Journal of Peptide Science
J. Peptide Sci. 2006; **12**: 65–71
Published online 9 June 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.683

JOURNAL OF PEPTIDE SCIENCE

Protamine: a unique and potent inhibitor of oligopeptidase B

AKIHIKO TSUJI,[a]* TADASHI YOSHIMOTO,[b] KEIZO YUASA[a] and YOSHIKO MATSUDA[a]

[a] Department of Biological Science and Technology, The faculty of Engineering, The University of Tokushima, 2-1 Minamijosanjima, Tokushima 770-8506, Japan
[b] School of Pharmaceutical Sciences, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan

Received 9 February 2005; Accepted 15 March 2005

Abstract: Oligopeptidase B is a serine endopeptidase found in prokaryotes, unicellular eukaryotes and higher plants. The enzyme has been shown recently to play a central role in the pathogenesis of several parasitic diseases such as African trypanosomiasis, and to be a potential therapeutic target. This study reports that protamine, a basic peptide rich in arginine, is a potent inhibitor at the nanomolar level of oligopeptidase B from *E. coli* and wheat. Protamines 1B, 2C, 3A and TP17 displayed similar inhibitory activities and were capable of binding strongly to oligopeptidase B without proteolytic cleavage. The concentration of protamine needed for 50% inhibition (IC_{50}) of oligopeptidase B was 10^4-fold lower than the IC_{50} of trypsin. Oligopeptidase B was highly sensitive to inhibition by protamines even in the presence of serum (IC_{50}, 1 μM). These data indicate that protamines might provide information useful for the design of more specific synthetic oligopeptidase B inhibitors.

Keywords: oligopeptidase B; protamine; protease inhibitor; serine protease; trypanocidal drug

INTRODUCTION

Oligopeptidase B (EC 3.4.21.83) is a member of the prolyl oligopeptidase family of serine peptidases belonging to clan SC, family S9 [1–3], which includes oligopeptidase B (OPB), prolyl oligopeptidase (POP) and dipeptidyl aminopeptidase. OPB catalyses the hydrolysis of synthetic substrates and oligopeptides consisting of no more than 30 amino acid residues at the carboxyl side of lysine and arginine. POP (EC 3.4.21.26) cleaves peptide bonds on the carboxyl side of prolyl residues within peptides [4]. Despite the difference in cleavage specificity, members of this family share significant amino acid sequence homology. Structural analysis shows that members are composed of two domains: an *N*-terminal β-propeller and a *C*-terminal catalytic domain [5,6]. The *N*-terminal domain has been suggested to exclude proteins from the enzyme, saving them from proteolysis [7,8]. OPB (previous name: protease II) is a cytosolic enzyme that was first cloned and characterized from *Escherichia coli* [2,9] and has been described in other prokaryotes [10], ancient eukaryotic unicellular organisms [11–14] and higher plants [15]. Genome analysis suggests that OPB is absent in mammals.

Peptidases are widely implicated as virulence factors and chemotherapeutic targets in parasitic diseases [16,17]. African trypanosomiasis caused by *Trypanosoma brucei*, commonly called sleeping disease, is a severe disease that is fatal if left untreated. American trypanosomiasis caused by *Trypanosoma cruzi* is known as Chagas' disease. OPB plays a key role in the pathogenesis of trypanosomiasis. OPB generates a calcium-signaling factor that interacts with a receptor on the mammalian cell surface, mobilizing Ca^{2+} from intracellular pools and promoting invasion by *Trypanosoma cruzi*. Targeted deletion of OPB in *Trypanosoma cruzi* causes significant impairment of their ability to infect mammalian cells. OPBs from *Trypanosoma brucei* and *Trypanosoma congolense* are released into the plasma and play a role in the pathogenesis of trypanosomiasis by digesting peptide hormones in the plasma [12,14]. Thus, OPB is a fascinating target for potential therapeutic agents to treat or prevent trypanosomiasis. To date, a number of drugs are available for the chemotherapy of trypanosomiasis, such as suramin, pentamidine and diminazene [18,19]. Although the biochemical basis of their trypanocidal action is unclear, these agents inhibit OPB and their inhibitory activities correlate with their trypanocidal efficacy against the trypanosoma OPB of a suramin analogue [18].

