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Oligopeptidase B from *Serratia proteamaculans*. III. Inhibition Analysis. Specific Interactions with Metalloproteinase Inhibitors

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Abstract—Inhibition of the novel oligopeptidase B from *Serratia proteamaculans* (PSP) by basic pancreatic trypsin inhibitor, Zn^{2+} ions, and *o*- and *m*-phenanthroline was investigated. A pronounced effect of calcium ions on the interaction of PSP with inhibitors was demonstrated. Inversion voltamperometry and atomic absorption spectrometry revealed no zinc ions in the PSP molecule. Hydrophobic nature of the enzyme inhibition by *o*- and *m*-phenanthroline was established.

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Key words: oligopeptidase B, *Serratia proteamaculans*, inhibition analysis, zinc-dependent enzymes, atomic absorption spectrometry, inversion voltamperometry

Oligopeptidases B (OpdB) belong to the family of serine prolyl oligopeptidases (clan SC, family S9) [1]. Like trypsin, OpdB hydrolyzes peptide bonds formed by the carbonyls of Arg/Lys residues, preferring Arg to Lys. Most preferable for hydrolysis residues in the P2 position of the substrate are also Arg or Lys [2]. The OpdB enzymes of ancient unicellular eukaryotes (*Trypanosoma cruzi* [3], *T. brucei* [4, 5], and *T. evansi* [6], as well as *Leishmania major* [5] and *L. amazonensis* [7]) are important virulent factors of these protozoan parasites. During trypanosomal infections (Chagas diseases, African sleeping sickness), OpdB is involved in the trypanosomal invasion of the cells [3] and abnormal degradation of peptide hormones of the host organism [6]. In mammals, genes encoding this enzyme have not been revealed. Thus, OpdB of protozoan parasites is a specific target for therapeutic treatment of these dangerous infections. The OpdB enzyme and/or the gene encoding this enzyme have been also found in prokaryotes, namely in such Gram-negative pathogenic bacteria as *Escherichia coli* [8-11], *Moraxella lacunata* [12], *Salmonella enterica* serovar *typhimurium* [13], mycobacteria *Mycobacterium tuberculosis* and *My. leprae* [13], as well as in the spirochete *Treponema denticola* [14]. Bacterial OpdB proteins are much less studied; however, they also can be important targets for antimicrobial therapy [13].

In the psychrotolerant Gram-negative microorganism *S. proteamaculans* 94 we revealed a novel trypsin-like proteinase (PSP) [15]. We obtained homogeneous (by PAGE) preparations of PSP (78 kDa), identified PSP as a previously unknown oligopeptidase B, sequenced the gene of OpdB *S. proteamaculans* 94, constructed a producer *E. coli* strain BL-21(DE3)/pOpdB, and developed a method for isolation of the recombinant enzyme His_6-PSP (part I in this series [16]). The substrate specificity of PSP and the effect of calcium ions, pH, and temperature on the enzyme activity were investigated in detail (part II in this series [17]). It was demonstrated that the enzyme is psychrophilic, i.e. adapted to low temperatures [17].

Previously we demonstrated [15] that natural PSP was efficiently inhibited by inhibitors specific for serine proteinases (diisopropyl fluorophosphate, tosyl-lysine

Abbreviations: AAS, atomic absorption spectroscopy; BAPNA, N_α-benzoyl-DL-arginine-*p*-nitroanilide; BPTI, bovine basic pancreatic trypsin inhibitor; buffer A, 0.1 M Tris-HCl (pH 8.0); DMSO, dimethyl sulfoxide; IVA, inversion voltamperometry; OpdB, oligopeptidase B; PSP, proteinase from *Serratia proteamaculans*; *p*-NA, *p*-nitroanilide.

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chloromethyl ketone, and phenylmethylsulfonyl fluoride) and at the same time by the known chelator and standard inhibitor of zinc-dependent enzymes *o*-phenanthroline, and also by zinc ions, which is characteristic for Zn-dependent metalloproteinases. Thus, the activity of the serine enzyme PSP can be additionally regulated if Zn^{2+} is located in close proximity to the active site of the protein. In the literature, such additional regulation of serine enzymes by metal ions has not been described. An additional regulation of the enzyme activity was shown for trypanosomal oligopeptidase B. One of the trypanosomal OpdB enzymes, namely the OpdB from *T. brucei*, is simultaneously a serine and SH-dependent enzyme: its activity is dependent on Cys256 of the N-terminal domain, as well as by residues Cys559 and Cys597 that are adjacent to the active site serine residue [18].

