System Gold chromatograph (USA). Peptides were eluted using 0.1% TFA with a 0-60% acetonitrile gradient at a rate of 0.3 ml/min. The results of the chromatography were analyzed using the Multikhrom 5.0 program (Ampersand, Russia). Products of the hydrolysis were identified using a MALDI-TOF spectrometer (Bruker).

Determination of kinetic parameters of hydrolysis of PSP substrates. The initial rate of the hydrolysis of *p*-nitroanilide substrates was determined from the initial linear part of the kinetic curve (extent of hydrolysis did not exceed 10%), monitoring the increase in the absorbance at 405 nm in 0.1 M Tris-HCl, pH 8.0 (buffer A), or in the same buffer containing 50 mM $CaCl_2$ (buffer B) at 25°C. Stock solutions of *p*-nitroanilide substrates (10 mM) were prepared using DMSO, except for the succinyl-peptides that were dissolved in water. To determine kinetic constants, no less than 10 concentrations of each substrate were used, usually in the range of 0.02-0.2 mM, and 3-20 μM in the case of Ac-Leu-Lys-Arg-pNA. In separate cases, to determine the k_{cat}/K_m value, low substrate concentrations were used (no less than three points) in the range of [S] << K_m (for example, in the range of 50-100 μM for the substrate Suc-Ala-Ala-Pro-Lys-pNA).

Assay of PSP preparations for enzymatic activity. The reaction mixture contained 1.44 ml of buffer B, 30 μl of 10 mM BAPNA solution in DMSO, and 30 μl of the enzyme preparation in a 1-cm quartz cuvette.

To determine the hydrolysis constants of thiobenzyl ether Z-Lys-S-Bzl (0.02-0.2 mM), an increase in the absorbance at 324 nm occurring due to the hydrolysis and subsequent reaction of the released SH-group with DTDP (0.2 mM) was monitored in buffer A or B at 25°C ($\Delta\varepsilon_{324}$ = 16,067 $M^{-1}\cdot cm^{-1}$). The initial rate of the reaction was determined as in the case of *p*-NA.

Kinetic parameters (k_{cat} and K_m) were calculated from the Michaelis–Menten equation using nonlinear regression. The standard error did not exceed 20%. In the case of substrate inhibition, the corresponding parameters (k_{cat}, K_m, K_i', and n) were determined using equations for substrate inhibition (2) and (4) (see further (Fig. 2)).

Determination of thermal stability of PSP. Aliquots of the enzyme (50 nM) were incubated in buffer A at different temperatures for 15 min, and then the activity was determined using the standard procedure with BAPNA as the substrate.

Dependence of enzyme activity on pH. To determine the initial rate of BAPNA hydrolysis, the substrate (0.03 mM) was incubated with PSP (9 nM) at pH 4.8-5.2 (0.1 M sodium-acetate buffer, 50 mM $CaCl_2$) or pH 6.0-

9.45 (0.1 M Tris-HCl, 50 mM $CaCl_2$). The experimental values of the initial rates of the hydrolysis were used to determine k_{cat}/K_m. Then the k_{cat}/K_m values were plotted against pH, and the resulting curve was analyzed by nonlinear regression (Origin 7.5; OriginLab, USA) using the equation:

$$\frac{k_{cat}}{K_m} = \frac{\frac{k_{cat}}{K_m}(\text{limit})}{1+10^{pK_1-pH}+10^{pH-pK_2}},$$

where pK_1 and pK_2 are the dissociation constants of the ionogenic groups of the free enzyme, and k_{cat}/K_m (limit) is the pH-independent rate constant [19].

Determination of temperature optimum of PSP. The initial rate of the hydrolysis of Ac-Leu-Lys-Arg-pNA (30.6 μM) by PSP in buffer A at 15-30°C was determined spectrophotometrically. At lower and higher temperatures the reaction was monitored using HPLC. The substrate was incubated with the enzyme at the corresponding temperature, and after certain time intervals (substrate conversion did not exceed 10%), six to eight aliquots (5-10 μl each) were taken from the reaction mixture, diluted 10-fold with 0.1% TFA and kept at −20°C. The initial rate of the hydrolysis was calculated from the substrate to product (*p*-nitroaniline) peak area ratio. To evaluate the reproducibility of the results obtained by different methods, the initial rate of the hydrolysis of the substrate at 15°C was determined simultaneously by HPLC analysis and spectrophotometrically; the results of the experiments coincided.

Melittin hydrolysis was monitored using HPLC. Melittin (50-90 μM) was incubated in the presence of 5-30 nM PSP in buffer A at pH 8.0 and 25°C, and aliquots were taken from the reaction mixture until the substrate was completely hydrolyzed. To identify the products of the hydrolysis, aliquots corresponding to separate peaks were analyzed using MALDI-TOF spectrometry (Ultraflex-TOF/TOF; Bruker).

RESULTS AND DISCUSSION

Substrate specificity of PSP was analyzed using a number of *p*-nitroanilide substrates (*p*-NA) and highly efficient for trypsin-like proteinases N_α-benzyloxycarbonyl-L-lysine thiobenzyl ester (Z-Lys-S-Bzl). Table 1 presents kinetic constants of the hydrolysis of various substrates by the natural oligopeptidase B from *S. proteamaculans* (PSP) and by the recombinant enzyme (His_6-PSP). It is known from the literature that the presence of the N-terminal hexahistidine sequence does not influence the enzymatic properties of the recombinant OpdB from different sources [4, 9, 11]. Actually, according to our data, His_6-PSP does not differ from natural PSP in its specificity and activity (Table 1).

The S3, S2, S1′, S2′, and S3′-specificity was analyzed in detail for OpdB from *T. cruzi* and *T. brucei* [14]. It should be noted that in virtually all investigations devoted to substrate analysis of different OpdB, the hydrolysis of the corresponding substrates was studied at 37°C in the absence of calcium ions (in part, the effect of calcium ions on the activity of OpdB from *E. coli* was studied by Polgar et al. [20]). For oligopeptidase B, we first analyzed the secondary specificity both in the absence and in the presence of Ca^{2+} (50 mM). Besides, as it was demonstrated earlier [16], PSP is a psychrophilic enzyme that is significantly inactivated at 37°C, so the substrate specificity was analyzed at the optimal temperature for this enzyme (25°C).