In our survey of OPB inhibitors from natural sources, it was found that protamines exhibit a marked inhibitory effect on *E. coli* and wheat OPB activities. It had been expected that protamines would be a good substrate for OPB because protamines are basic peptides with high arginine content. However, in contrast to our expectation, protamine was not cleaved by OPB, but inhibited OPB activity at the nanomolar level. Although leupeptin, a specific inhibitor of trypsin-like serine proteases, also inhibited OPB, the concentration of leupeptin required to achieve 50%

*Correspondence to: A. Tsuji, Department of Biological Science and Technology, Faculty of Engineering, The University of Tokushima, 2-1 Minamijosanjima, Tokushima 770–8506, Japan;
e-mail: tsuji@bio.tokushima-u.ac.jp

inhibition (IC_{50}) of *E. coli* OPB was 1000-fold higher than the IC_{50} of protamine *in vitro*. The interaction of protamine with OPB was examined further using immobilized protamine–Sepharose.

MATERIALS AND METHODS

Materials

Peptide-4-methylcoumaryl-7-amide (MCA) substrates, antipain, leupeptin and water-soluble carbodiimide [1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride] were purchased from the Peptide Institute (Osaka, Japan). Protamine sulfate was obtained from Nacalai Tesque Inc. (Kyoto, Japan). Histone from calf thymus was purchased from MP Biomedicals (Aurora, OH, USA). Suramin was from Wako Pure Chemical (Osaka, Japan). Hepta-L-arginine was from Bachem AG (Bubendorf, Switzerland). All other chemicals used were of analytical grade.

Preparation of Proteases

E. coli and wheat oligopeptidase B were purified from *E. coli* transformed with the *E. coli* OPB expression vector [2] and wheat germ [15], respectively, as described previously. Recombinant furin was expressed transiently in HEK293 cells transfected with the expression vector containing cDNA encoding a truncated mouse furin. The resulting conditioned medium was concentrated by ultrafiltration and used as an enzyme source, as described previously [20]. Bovine trypsin was purchased from Sigma Chemical Co (St Louis, MO, USA) and *Flavobacterium* prolyl endopeptidase was obtained from Seikagaku Corporation (Tokyo, Japan).

Enzyme Assay

The OPB and trypsin activities were measured using carbobenzoxy-Phe-Arg-4-methylcoumaryl 7-amide (Z-Phe-Arg-MCA) unless otherwise stated. The reaction mixture contained 0.1 M Tris-HCl (pH 8.0) and 50 μM Z-Phe-Arg-MCA. The reaction was initiated by adding the enzyme solution and monitored by recording the changes in fluorescence at 460 nm upon excitation at 380 nm at 25°C using a Hitachi F-3010 fluorescence spectrophotometer. Furin activity was measured using L-pyroglutamyl (pyr)–Arg–Thr–Lys–Arg–MCA at pH 7.0 in 100 mM Tris-HCl and 2 mM $CaCl_2$ as described previously [20]. Prolyl endopeptidase activity was measured using Gly–Pro–MCA at pH 7.0 in 0.1 M Tris-HCl. Following incubation at 37°C for 10–20 min, the reaction was terminated by adding acetic acid and the liberated MCA was measured fluorometrically as previously described [21]. The protein concentration was measured by the method of Bradford using BSA as a standard [22].

Purification of Protamine Sulfate

Protamine sulfate was dissolved in water and subjected to gel filtration on Sephadex G-25 equilibrated with water to remove low-molecular weight materials. After lyophilization, dry weight was measured and the lyophilized purified material was used.