We investigated PSP using the most important and interesting inhibitors (bovine basic pancreatic trypsin inhibitor that was used as the ligand during affinity chromatography of this enzyme [16], Zn^{2+}, and *o*- and *m*-phenanthroline) to determine whether PSP is a Zn-dependent enzyme. Atomic absorption spectrometry (AAS) and inversion voltamperometry (IVA) were used to determine the content of Zn^{2+} in the enzyme molecule.

MATERIALS AND METHODS

In the present work we used N_α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), bovine basic pancreatic trypsin inhibitor (BPTI), *o*- and *m*-phenanthroline (Sigma, USA); Protein Assay kit (BioRad, USA); Tris, NaCl, glycerol (ICN, USA); *p*′-guanidinobenzoic acid *p*-nitrophenyl ester, dimethyl sulfoxide (DMSO) (Fluka, Germany); Centricon ultrafiltration filters (Millipore, USA). The substrate Ac-Leu-Leu-Arg-pNA (Ac-LLR-pNA) was synthesized in the laboratory of Proteolytic Enzyme Chemistry using standard peptide synthesis procedures. The homogeneity of the peptide was confirmed by HPLC analysis, and the compound was identified by mass-spectrometry assay (Ultraflex TOF/TOF; Bruker, Germany). Other chemicals were of domestic production (high purity grade).

The natural oligopeptidase B from *S. proteamaculans* (PSP) and the corresponding recombinant enzyme (His_6-PSP) were obtained according to the earlier developed methods [16].

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

Protein concentration was determined by the Bradford method using the Protein Assay Kit (BioRad) and bovine serum γ-globulin as the standard. Molar concentration of enzyme solutions was determined by the titration of the enzyme active sites with *p*′-guanidinobenzoic acid *p*-nitrophenyl ester [19].

Kinetic measurements. The initial rate of the hydrolysis of *p*-nitroanilide substrates was determined from the initial linear part of the kinetic curve (the extent of the hydrolysis did not exceed 10%), monitoring the increase in absorbance at 405 nm in buffer A at 25°C using 10 mM stock solutions of *p*-nitroanilide substrates in DMSO. To determine kinetic constants, no less than 10 concentrations of each substrate were used, usually in the range of 0.02-0.2 mM.

Kinetic parameters (k_{cat} and K_m) were calculated from the Michaelis–Menten equation using nonlinear regression. The standard error did not exceed 20%. The inhibition constants (K_i) were determined from the linear dependences v/v_i versus [I] in the cases of competitive and noncompetitive inhibition (Fig. 1) and using