To prepare stock solutions (10 mM), the synthetic PSP substrates were dissolved in DMSO. Previously, it was found that the catalytic activity of OpdB from *E. coli* is significantly inhibited by organic solvents [19]. We tested the effect of DMSO on the kinetics of the BAPNA hydrolysis and found that 10% DMSO inhibited the enzyme activity more than twofold, but the presence of 2% DMSO had virtually no affect on the kinetic constants of BAPNA hydrolysis. So, the values of the kinetic constants presented in Table 1 (except for highly soluble substrates with the N-terminal succinyl groups) were obtained in the presence of 2% DMSO.

Primary specificity. PSP, like other oligopeptidases B, exhibits trypsin-like primary specificity, hydrolyzing substrates containing Arg or Lys residues in the P1 position. Site-directed mutagenesis of the oligopeptidase B from *S. enterica* allowed determination of two glutamic acid residues (Glu576 and Glu578) that are responsible for the trypsin-like P1-specificity of this enzyme [11]. These residues are conserved for all investigated OpdB. In the PSP molecule, this negatively charged pair of residues (Glu576 and Glu578) is also present [17].

As seen from Table 1, PSP prefers arginine residues. Comparison of the efficiency of the hydrolysis of lysine and arginine substrates (especially in the case of the pair Suc-AAPR-pNA and Suc-AAPK-pNA) shows a 10-fold preference for Arg substrates to Lys ones, both in the presence and in the absence of calcium ions. According to literature data, for oligopeptidases B from other sources (trypanosomes, *E. coli*, *S. enterica*), this preference of arginine over lysine is less pronounced and does not exceed twofold; in some cases, arginine and lysine substrates with the same composition do not differ in the efficiency of their hydrolysis [4, 8, 11]. However, in the case of long peptides and proteins containing both arginine and lysine residues, OpdB usually hydrolyses only the bonds formed by the carboxyl groups of Arg residues [14].

Secondary specificity. Effect of [Ca^{2+}]. Most studied OpdB, besides the pair Glu576 and Glu578 determining the trypsin primary specificity, also contain a second pair of negatively charged residues, for example Asp460 and Asp462 in OpdB of *E. coli* and *S. enterica* [11]. It has been

Table 1. Kinetic constants of hydrolysis of different substrates by oligopeptidase B from *S. proteamaculans* (PSP) (0.1 M Tris-HCl, pH 8.0; 2% DMSO, 25°C)

Substrate	k_{cat}, min^{-1}		K_m, mM		k_{cat}/K_m, $\mu M^{-1} \cdot min^{-1}$	
	$[Ca^{2+}] = 0$	$[Ca^{2+}] = 50$ mM	$[Ca^{2+}] = 0$	$[Ca^{2+}] = 50$ mM	$[Ca^{2+}] = 0$	$[Ca^{2+}] = 50$ mM
[1]BAPNA	870	2260	0.09	0.302	9.65	7.5
[2]BAPNA	–	2210	–	0.309	–	7.2
[3]BAPNA	–	2230	–	0.300	–	7.5
[4]BAPNA	965	1810	0.145	0.663	6.65	2.7
[1]Z-Lys-S-Bzl	3330	–	0.096	–	35	9.09
[2]Z-Lys-S-Bzl	–	3230	–	0.3220	–	10.4
[1]Ac-LKR-pNA	1460	1920	0.0226	0.265	65	7.2
[2]Ac-LKR-pNA	1430	–	0.0196	–	73	–
[1]Ac-LLR-pNA	–	1010	–	0.559	–	1.8
[2]Ac-LLR-pNA	457	834	0.224	0.457	2.04	1.83
[1]Z-FR-pNA	1680	2110	0.058	0.0513	29.1	41.2
[2]Z-FR-pNA	–	3460	–	0.0732	–	47.2
[1]Bz-PFR-pNA	–	1090	–	0.062	–	17.6
[2]Bz-PFR-pNA	12 100	1270	0.54	0.084	22.6	15.07
[1]Suc-AAPR-pNA	–	72	–	0.323	–	0.22
[2]Suc-AAPR-pNA	30	62	1.05	0.306	0.029	0.205
[1]Suc-AAPK-pNA	–	–	–	–	–	0.018
[2]Suc-AAPK-pNA	–	56	–	2.2	0.004	0.0255
[1]Z-AAR-pNA	–	–	–	–	–	0.79
[1]Suc-GPK-pNA	–	–	–	–	–	0.067

Note: Error does not exceed 20%.
[1] PSP; [2] His_6-PSP; [3] 0.2-2% DMSO; [4] 10% DMSO.

shown that they determine the P2 specificity of the enzyme to main residues, thus providing preferential hydrolysis of a polypeptide chain after the pair of main residues [11].

As we demonstrated previously [17], one of the peculiarities of the primary structure of the oligopeptidase B from *S. proteamaculans* is the absence of one of two Asp/Glu residues controlling P2 specificity of the enzyme: the residue Asp462 is replaced by Ala. However, both substrate-binding sites of PSP (S1 and S2) remain negatively charged, this providing the efficient hydrolysis of peptides with positively charged residues in the P1 and P2 positions. Actually, Ac-LKR-pNA (in the absence of Ca^{2+}) is the most efficient substrate of those investigated in this work (Table 1). The efficiency of hydrolysis of substrate with positively charged P2 residue in the presence of Ca^{2+} that is capable of blocking the carboxylic groups of the Asp/Glu residues of S1 and S2 sites decreases by an order of magnitude due to the worsening in the substrate binding: in the presence of 50 mM Ca^{2+}, the K_m value for Ac-LKR-pNA increases by an order of magnitude, the k_{cat} value being virtually unchanged (Table 1).