Isolation of Protamine Components

Purified protamine sulfate was dissolved in 0.1% trifluoroacetic acid and separated by reversed-phase high-performance liquid chromatography (RP-HPLC) on a μRPC C2/C18 pc3.2/3 column (Pharmacia, Uppsala, Sweden). Protamines (protamine 3A, TP17, 2C and 1B) were eluted at 0.24 ml/min with a linear gradient of acetonitrile (7.5%–25% in 35 min) in 0.1% trifluoroacetic acid. The peaks were collected and further purified by rechromatography on the same column. Purified protamine components were dissolved in water and their amino acid sequences were determined using a Shimadzu amino acid sequencer (Model PPSQ-10). The concentration of the peptide was determined by quantitative amino acid analysis after hydrolysis in 6 N HCl at 110°C for 24 h.

Preparation of Protamine–Sepharose 4B

Protamine (100 mg) was dissolved in 20 ml of water, its pH was adjusted to pH 4.5 with 6 N HCl. The protamine was immobilized with 8 ml of AH-Sepharose (Pharmacia) using water-soluble carbodiimide and washed according to the manufacturer's protocol. *E. coli* oligopeptidase B (20 μg) was incubated with additives in 0.1 M Tris-HCl, pH 7.5 at 25°C. After 10 min, protamine–Sepharose (20 μl) was added and incubated at 25°C. After 30 min, the aliquots were centrifuged at 12 000 rpm for 30 s and the supernatants were removed. The gel was washed twice with 0.5 ml of 50 mM Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl by centrifugation. Finally, the gel was suspended in 25 μl of SDS-PAGE loading buffer and treated at 95°C for 5 min. SDS-PAGE was performed by the method of Laemmli [23] and the gels were stained with Coomassie Brilliant Blue R-250.

RESULTS AND DISCUSSION

Inhibition of OPB by Protamine

OPB cleaves peptide bonds at the carboxyl side of arginine or lysine residues. Previous studies have shown that OPB hydrolysis the multibasic amino acids site faster than the monobasic site and that the resultant carboxyl-terminal dibasic amino acid is further cleaved by carboxypeptidase activity of OPB [13,15,24]. Because protamine contains polyarginine sequences (e.g. tetra-, penta- and hexa-arginine sequences), it was expected that protamine would be a good substrate for OPB. However, it was found that the HPLC profile of protamine did not change after digestion with OPB. Further analysis demonstrated that protamine was an uncleavable competitive inhibitor of OPB. Figure 1A shows the comparison of the effects of protamine on the activity toward synthetic substrate of OPBs, furin, trypsin and prolyl endopeptidase. Because the Arg–X–X–Arg motif is a recognition sequence of furin [20], it was expected that protamine would be a competitive inhibitor of furin. Although the cleavage specificity of prolyl endopeptidase differed from that of OPB, they have similar three-dimensional structures composed of homologous *N*-terminal β-propeller

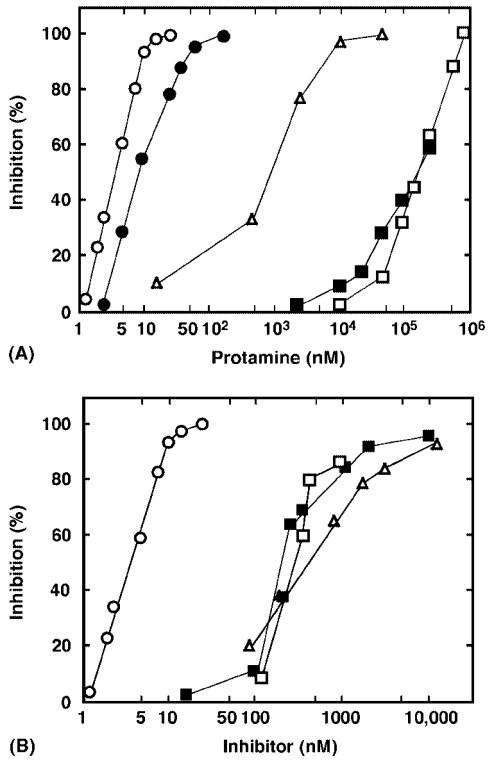
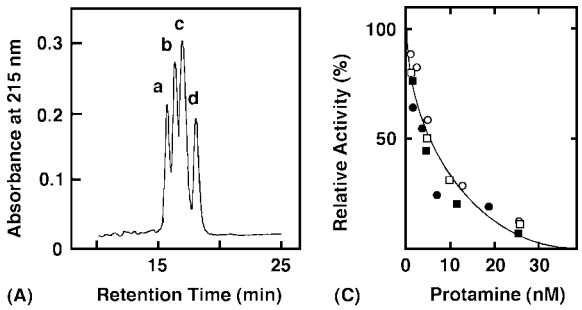