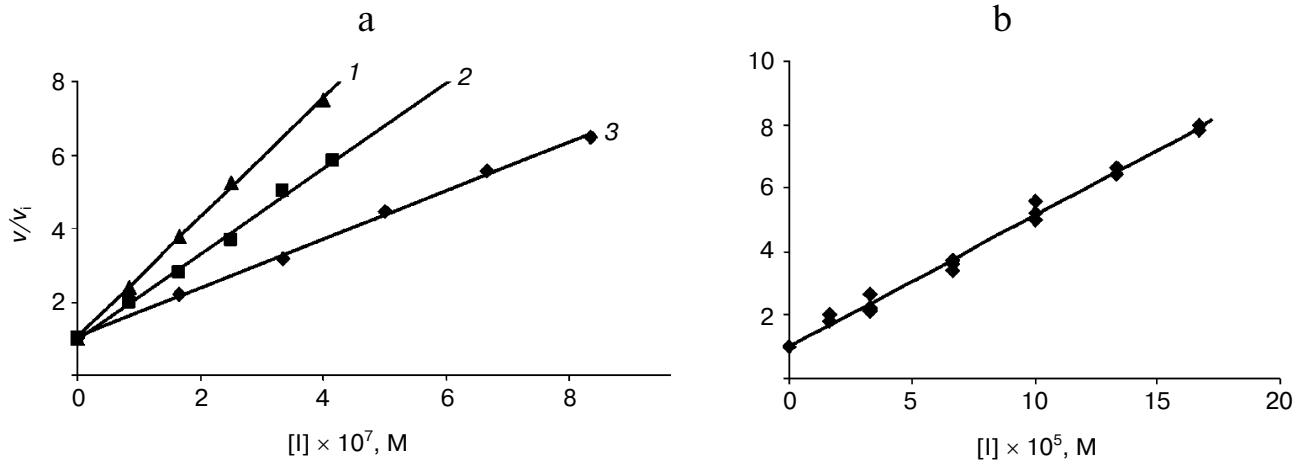

Fig. 1. Inhibition of BAPNA hydrolysis by BPTI (a) and Zn^{2+} (b). Substrate concentrations were 0.05, 0.1, and 0.2 mM (*1-3*, respectively) (a) or 0.05-0.2 mM (b).