Worsening in the substrate binding in the presence of calcium ions is also observed in the case of shorter substrates, esters and amides of *N*-acyl-arginine (or lysine). In the case of Z-Lys-S-Bzl, the effect is the same as in the case of Ac-LKR-pNA but less pronounced: 50 mM Ca^{2+} increases the K_m value threefold, not affecting the k_{cat}

value. Thus, in total, the efficiency of Z-Lys-S-Bzl hydrolysis in the presence of Ca^{2+} decreases threefold compared to the 10-fold decrease in the case of Ac-LKR-pNA (Table 1). The k_{cat}/K_m value for N-tosyl-L-arginine methyl ester hydrolysis catalyzed by oligopeptidase B from *T. brucei* decreases in the presence of calcium ions to 30% of its original value [21]. Presumably, for this ester substrate the similar worsening of the substrate binding is observed due to the blocking of the carboxylic groups of the substrate-binding sites by calcium ions.

For the amide substrate BAPNA, threefold increase in the K_m value was observed in the presence of Ca^{2+}; however, this was also accompanied by twofold increase in the k_{cat} value (Table 1). Similar behavior of these constants is usually accounted for the nonproductive binding. Oligopeptidase B contains several negatively charged amino acid residues in its substrate-binding sites, so such substrates as BAPNA can bind to the enzyme in different ways, but only one of these enzyme–substrate complexes yields the reaction products. The k_{cat} value decreases since only a part of the substrate binds productively, and K_m value also decreases due to the additional (nonproductive) substrate binding. Since k_{cat} and K_m values change in a similar way, the nonproductive binding usually does not change the k_{cat}/K_m ratio [20]. In the case of PSP, the increase in the catalytic efficiency in the presence of Ca^{2+} does not compensate the worsening of the binding, and so during the reaction of BAPNA hydrolysis the k_{cat}/K_m ratio in the presence of 50 mM Ca^{2+} is somewhat lower than in the absence of Ca^{2+} (Table 1).

A classic example of the nonproductive binding in the absence of Ca^{2+} is observed in the case of Ac-LLR-pNA: addition of 50 mM Ca^{2+} increases twofold both the k_{cat} and K_m values, not affecting the total efficiency (k_{cat}/K_m) of the hydrolysis (Table 1). Presumably, in the case of the presence of two carboxyl amino acid residues (Glu576 and Glu578) in the S1 site and the third (Asp460) in the S2 site of PSP, the probability of the nonproductive binding of the substrates containing only one positively charged Arg residue is rather high, and the binding of Ca^{2+} decreases this probability.

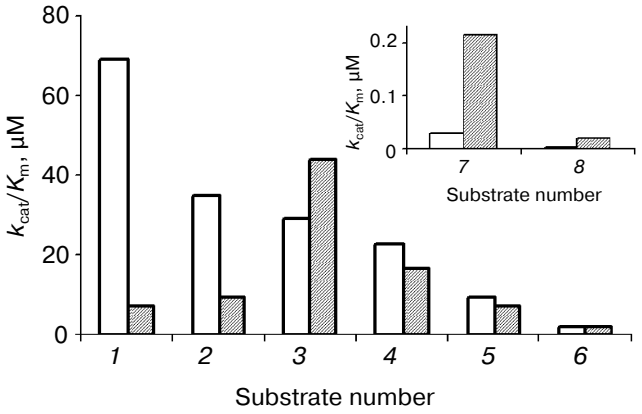

Fig. 1. Activity of PSP in the absence and in the presence of Ca^{2+}: *1*) Ac-LKR-pNA; *2*) Z-Lys-S-Bzl; *3*) Z-FR-pNA; *4*) Bz-PFR-pNA; *5*) BAPNA; *6*) Ac-LLR-pNA; *7*) Suc-AAPR-pNA; *8*) Suc-AAPK-pNA. [Ca] = 0 mM (white columns); [Ca] =50 mM (hatched columns).

The substrates Suc-AAPR-pNA and Suc-AAPK-pNA containing negatively charged succinyl group are the least efficient of the investigated compounds (Table 1). The succinyl group is apart (three amino acid residues) from the bond to be hydrolyzed, but its negative effect on the substrate-binding sites of PSP is obvious: both low binding of the substrate (high K_m values) and low catalytic efficiency are observed: the k_{cat} values determined for Suc-AAPR-pNA and Suc-AAPK-pNA are lower by virtually two orders of magnitude than the corresponding constants for all other substrates (Table 1). It should be noted that these *p*-nitroanilides are highly efficient substrates for trypsin, an enzyme also containing an Asp residue in the S1 site. Presumably, the Suc-group of these substrates conflicts with Asp460 of the S2 site of PSP. In contrast to Ac-LKR-pNA, the addition of calcium ions blocking the carboxylic groups of the enzyme and/or the substrate increases the efficiency of the hydrolysis of Suc-AAPR-pNA and Suc-AAPK-pNA by an order of magnitude, resulting from increase in the k_{cat} value and decrease in the K_m value (Table 1).

Thus, the values of the kinetic constants of the hydrolysis of the investigated substrates by PSP depend on the presence or absence of calcium ions in the incubation medium, but for substrates with different substrate specificity, the effects are often oppositely directed (Table 1 and Fig. 1). Therefore, the sequence of specificity for the substrates is different in the absence of calcium and in the presence of 50 mM Ca^{2+}.

It should be noted that the Ca^{2+}-dependence of the efficiency of the hydrolysis of BAPNA and peptide substrates with one Arg residue by oligopeptidase B from *E. coli* containing four Asp/Glu residues in the S1 and S2 sites significantly differs from that of PSP: in the presence of calcium ions, the efficiency of the hydrolysis (k_{cat}/K_m) increases threefold, not only at the expense of the enhancement of the catalytic efficiency (k_{cat}), but to the most extent, at the expense of the facilitation of the binding (decrease in K_m) [20]. Evidently, such a difference in the properties of these two oligopeptidases B is due to the different structure of their S2 site.