Figure 1 (A) Effect of protamine on activities of OPB, furin, prolyl endopeptidase and trypsin. Increasing amounts of protamine were added to the reaction mixture and the reactions were started by the addition of *E. coli* OPB (170 ng, open circles), wheat OPB (34 ng, closed circles), furin (10 µl of the concentrated conditioned medium, open triangles), prolyl endopeptidase (10 µg, closed squares) and trypsin (0.2 µg, open squares). The rate of hydrolysis of the MCA substrate was monitored by recording the change in fluorescence at 460 nm upon excitation at 380 nm. The concentration of protamine was calculated assuming an average molecular weight of protamine of 4200 Da. (B) Inhibition of *E. coli* OPB by protamine, histone, suramin and leupeptin. Protamine (open circles), histone (opened squares), suramin (closed squares), leupeptin (open triangles). The concentration of histone was calculated by assuming an average molecular weight of 15 000 Da. Each value is the mean of three or more independent experiments.



peak	sequence	protamine
a	PRRRRRSSSRPIRRRRRPRVSRRRRRGGRRRR	3A
b	PRRRRSSSRPVRRRRRPRVSRRRRRRGRRRRR	TP17
c	PRRRRSSRRPVRRRRRPRVSRRRRRRGGRRRR	2C
d	PRRRRASRRIRRRRRPRVSRRRRRGGRRRR	1B

(B)

Figure 2 Effects of protamine 3A, TP17, 2C and 1B on *E. coli* OPB. (A) Purification and sequences of protamine components. Protamines were purified using RP-HPLC described in Materials and Methods. (B) The sequences of protamine 3A, TP17, 2C and 1B are shown. (C) Inhibition of *E. coli* OPB by protamine 3A (open circles), TP17 (closed circles), 2C (open squares) and 1B (closed squares).

and *C*-terminal catalytic domains. Starting with equal amounts of MCA substrate cleavage activity, *E. coli* and wheat OPB were most sensitive to protamine inhibition (Figure 1A). The concentration of protamine needed to inhibit 50% of the activity (IC_{50}) was 3 nM for *E. coli* OPB, 7 nM for wheat OPB, 1 µM for furin and 150 µM for trypsin or prolyl endopeptidase. Both OPBs were inhibited at 140–330-fold lower concentrations of protamine than was furin. Inhibition of trypsin and prolyl endopeptidase required $2–5 \times 10^4$-fold higher concentrations of protamine than OPBs. The inhibitory effects on *E. coli* OPB activity of protamine were compared with those of histone, leupeptin and suramin. Like protamine, histone is also a basic protein and its molecular mass is five times larger than that of protamine. Suramin is a symmetrical, polysulfonated naphthylamine polyanionic compound, which is commonly used as a trypanocidal drug because of its inhibitory activity against trypanosoma OPB [18]. As shown in Figure 1B, the IC_{50} of histone was 100-fold higher than that of protamine. Morty *et al.* reported that suramin inhibited OPB from *Trypanosoma brucei* in a partial, uncompetitive manner [18]. Although suramin inhibited both *E. coli* OPB and trypanosoma OPB, its IC_{50} was 100-fold higher than that of protamine. The IC_{50} of leupeptin was 400-fold higher than that of protamine in *E. coli* OPB. Salmon protamine consists of a family of highly homologous proteins with an average molecular mass of 4200 Da (30–32 amino acid residues) [25]. These components were separated by HPLC and their inhibitory activities were examined using the *E. coli* enzyme. As shown in Figure 2A, four major components of protamine were isolated. Peaks 1, 2, 3 and 4 were identified as protamine 3A, TP17, 2C and 1B, respectively, by sequence and amino acid composition (Figure 2B). Their amino acid sequences are highly homologous. Protamine 3A, TP17 and 2C are composed of 32 residues, whereas protamine 1B is composed of 30 residues. All protamines are two-thirds arginine, and contain proline, serine, alanine, isoleucine, valine and glycine. The notable difference between these protamines is the number of serine residues, which range from two in protamine 1B to three in protamine 2C, and four in protamine 3A and TP17. Although these protamines differed slightly in amino acid sequences, all had similar inhibitory activities (Figure 2C).