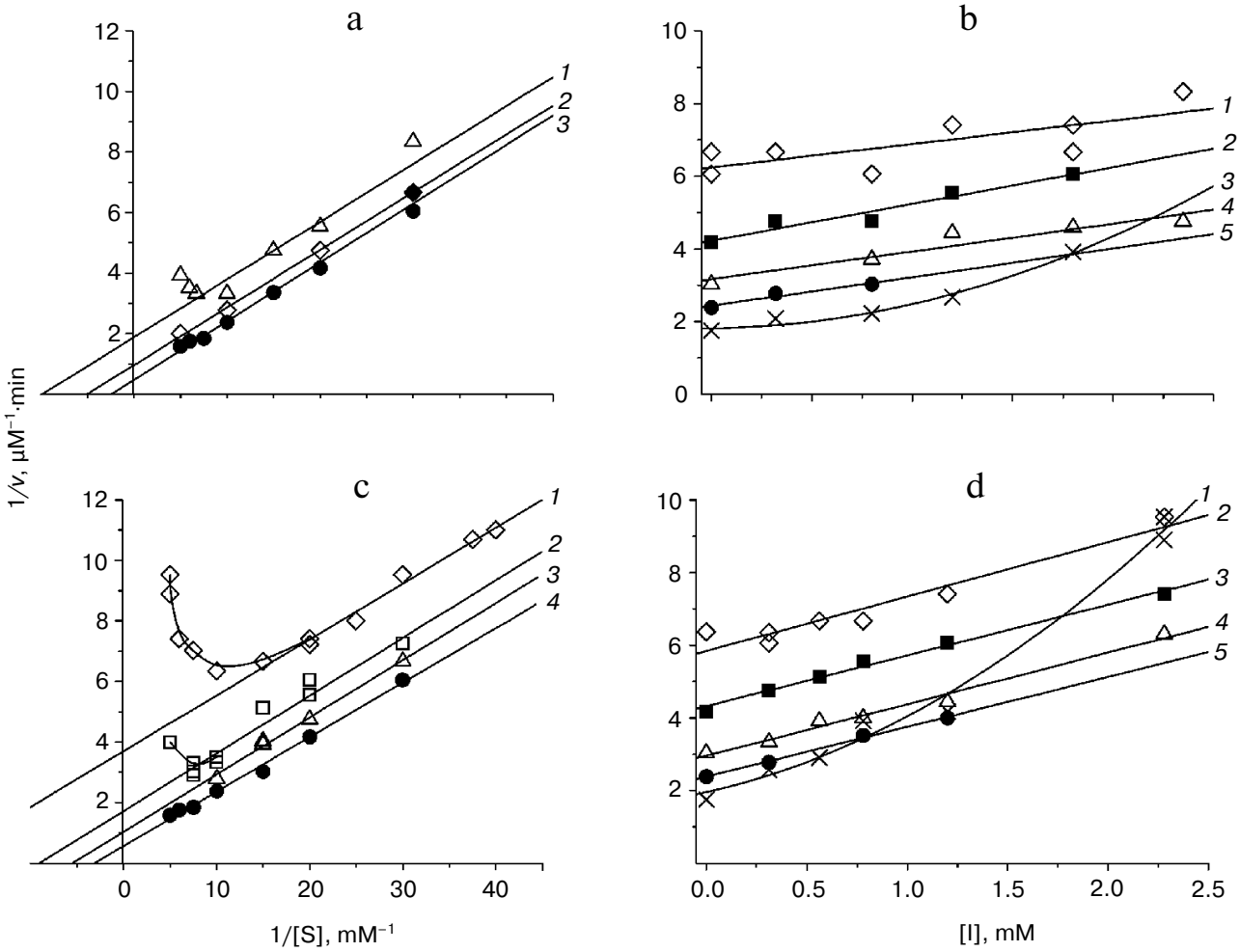

Fig. 2. Inhibition of Ac-LLR-pNA hydrolysis by *o*-phenanthroline (a, b) and *m*-phenanthroline (c, d). The reaction mixture contained 2.58 nM His_6-PSP. Constant concentrations of inhibitors (a, c) or substrates (b, d) constituted: a) 3.2, 1.8, and 0 mM (*1-3*, respectively); b, d) 0.033, 0.05, 0.067, 0.1, and 0.2 mM (*1-5*, respectively); c) 2.28, 0.78, 0.56, and 0 mM (*1-4*, respectively).

Eq. (2) in the case of the anticompetitive inhibition (Fig. 2). The K_S' and n values were determined by nonlinear approximation of the experimental points to Eqs. (3) and (4).

Determination of Zn^{2+} content in PSP. A homogeneous (by PAGE) preparation of His_6-PSP (10 μM) in buffer A was concentrated 5-fold using Centricon-30 centrifugal filters and then diluted with an equal volume of glycerol. In a blank experiment, buffer A without protein was treated in the same way. The experimental and blank samples were stored at −20°C.

The protein solution (1 ml) was dispensed in quartz 20-ml cups and evaporated to dryness. To the cooled samples, 0.3-1 ml of concentrated HNO_3 was added, and the samples were charred using a hot plate. The charred samples were placed into a muffle furnace for 30 min at 350°C and then for 30 min at 550°C. If ash particles were present, the treatment with HNO_3 was repeated; the solution was evaporated to dryness and placed into the muffle furnace for 1.5 h at 550°C. To determine the content of Zn^{2+} using AAS analysis, the residue was dissolved in 5 ml of concentrated HNO_3 or in 0.5 ml of concentrated formic acid and incubated at room temperature for 10 min. Then the volume was adjusted to 5 ml with water, and the samples were assayed for Zn^{2+} content. The blank experiment was carried out in the same way using 1 ml of buffer without protein.

The content of Zn^{2+} was determined using an atomic absorption spectrometer (Kvant-AFA, Russia) at 211.4 nm. Calibration curves were created using standard mixtures (Ekoanalitika, Russia) according to the supplier's methodological recommendations (measuring accuracy, 5%). Part of the samples was analyzed by IVA using a TA-4 device (Tom'analit, Russia). The obtained voltamperograms were processed using the VaLabTx program. The result is presented as the mean of two values obtained for the same sample differing by no more than 36%.

RESULTS AND DISCUSSION

Inhibitor analysis. Previously, the inhibitor analysis of PSP was performed using a partially purified preparation of the natural enzyme [15]. In the present work, the effect of the most important of the found inhibitors was investigated in detail with identification of the inhibition type and determination of inhibition constants (Table 1) using homogeneous PSP preparations (natural and recombinant ones). As known from the literature, the presence of the N-terminal hexahistidine sequence does not affect the enzymatic properties of the recombinant OpdB from different sources [6, 11, 13]. Previously, we demonstrated that the substrate characteristics of the natural and recombinant enzymes are the same [17]. In the present work, inhibition constants were determined using the recombinant enzyme, but some experiments with a certain concentration of the inhibitors were repeated using the natural enzyme (the data coincided).

A general scheme of the inhibition suggesting that the enzyme–inhibitor complexes are unable to yield reaction products can be presented as follows:

$$\begin{array}{ccccc} & & \alpha K_m & & k_{cat} \\ E + S & \rightleftharpoons & ES & \longrightarrow & E + P \\ +I \uparrow\downarrow & \alpha K_i' & +I \uparrow\downarrow & \beta K_i' & \\ & & \beta K_m & & \\ EI + S & \rightleftharpoons & ESI, & & \end{array} \qquad (1)$$

where E is the enzyme; S is the substrate; P is the product; I is the inhibitor; αK_m and βK_m are the Michaelis constants for the free enzyme and its complex with the inhibitor, respectively; $\alpha K_i'$ and $\beta K_i'$ are the inhibition constants of the free enzyme and the enzyme–substrate complex, respectively, and k_{cat} is the catalytic constant.