A number of substrates of PSP exhibit similar K_m values (~0.3 mM) in the presence of 50 mM Ca^{2+} (Table 1). This includes both the best (Ac-LKR-pNA) and the worst (Suc-AAPR-pNA) of the investigated substrates, as well as BAPNA. According to this, it can be assumed that this value characterizes the binding of the Arg residue in the primary substrate-binding site (S1) of PSP when the secondary site (S2) is blocked by calcium ions. In the absence of Ca^{2+}, both sites (S1 and S2) take part in the substrate

binding, this increasing the efficiency of the hydrolysis by an order of magnitude in the case of Ac-LKR-pNA, and on the contrary, decreasing the efficiency in the case of Suc-AAPR-pNA. During the hydrolysis of BAPNA and Ac-LLR-pNA, nonproductive binding is observed.

In the presence of Ca^{2+}, the K_m value for the lysine substrate Z-Lys-S-Bzl also is approximately 0.3 mM. However, in this case it cannot be included in the sequence of arginine substrates since this thiobenzyl ester is known to be a highly effective substrate for all trypsin-like enzymes. The k_{cat} value of its hydrolysis by PSP exceeds the corresponding values for all substrates in Table 1 including Ac-LKR-pNA.

Comparison of the efficiency of the hydrolysis of the substrates Ac-LKR-pNA and Ac-LLR-pNA demonstrates that the leucine residue in the P2 position of the substrate is unfavorable for PSP (Table 1). The efficiency of the hydrolysis of the best PSP substrate, Ac-LKR-pNA, exceeds that of Ac-LLR-pNA 30-fold in the absence of Ca^{2+} and 4-fold in the presence of 50 mM Ca^{2+}. Compared to BAPNA, the efficiency of hydrolysis Ac-LLR-pNA is reduced 3-5-fold (mainly at the expense of k_{cat}, but the K_m value for this arginine tripeptide substrate is 1.5-2.0-fold higher in the presence of Ca^{2+} than that for BAPNA and other arginine substrates) (Table 1).

In contrast, according to literature data, a Leu residue at the P2 position is rather favorable for trypanosomal oligopeptidases B: the efficiency of the hydrolysis of such peptides is no more than 5-fold lower compared to the best substrates containing a pair of positively charged residues in the P1 and P2 positions, and in separate cases it is still higher [14]. However, the first place among hydrophobic residues in the P2 position corresponds to Met and Phe; corresponding peptides (with Arg in the P1 position) take the second place in the efficiency of the hydrolysis after the substrates with a pair of positively charged amino acid residues [14].

We also demonstrated that in the absence of calcium ions, the second place in the efficiency of the hydrolysis after the substrate with two positively charged residues is taken by Z-FR-pNA (a substrate with a hydrophobic residue in the P2 position). In the presence of calcium ions, the k_{cat}/K_m value for Z-FR-pNA is the highest among the investigated substrates (Table 1 and Fig. 1). The K_m value is virtually independent of the presence of calcium ions, and the k_{cat} value slightly increases (1.3-2.0-fold) in the presence of 50 mM Ca^{2+}. As a result, the k_{cat}/K_m value characterizing the efficiency of the hydrolysis of this substrate is only slightly (~1.5-fold) higher in the presence of 50 mM Ca^{2+} than in its absence. The K_m value for Z-FR-pNA is significantly lower than for other arginine substrates (0.3 mM in the presence of 50 mM of Ca^{2+}, when the S2 site is blocked for substrate binding) (Table 1). So, we assumed that PSP contains an additional hydrophobic site that is responsible for the binding of aromatic residues of the substrate.

Another investigated substrate with aromatic P2 residue, Bz-PFR-pNA, contains in the P3 position a proline residue, which is unfavorable (according to literature data) for the peptide hydrolysis by trypanosomal OpdB [21]. In the case of PSP, the efficiency of the hydrolysis of this substrate (k_{cat}/K_m) is also lower than for the shorter substrate Z-FR-pNA, especially in the presence of calcium ions. The hydrolysis of Bz-PFR-pNA is characterized by a strong nonproductive binding: the k_{cat} and K_m values change simultaneously by an order of magnitude depending on the presence of Ca^{2+} (Table 1). However, in contrast to the abovementioned nonproductive binding of Ac-LLR-pNA and BAPNA, Ca^{2+} promotes the nonproductive binding of Bz-PFR-pNA, which decreases by an order of magnitude in the absence of calcium ions.

The most interesting feature of the substrates with an aromatic amino acid residue in the P2 position is the inhibition by high substrate concentration that is significantly pronounced in the absence of calcium ions (Fig. 2 and Table 2). In the case of the hydrolysis of Bz-PFR-pNA, an S-shaped kinetic curve is observed at some concentrations of the enzyme and the substrate: the effect of the inhibition by the substrate is so strong that 10-20% hydrolysis increases the rate of the product formation

Table 2. Substrate inhibition of PSP (0.1 M Tris-HCl, pH 8.0, 2% DMSO, 25°C)

Substrate	$[Ca^{2+}]$	n	K_S', μM
Ac-LKR-pNA	0	1.0 ± 0.2	400 ± 200
	50 mM	(no inhibition)	(no inhibition)
Z-FR-pNA	0	5.2 ± 0.8	45 ± 4
Z-FR-pNA	50 mM	5.9 ± 0.4	126 ± 4
Bz-PFR-pNA	0	2.7 ± 0.1	27 ± 2
Bz-PFR-pNA	50 mM	2.0 ± 0.4	190 ± 50