Mechanism Responsible for the Inhibition of OPB by Protamine

A Lineweaver–Burk plot was used to determine the possible mechanism responsible for the inhibition of OPB by protamine. Kinetic analyses showed that protamine displays strictly competitive inhibition (Figure 3). *E. coli* and wheat OPBs were competitively inhibited by protamine giving K_i values of 11 nM and 24 nM, respectively. To know whether protamine was cleaved by OPB, the reaction products of protamine with OPB were analysed. Purified protamine 1B was incubated with *E. coli*, wheat OPB (protamine-to-OPB molar ratio, 100 : 1) and trypsin (molar ratio 2500 : 1) at 37 °C for 3 h, and subjected the products to RP-HPLC. As shown in Figure 4A, no differences of elution profiles were observed between control and OPB-digested protamine 1B, and protamine's inhibitory activity was not affected by OPB treatment. No differences were detected in the amino acid compositions of the control and OPB-digested protamine 1B. Like purified protamine 1B, the elution profile and the inhibitory activity of unfractionated protamines were unchanged by OPB treatment (data not shown). Protamine was highly sensitive to trypsin treatment (Figure 4A). Protamine was rapidly digested and its inhibitory activity was completely lost after treatment with trypsin. In contrast, treating protamine with excess *E. coli* OPB produced inverse hyperbolic progress curves for the hydrolysis of Z–Phe–Arg–MCA by OPB (Figure 4B). As the concentration of protamines increased, the linear phase of the progress curve shortened and the acceleration of hydrolysis of Z–Phe–Arg–MCA was achieved faster, suggesting that OPB had cleaved protamine. As shown in Figure 4C, a titration experiment suggested that binding of two molecules of protamine to OPB is necessary to inhibit OPB activity. Binding of protamines to

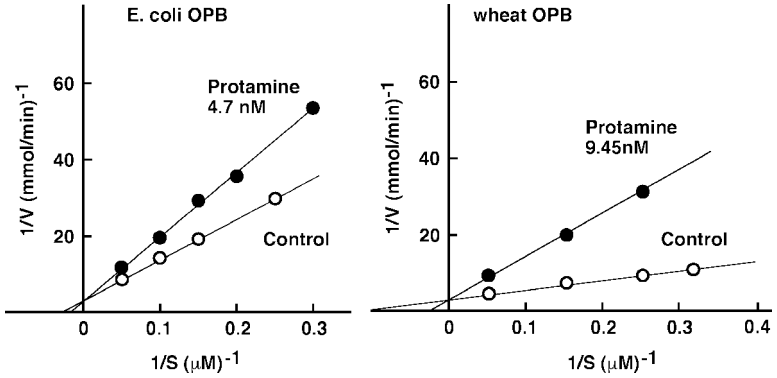


Figure 3 Kinetic analysis of the inhibitory effects of protamines on *E. coli* and wheat OPB. OPB activity was measured in the absence (open circles) and presence (closed circles) of protamine using Z–Phe–Arg–MCA and purified OPB (*E. coli*: 170 ng and wheat: 34 ng).