In contrast to all known OpdB forms from other sources, PSP is efficiently inhibited by bovine basic pancreatic trypsin inhibitor (BPTI). This allowed elaboration of an efficient method for the purification of this enzyme based on affinity chromatography, which significantly simplified complex multistep procedures described for other natural oligopeptidases B (for example, from *E. coli* [8] and *T. brucei* [4, 5]).

BPTI is a competitive inhibitor of PSP ($\beta \to \infty$, $K_i = \alpha K_i'$), both in the absence of Ca^{2+} and in the presence of 50 mM Ca^{2+}. In the latter case the inhibition effect is reduced by more than two orders of magnitude (Fig. 1a and Table 1). Trypsin and other trypsin-like serine proteinases (thrombin, kallikrein, enteropeptidase, etc.) are also inhibited by BPTI through the competitive mechanism.

Earlier we demonstrated that natural PSP is efficiently inhibited by Cu^{2+}, Cd^{2+}, and Zn^{2+} [15]. It could be assumed that these bivalent metal ions bind to the carboxyl groups of the aspartic and glutamic acid residues in the substrate-binding site of PSP (S1 and S2). However, it appeared that Zn^{2+} is a noncompetitive inhibitor of this enzyme (Fig. 1b and Table 1): $\alpha K_i' = \beta K_i' = K_i$. Thus, presumably, zinc ions interact not with the S1 and S2 sites, but with some other sites affecting the enzyme activity.

As demonstrated for different zinc-dependent metalloenzymes, an excess of zinc ions inhibits their catalytic activity [20-22]. Besides, we revealed inhibition of PSP by *o*-phenanthroline ([15], Table 1), a well-known chelator and standard inhibitor of zinc-dependent enzymes [23, 24]. This suggests that PSP is a serine and simultaneously a Zn^{2+}-dependent enzyme.

In the case of Zn^{2+}-dependent metalloenzymes, both *o*-phenanthroline and Zn^{2+} are competitive inhibitors, since the active site of these enzymes contains a zinc ion [20-22]. In the range of 0.05-0.2 mM BAPNA, the I_{50} value for the inhibition of PSP by *o*-phenanthroline did not increase with increase in the substrate concentration. This suggests noncompetitive character of the enzyme inhibition, and also points to the fact that the Zn^{2+}-binding site of the enzyme is not at its active site.

To reveal the firmly bound zinc in the PSP molecule, the enzyme was incubated with *o*-phenanthroline with

Table 1. Inhibition of PSP by some compounds

Inhibitor	Substrate	Mechanism of inhibition	$[Ca^{2+}]$, mM	K_i, μM
BPTI	BAPNA	competitive	0 50	0.035 7.35
$ZnCl_2$	–"–	noncompetitive	0	24.4
o-Phenanthroline	BAPNA Ac-LLR-pNA	anticompetitive –"–	0 0	1590 820
m-Phenanthroline	BAPNA Ac-LLR-pNA	–"– –"–	0 0	310 440

Note: Error for K_i does not exceed 20%.

subsequent dialysis according to the procedures described for the preparation of the apoenzymes of astacin [23] and serralysin [24]. However, after the removal of *o*-phenanthroline, the activity of PSP was recovered. This suggests that PSP does not contain Zn^{2+}, or it is bound to the protein so tightly that it is impossible to remove it by the treatment with *o*-phenanthroline [25].

To distinguish between these two alternatives, Zn^{2+} content was determined by IVA and AAS. According to the IVA data, the content of Zn^{2+} in the sample containing PSP did not differ significantly from that in the blank sample and did not exceed 0.03 atom per molecule of protein. AAS also did not reveal significant difference in Zn^{2+} content between PSP and blank samples. Thus, no zinc was detected in the PSP molecule. The efficient inhibition (K_i = 24 μM) of the enzyme activity by zinc ions can be explained by their binding to the His cluster ^{277}HYHQH^{281}. Such a cluster is absent in all previously studied oligopeptidases B from other sources.