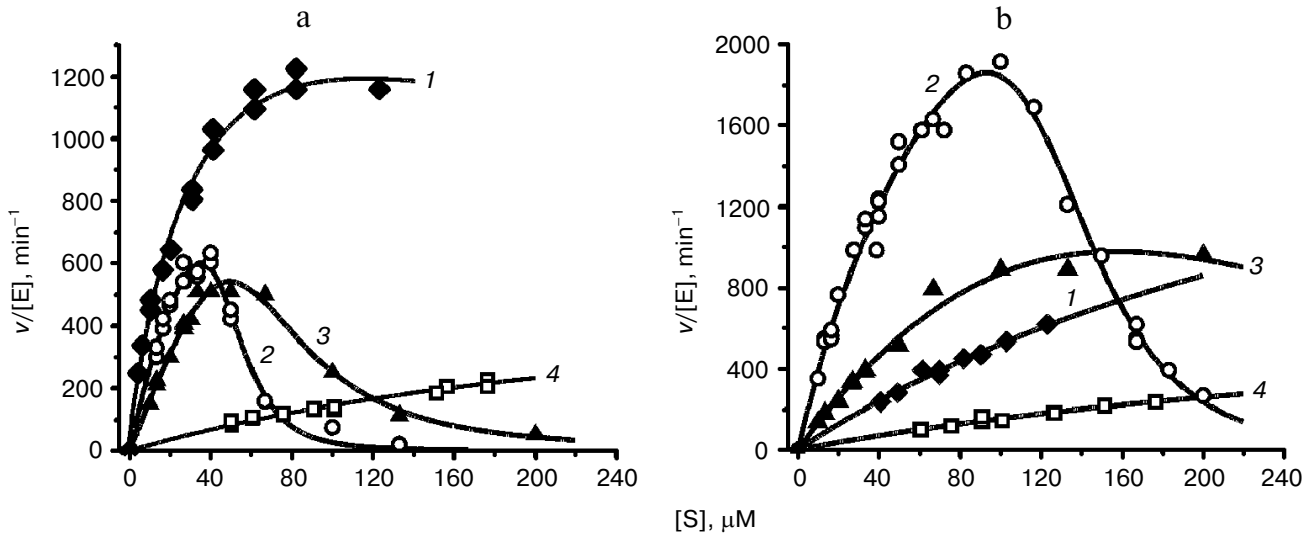

Fig. 2. Different character of dependences of initial rates of hydrolysis of some PSP substrates on their concentration and on the presence of calcium ions (0.1 M Tris-HCl, pH 8.0; 25°C, $[Ca^{2+}] = 0$ (a) or $[Ca^{2+}] = 50$ mM (b)). *1*) Ac-LKR-pNA; *2*) Z-FR-pNA; *3*) Bz-PFR-pNA; *4*) Ac-LLR-pNA. For correct comparison, the rates are normalized to the enzyme concentration.

(Fig. 3). Such an inhibition by the substrates with an aromatic residue in the P2 position was not observed for any OpdB from other sources.

In the cases of the hydrolysis of both BAPNA and Ac-LLR-pNA, in the investigated range of concentrations (below 0.2 mM) we did not observe any deviation from the standard Michaelis–Menten kinetics. However, for the substrate with a pair of positively charged amino acid residues Ac-LKR-pNA at $[S] \geq 50$ μM, such a deviation was observed, but only in the absence of calcium ions (Fig. 2). However, substrate inhibition is described by a usual mechanism suggesting that the binding of the second substrate molecule results in the formation of the nonproductive complex:

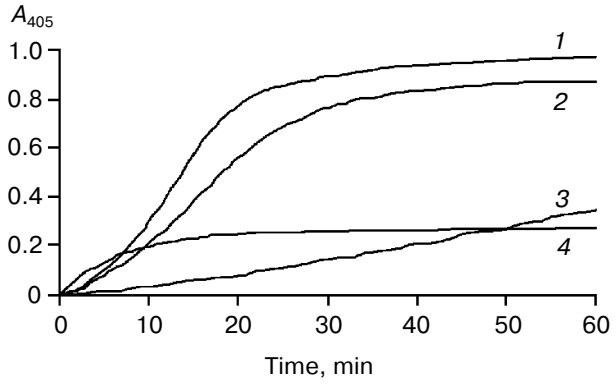

Fig. 3. Kinetic curves of hydrolysis of Bz-PFR-pNA by PSP (0.1 M Tris-HCl, pH 8.0; 25°C). Concentrations of substrate and enzyme, respectively, constituted 0.1 mM and 10 nM (*1*), 0.1 mM and 5 nM (*2*), 0.2 mM and 5 nM (*3*), 0.03 mM and 5 nM (*4*).

$$\begin{array}{ccc} E + S \underset{}{\overset{K_m}{\rightleftharpoons}} ES & \xrightarrow{k_{cat}} & E + P \\ +S \downarrow \uparrow K_S' & & \\ ES_2 & & \end{array} \qquad (1)$$

$$v = k_{cat}[E][S]/(K_m + [S] + [S]^2/K_S'), \qquad (2)$$

where v is the initial rate of the hydrolysis, and [S] is the original substrate concentration.

Kinetic constants for the hydrolysis of Ac-LKR-pNA (Table 1) are calculated using the full dependence v/[E] versus [S] (Fig. 1), as well as the usual Michaelis–Menten kinetics in the range of low substrate concentrations. The value of the substrate inhibition constant (K_s') is approximately 400 μM. Substrate inhibition according to mechanism (1) was observed in the case of the hydrolysis of the substrates as X-Arg-Arg-Y by oligopeptidase B from *E. coli* [7, 20]. In contrast to our data, the substrate inhibition of the enzyme from *E. coli* was also observed in the case of the hydrolysis of BAPNA and peptides containing an arginine residue in the P1 position and a citrulline (uncharged analog of arginine) residue in the P2 position. However, the kinetics of this inhibition did not follow mechanism (1), and Eq. (2) did not allow calculation of the corresponding constants [7, 20].