However, the mechanism of inhibition of the PSP activity by the metal chelator *o*-phenanthroline remained unclear. It is known that the inhibition of enzymes by *o*-phenanthroline can be explained by not only chelation with metal ions, but also by hydrophobic interactions of the reagent with the protein molecule [25]. To distinguish between these two possibilities, we used the nonchelating analog of the reagent, *m*-phenanthroline. It was found that *m*-phenanthroline inhibited PSP more efficiently than *o*-phenanthroline, but in the presence of 50 mM Ca^{2+} the effect of both *o*- and *m*-phenanthroline disappeared. Detailed investigation of the kinetics of the PSP inhibition by *o*- and *m*-phenanthroline showed that the I_{50} value is inversely related to the substrate concentration: in contrast to the case of competitive inhibition, the inhibitory effect (v/v_i) grows with the increase in the substrate concentration. Such dependence suggests an anticompetitive type of inhibition [26], a rarer inhibition type, where the inhibitor interacts only with the enzyme–substrate complex. According to scheme (1), in the case of the anticompetitive inhibition, $\alpha K_i = \infty$, $K_i = \beta K_i'$, and the dependence of the inverse value of the initial hydrolysis rate on substrate concentration is the following:

$$1/v_i = (1 + [I]/K_i)/(k_{cat}[E]) + K_m/(k_{cat}[E][S]). \quad (2)$$

At different concentrations of the inhibitor and the substrate, Eq. (2) yields a series of parallel straight lines in both Lineweaver–Burk ($1/v$ versus $1/[S]$) and Dixon ($1/v$ versus [I]) coordinates. To distinguish the anticompetitive inhibition from some other complex types of inhibition and to determine the inhibition constant, it is necessary to create both of these types of plots [26]. We plotted the corresponding dependences for the hydrolysis of the substrates BAPNA and Ac-LLR-pNA catalyzed by PSP in the presence of *o*- and *m*-phenanthroline. It should be noted that the chosen substrates do not exhibit the effect of substrate inhibition within the used concentration range [17]. Figure 2 demonstrates corresponding plots in the Lineweaver–Burk (a, c) and Dixon (b, d) coordinates for the substrate Ac-LLR-pNA. The presented data point to the anticompetitive character of inhibition of PSP by *o*- and *m*-phenanthroline. The enzyme interacts with one molecule of the inhibitor. The inhibition constants corresponding to Eq. (2) are given in Table 1.

However, as seen in Fig. 2, the straight lines in the Lineweaver–Burk and Dixon plots are parallel only at low concentrations of the substrates and inhibitors. At high concentrations of [S] and [I], the kinetics of the hydrolysis deviates from the linear dependence, which is clearly seen in the coordinates $1/v$ versus $1/[S]$: similar deviation is typical for Michaelis kinetics in the case of inhibition by the substrate.

As we demonstrated earlier for the substrate inhibition [17], the inhibition constant K_S' and the number of the additional substrate molecules binding to the enzyme–substrate complex (n) can be calculated from Eq. (3):

$$v = k_{cat\ ef}[E][S]/\{(K_{m\ ef} + [S] + [S]^{n+1}/(K_S')^n\}, \quad (3)$$

where the parameters $K_{m\ ef}$ and $k_{cat\ ef}$ according to Eq. (2) for the anticompetitive inhibition are described by the following equations:

$$K_{m\ ef} = K_m/(1 + [I]/K_i),\ k_{cat\ ef} = k_{cat}/(1 + [I]/K_i). \quad (4)$$

In all considered cases, the parameter n constituted ~2, and the substrate inhibition constant was ~100 μM (Table 2). Thus, the complex of PSP with the substrate is capable of binding large hydrophobic molecules of *o*- and *m*-phenanthroline, and the formed triple enzyme–substrate–phenanthroline complexes additionally bind two substrate molecules:

$$\begin{array}{ccc} & \overset{K_m}{\rightleftharpoons} & \overset{k_{cat}}{\longrightarrow} \\ E + S & & ES \quad E + P \\ & +I \downarrow \uparrow K_i & \\ & ESI & \\ & +2S \downarrow \uparrow K_S' & \\ & ES_3I. & \end{array} \quad (5)$$