The kinetics of the substrate inhibition of the hydrolysis of Z-FR-pNA and Bz-PFR-pNA by PSP also does not correspond to the classic kinetics of substrate inhibition, where the binding of the second substrate molecule to the productive enzyme–substrate complex ES results in the formation of the nonproductive complex ES_2. The

corresponding dependences v/[E] versus [S] (Fig. 2) cannot be approximated by Eq. (2). We assumed that the observed sharp drop in the initial rate of the hydrolysis of Z-FR-pNA and Bz-PFR-pNA with the increase in substrate concentration could be caused by the binding of several (not one) additional substrate molecules (n) to the ES complex:

$$\begin{array}{c} E + S \underset{}{\overset{K_m}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P \\ \qquad\quad +nS \downarrow \uparrow K_S' \\ \qquad\quad ES_{n+1} \end{array} \qquad (3)$$

It was shown [22, 23] that the initial rate of such a hydrolysis can be calculated using Eq. (4):

$$v = k_{cat}\,[E]\,[S]/\{(K_m + [S] + [S]^{n+1}/(K_S')^n\}, \qquad (4)$$

where each of n additional molecules of the substrate are supposed to bind independently with the same constant K_s'. The values of K_s' and n can be determined from the experiment.

Theoretical curves of v/[E] versus [S] for Z-FR-pNA and Bz-PFR-pNA created using Eq. (4) approximate the experimental data reasonably well (Fig. 2). The results of calculations showed that in the absence of calcium ions in the enzyme–substrate complex of PSP with Bz-PFR-pNA, $n = 2.7$, i.e. the enzyme is capable of binding additionally two-three molecules of this substrate, resulting in the formation of the nonproductive complex. The number of additional molecules of Z-FR-pNA was five-six (Table 2). The values of the kinetic constants for the hydrolysis of these substrates, k_{cat} and K_m, virtually coincide with the values presented in Table 1 obtained using the Michaelis–Menten equation in the range of low concentrations.

In the presence of 50 mM Ca^{2+}, no substrate inhibition was observed in the case of Ac-LKR-pNA, and the effect was less pronounced for other substrates (Fig. 2). Actually, the calculations showed that the values of the substrate inhibition constants (K_s') for Z-FR-pNA and Bz-PFR-pNA in the presence of calcium ions are significantly higher than in their absence, but the number of additional substrate molecules that bind to the enzyme–substrate complex remains constant both in the presence and in the absence of Ca^{2+} (Table 2).

The ability of PSP to bind a large number of molecules of arginine-containing di- and tripeptides with Phe residue in the P2 position supports our assumption on the presence of the hydrophobic substrate-binding site in the molecule of this oligopeptidase B and indicates that this site is rather large.

It is interesting to compare the effects of the nonproductive binding and substrate inhibition. For PSP, these effects are often observed simultaneously, especially in the case of Bz-PFR-pNA, when one of two effects prevails depending on the presence of calcium ions. In the presence of 50 mM Ca^{2+}, the effect of the nonproductive binding of this substrate is 10-fold higher than in the absence of Ca^{2+}, but inhibition by the substrate is virtually not observed. In the absence of calcium ions, the additional binding of 2-3 molecules of Bz-PFR-pNA to the ES complex results in a strong inhibition of the hydrolysis. Obviously, the conformation of the active site of the enzyme and its substrate-binding sites is not the same in the absence and in the presence of Ca^{2+}.

Hydrolysis of oligopeptides. Natural peptides with a high content of basic residues are efficiently hydrolyzed by OpdB after Arg/Lys residues. This was demonstrated using peptide hormones such as atrial natriuretic factor [2, 4], ACTH [8, 24], bradykinin [25], angiotensins I and II [24, 26], VIP peptide, compound P [24], neurotensin [2, 8], reduced Arg^8- and Lys^8-vasopressins [2], glucagon, dynorphin A, and somatostatin-28 [27].

It seems possible that OpdB takes part in the degradation of antimicrobial peptides excreted by plants, invertebrates, and mammals in response to a bacterial infection. There are about 600 antimicrobial peptides with high content of positively charged amino acid residues (Arg and Lys) destroying cells of different microorganisms [28-35]. They include buforin II, thanatin, melittin, magainin II, tachyplesin I, etc. [32-35] containing sequences of two or more Arg/Lys residues, which making them potential substrates of OpdB. These peptides that are called cation antimicrobial peptides possess an affinity to lipopolysaccharides, main components of the external sheath of the gram-negative bacteria [29]. A part of these arginine-reach peptides, being positively charged, can penetrate into the cell and influence physiological processes. For example, buforin II and tachyplesin I bind to nucleic acids [30].

To investigate the action of PSP on oligopeptides, we have chosen the 26-membered peptide melittin (GIGAVLK^7-VLTTGLPALISWIK-R^{22}-KRQQ-NH_2), a biologically active peptide from bee venom [35]. Melittin contains several potential sites of hydrolysis by OpdB: the sequence 21-24 of four positively charged amino acid residues (KRKR-), as well as the residue Lys7. It was shown that under the action of PSP, the first two simultaneously formed products corresponded to the hydrolysis of melittin after residues Arg22 and Lys7: fragments 1-22 (GIGAVLKVLTTGLPALISWIKR) and 8-22 (VLTTGLPALISWIKR). Evidently, the fragment 8-22 is formed due to the additional cleavage of the fragment 1-22, and the rate of the hydrolysis of the polypeptide chain after the lysine residue at least twofold lower than that after the arginine residue. No products corresponding to the cleavage after residues Lys23 and Arg24 were revealed. Thus, in the hydrolysis of both *p*-nitroanilide substrates and oligopeptides catalyzed by PSP, the arginine residue in the P1 position is more favorable than the lysine residue.