In the absence of *o*- and *m*-phenanthroline, the kinetics of the hydrolysis of BAPNA and Ac-LLR-pNA in the range of high substrate concentrations corresponds to the Michaelis–Menten equation, but in the presence of the hydrophobic aromatic inhibitors interacting with the enzyme–substrate complex, the hydrolysis of these compounds is inhibited by high substrate concentrations

Table 2. Substrate inhibition of PSP in the presence of phenanthroline (reaction medium contained buffer A and 2% DMSO)

Substrate	Effector	Phenanthroline concentration, mM	*n*	K'_S, μM
Ac-LLR-pNA	*m*-phenanthroline	2.28	1.6 ± 0.4	90 ± 40
		1.20	2.7 ± 0.5	110 ± 30
	o-phenanthroline	1.80	2.0 ± 0.5	113 ± 5
		1.20	2.0 ± 0.6	100 ± 20
BAPNA	*m*-phenanthroline	2.28	2.0 ± 0.5	130 ± 40
		1.20	2.0 ± 0.2	190 ± 30
		0.78	2.1 ± 0.4	60 ± 10
		0.56	2.6 ± 0.9	100 ± 55
		0.31	2.3 ± 0.6	120 ± 20
	o-phenanthroline	2.35	3.0 ± 0.8	130 ± 30
		1.20	2.0 ± 0.6	220 ± 50

(substrate inhibition), which is characteristic for substrates containing an aromatic amino acid residue in the P2 position [17].

This ability of PSP to supplement the "construction" of the substrate with a hydrophobic aromatic residue of *o*- and *m*-phenanthroline is typical for the enzyme only in the absence of calcium ions. In the presence of 50 mM Ca^{2+}, even at maximal concentration of the substrate (0.2 mM) and phenanthroline (2 mM), the rate of the hydrolysis of the substrate decreases by no more than 20-30%.

The secreted OpdB from *T. cruzi* [27] was also shown to be inhibited by zinc ions and *o*-phenanthroline. Based on these results, it was suggested that this OpdB was a Zn-dependent enzyme. Presumably, OpdB from *T. cruzi* is similar to PSP, and its activity is also controlled by hydrophobic effectors.

In conclusion, it should be noted that the observed regulation of the PSP enzymatic activity by hydrophobic effectors may serve for the prevention of hydrolysis of certain undesirable substrates, as well as for the promotion of hydrolysis of some natural substrate containing a specific hydrophobic site.

The substrate analysis demonstrated that PSP significantly differs from other oligopeptidases B in the character of the influence of calcium ions [17]. Calcium ions significantly influence the activity of PSP, affecting the efficiency of hydrolysis of different substrates and substrate inhibition [17]. In the present investigation, the inhibitor analysis revealed a pronounced dependence of the efficiency of PSP inhibition on the presence of Ca^{2+}. For example, the K_i value for BPTI in the presence of 50 mM Ca^{2+} increases by more than two orders of magnitude, and the efficiency of the inhibition sharply decreases (Table 1). The inhibition of the hydrolysis of the substrates by *o*- and *m*-phenanthroline is completely suppressed in the presence of 50 mM Ca^{2+}. Obviously, the conformation of the complex of PSP with Ca^{2+} differs from that of the free enzyme.

Our data also suggest that on binding one molecule of the substrate in S1 and S2 sites, the PSP molecule also experiences significant conformational rearrangements, since in contrast to the free enzyme, the ES complex acquires an ability to bind additional molecules of aromatic substrates and inhibitors.

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REFERENCES

1. Polgar, L. (2002) *Cell Mol. Life Sci.*, **59**, 349-362.
2. Hemerly, J. P., Oliveira, V., Del Nery, E., Morty, R. E., Andrews, N. W., Juliano, M. A., and Juliano, L. (2003) *Biochem. J.*, **373**, 933-939.
3. Burleigh, B. A., Caler, E. V., Webster, P., and Andrews, N. W. (1997) *J. Cell Biol.*, **136**, 609-620.
4. Troeberg, L., Pike, R. N., Morty, R. E., Berry, R. K., Coetzer, T. H., and Lonsdale-Eccles, J. D. (1996) *Eur. J. Biochem.*, **238**, 728-736.
5. Morty, R. E., Lonsdale-Eccles, J. D., Morehead, J., Caler, E. V., Mentele, R., Auerswald, E. A., Coetzer, T. H., Andrews, N. W., and Burleigh, B. A. (1999) *J. Biol. Chem.*, **274**, 26149-26156.
6. Morty, R. E., Pelle, R., Vadasz, I., Uzcanga, G. L., Seeger, W., and Bubis, J. (2005) *J. Biol. Chem.*, **280**, 10925-10937.
7. De Matos Guedes, H. L., Carneiro, M. P. D., Gomes, D. C. O., Rossi-Bergmanmn, B., and de Simone, S. G. (2007) *Parasitol. Res.*, **101**, 853-863.
8. Pacaud, M., and Richaud, C. (1975) *J. Biol. Chem.*, **250**, 7771-7779.
9. Polgar, L. (1997) *Proteins*, **28**, 375-379.

10. Kanatani, A., Masuda, T., Shimoda, F., Misoka, X. S., Lin, T., Yoshimoto, D., and Tsuru, D. (1991) *J. Biochem.*, **110**, 315-320.
11. Yan, J.-B., Wang, G.-Q., Du, P., Zhu, D.-X., Wang, M.-W., and Jiang, X.-Y. (2006) *Prot. Expr. Purif.*, **47**, 645-650.
12. Yoshimoto, T., Tabira, J., Kabashima, T., Inoue, S., and Ito, K. (1995) *J. Biochem.*, **117**, 654-660.
13. Morty, R. E., Fulop, V., and Andrews, N. W. (2002) *J. Bacteriol.*, **184**, 3329-3337.
14. Fenno, J. C., Lee, S. Y., Bayer, C. H., and Ning, Y. (2001) *Infect. Immun.*, **69**, 6193-6200.
15. Mikhailova, A. G., Likhareva, V. V., Khairullin, R. F., Lubenets, N. L., Rumsh, L. D., Demidyuk, I. V., and Kostrov, S. V. (2006) *Biochemistry (Moscow)*, **71**, 563-570.
16. Khairullin, R. F., Mikhailova, A. G., Sebyakina, T. Yu., Lubenets, N. L., Ziganshin, R. Kh., Demidyuk, I. V., Gromova, T. Yu., Kostrov, S. V., and Rumsh, L. D. (2009) *Biochemistry (Moscow)*, **74**, 1164-1172.
17. Mikhailova, A. G., Khairullin, R. F., Demidyuk, I. V., Gromova, T. Yu., Kostrov, S. V., and Rumsh, L. D. (2011) *Biochemistry (Moscow)*, **76**, 480-490.
18. Juhasz, T., Szeltner, Z., Renner, V., and Polgar, L. (2002) *Biochemistry*, **41**, 4096-4106.
19. Walsh, K. F., and Wilcox, P. E. (1970) *Meth. Enzymol.*, **19**, 31-41.
20. Mikhailova, A. G., Vorotyntseva, T. I., Bessmertnaya, L. Ya., and Antonov, V. K. (1984) *Biokhimiya*, **49**, 1733-1738.
21. Larsen, K. S., and Auld, D. A. (1989) *Biochemistry*, **28**, 9620-9625.
22. Larsen, K. S., and Auld, D. A. (1991) *Biochemistry*, **30**, 2613-2618.
23. Stocker, W., Wolz, R. L., and Zwilling, R. (1988) *Biochemistry*, **27**, 5026-5032.
24. Park, H. I., and Ming, L.-J. (2002) *J. Biol. Inorg. Chem.*, **7**, 600-610.
25. Drum, D. E., and Vallee, B. L. (1970) *Biochemistry*, **9**, 4078-4086.
26. Berezin, I. V., and Klesov, A. A. (1976) *Practical Course in Chemical and Enzyme Kinetics* [in Russian], MGU Publisher, Moscow, pp. 82-84.
27. Da Silva-Lopez, R. E., Morgado-Diaz, J. A., dos Santos, P. T., and de Simone, S. G. (2008) *Acta Trop.*, **107**, 159-167.