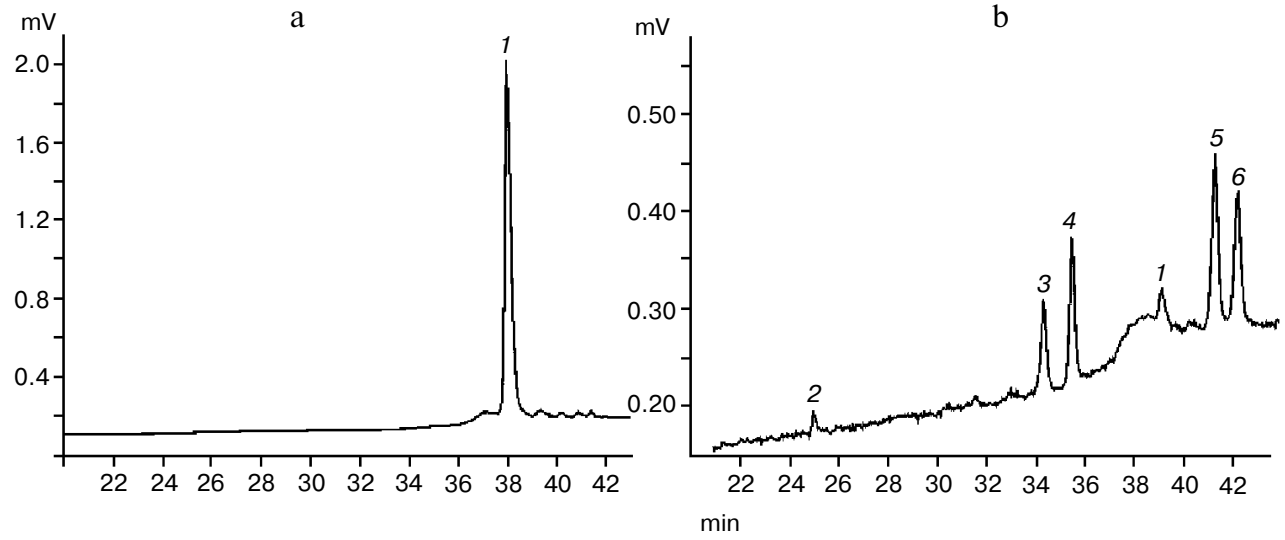

Fig. 4. Chromatogram of products of melittin hydrolysis by PSP ([S] = 90 μM, [E] = 105 nM, 0.1 M Tris-HCl, pH 8.0, 25°C; 3-h incubation). Chromatography conditions: a Luna C8 column (2 × 250 mm; Phenomenex); products were eluted with 0.1% TFA with a 0-100% acetonitrile gradient for 60 min (0.3 ml/min). a) Original preparation of melittin; b) products of melittin hydrolysis by PSP: *1*) melittin (GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2); *2*) fragment 1-7 (GIGAVLK, 657.4 Da); *3*) fragment 8-22 (VLTTGLPALISWIKR, 1668 Da); *4*) fragment 8-21 (VLTTGLPALISWIK, 1512.2 Da); *5*) fragment 1-22 (GIGAVLKVLTTGLPALISWIKR, 2308 Da); *6*) fragment 1-21 (GIGAVLKVLTTGLPALISWIK, 2151.8 Da).

The subsequent incubation together with fragments 1-22 and 8-22 results in the accumulation of fragments 1-21 (GIGAVLKVLTTGLPALISWIK) and 8-21 (VLTTGLPALISWIK) (Fig. 4). For trypanosomal oligopeptidases B, an unusual carboxypeptidase activity was observed: the cleavage of the C-terminal Arg residues (other amino acid residues including Lys were not revealed) [4, 14], but only in the case of the presence of Arg or Lys residues in the P2 position.

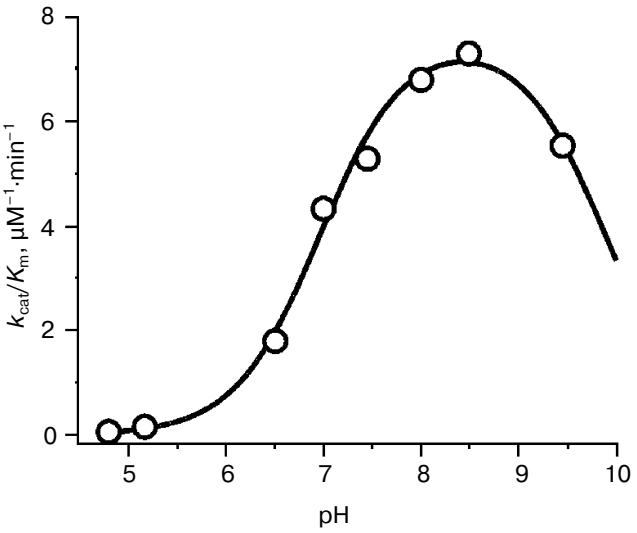

Fig. 5. Effect of pH on activity of PSP. The medium contained 50 mM Ca^{2+}, 0.03 mM BAPNA, and 8.96 nM PSP in 0.1 M acetate buffer (pH 4.8-5.2) or 0.1 M Tris-HCl buffer (pH 6.0-9.45).

Melittin fragments 1-21 and 8-21 are products of such a cleavage of C-terminal Arg residues in fragments 1-22 and 8-22. Thus, PSP also exhibits carboxypeptidase activity of narrow specificity that is characteristic for oligopeptidases B from other sources. The hydrolysis of the antimicrobial oligopeptide melittin by PSP is a model of possible biological functions of this enzyme.

Study of effects of pH and temperature on activity of PSP. The effect of pH on the hydrolysis of the standard trypsin substrate BAPNA was studied. The pH dependence of the hydrolysis exhibits a standard for serine proteinases including OpdB bell-shaped form (Fig. 5). The pK_a values calculated by nonlinear approximation of the experimental data (6.96 ± 0.06 and 9.9 ± 0.1) are similar to the corresponding constants for OpdB from *E. coli*. It was demonstrated that the lower pK_a value (~7) corresponds to the ionization of the active site imidazole residue, and the upper pK_a value (~9-10) is accounted for by the ionization of an unidentified amino acid residue (Lys or Tyr) controlling the active enzyme conformation [20].

Previously, in preliminary experiments with partially purified PSP preparations, we determined that this enzyme is psychrophilic [16]. In the present work, we investigated in detail the influence of temperature on the stability (Fig. 6) and activity (Fig. 7) of highly purified preparations of PSP. It was demonstrated that the enzyme

is rather quickly inactivated: incubation at 43°C for 15 min resulted in 90% inactivation. At 30°C, after 30 min of incubation the enzyme retained only 50-60% of its activity. All earlier investigated oligopeptidases B, both bacterial and trypanosomal, were rather thermostable at pH 8.0. For example, OpdB from *E. coli* retained more than 95% of its activity after 5 h of incubation at 40°C, the inactivation beginning only at 45-50°C [9].

The apparent optimal temperature of the PSP activity is 25°C. At 10°C the activity constitutes more than 60% of its maximal value. Such temperature characteristics of PSP (high activity at low temperatures and inactivation of the enzyme at temperature above 25°C) (Figs. 6 and 7) are typical of psychrophilic enzymes [36].

The amino acid sequence of OpdB determined from the corresponding gene of *S. proteamaculans* [17] showed that in contrast to the corresponding enzymes of other gram-negative bacteria, the protein contains no cysteine residues. The absence of disulfide bonds is one of the factors that are responsible for the enhanced flexibility and, consequently, low temperature stability of enzymes adapted to decreased temperatures [36].

Thus, we found that the oligopeptidase B from *S. proteamaculans* (PSP), exhibiting a number of common properties with OpdB from other sources (pH dependence of hydrolysis, preferable hydrolysis after negatively charged amino acid residues (Arg, Lys), highly specific carboxypeptidase activity), at the same time exhibits some specific features. First of all, this is psychrophilic character of PSP, which is characterized by the low apparent temperature optimum of this enzyme (25°C) and a rather high activity at 5-10°C that is typical for none of the earlier known OpdB. The secondary specificity of PSP also significantly differs from the specificity of OpdB of microbial or trypanosomal origin (substrate inhibition of PSP by peptides with a hydrophobic residue in the P2 position). It should be noted that other OpdB with well-studied enzymatic properties contain two Asp/Glu residues in the S2 center, but PSP contains only one (Asp460). Besides, PSP significantly differs from other oligopeptidases B by the character of the influence of calcium ions. This influence on the PSP activity is so pronounced that at $[Ca^{2+}] = 0$ and at $[Ca^{2+}] = 50$ mM, the same preparation of PSP behaves as two enzymes differing in the efficiency of hydrolysis of various substrates (Table 1 and Fig. 1) and in efficiency of the inhibition by substrates (Table 2 and Fig. 2). Calcium ions affect the temperature stability of PSP, as well as the efficiency of its inhibition by a number of compounds, for example, basic pancreatic trypsin inhibitor [16]. It can be assumed that the complex of PSP with Ca^{2+} has a conformation that differs from that of the free enzyme.

Our experimental data suggest that the binding of one substrate molecule in substrate-binding sites S1 and S2 also results in significant conformational rearrangements in the PSP molecule, since the ES complex acquires an ability to bind additional molecules of aromatic substrates.

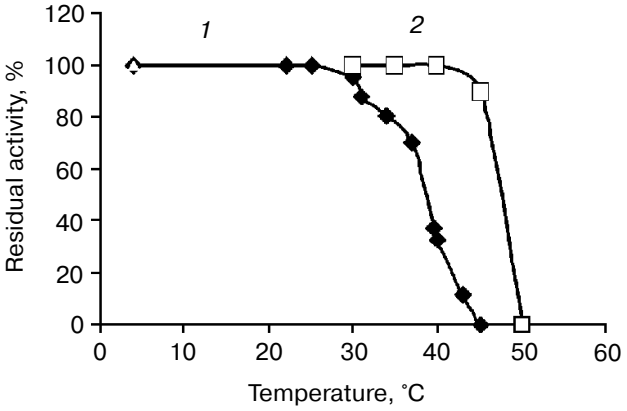

Fig. 6. Thermostability of PSP compared with a mesophilic enzyme. *1*) PSP was preincubated for 15 min at a corresponding temperature in 0.1 M Tris-HCl, pH 8.0. The activity was determined by the hydrolysis of BAPNA (0.2 mM); *2*) OpdB from *E. coli* [9].

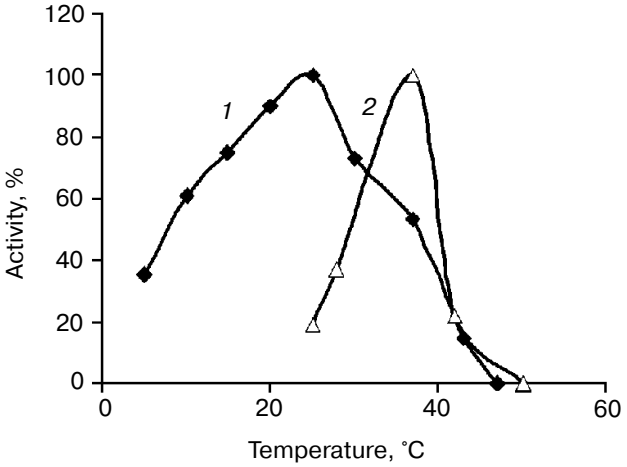

Fig. 7. Effect of temperature on activity of PSP compared with a mesophilic enzyme. *1*) 30.6 μM AcLKR-pNa and 0.53 nM PSP in 0.1 M Tris-HCl, pH 8.0; *2*) OpdB from *T. cruzi* [21].

The importance of conformational rearrangements in the catalytic mechanism of OpdB is confirmed by an unusual isotopic effect of deuterium on the kinetics of the hydrolysis of substrates by OpdB from *E. coli* [19]. The authors of that work suggest that the limiting step of the catalysis in the case of this enzyme is not chemical, but physical process of isomerization of the ES complex into ES′, this preceding the formation of the tetrahedral transition compound (ET) that yields further the acyl-enzyme (EA): $E + S \leftrightarrow ES \leftrightarrow ES' \leftrightarrow ET \leftrightarrow EA \rightarrow E + P$. However, the tertiary structure of oligopeptidases B have not been determined, so it is difficult to speculate concerning the nature of the structural rearrangements and the localization of the potential hydrophobic site.

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