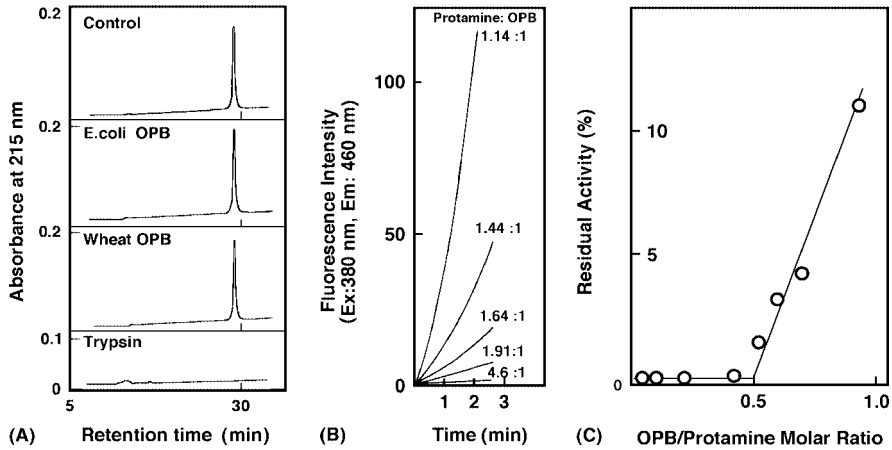


Figure 4 Digestion of protamine by *E. coli* and wheat OPB. (A) Protamine 1B was incubated with *E. coli* OPB (protamines-to-OPB molar ratio, 100 : 1), wheat OPB (100 : 1), and trypsin (2500 : 1). The digests were subjected to RP-HPLC and eluted by linear gradient of acetonitrile (7.5%–25%) in 0.1% trifluoroacetic acid. (B) Progress curve for the hydrolysis of Z–Phe–Arg–MCA by *E. coli* in the presence of the indicated molar ratio of protamine. *E. coli* OPB (10.3, 20.5, 24.6, 28.7, 32.8 and 41 pmol) was reacted with 47 pmol protamine at 25 °C in 1.0 ml of 0.1 M Tris-HCl, pH 8.0 and 50 mM Z–Phe–Arg–MCA. The reactions were monitored by recording the changes in fluorescence at 460 nm upon excitation at 380 nm. (C) Plot of residual OPB activity versus the ratio of OPB to protamine.

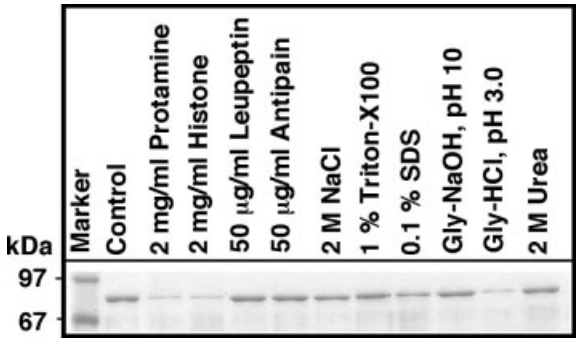


Figure 5 Binding of *E. coli* OPB to protamine–Sepharose. OPB (20 μg) was incubated with 0.1 M NaCl (control), protamine, histone, leupeptin, antipain, 2 M NaCl, Triton X-100, and SDS in 0.5 ml of 50 mM Tris-HCl, pH 7.5, 50 mM Gly-NaOH, pH 10, and 50 mM Gly-HCl, pH 3.0, at 25 °C. The reaction mixtures were treated as described in Materials and Methods.

OPB was confirmed using protamine–Sepharose. OPB was incubated with protamine–Sepharose under various conditions and the bound OPB was quantified by SDS-PAGE. As shown in Figure 5, *E. coli* OPB bound to protamine–Sepharose at pH 7.5. The binding of OPB to arginine–Sepharose was also examined as a negative control; OPB did not bind to arginine–Sepharose. Binding specificity was confirmed by competition with protamine and histone, both of which inhibited OPB binding. Heparin (1 mg/ml) also inhibited the binding of OPB to the gel (data not shown). Although NaCl (2 M), serine protease inhibitors (leupeptin and antipain), Triton X-100 (0.1%) and urea (2 M) had no significant effect on the binding of OPB to protamine–Sepharose, its binding was markedly affected by acidic pH. A little inhibition of its binding by SDS (0.1%) was also observed. In contrast, OPB did not elute from protamine–Sepharose with heparin (1 mg/ml) and NaCl (2 M). These results indicate tight binding of protamine to OPB. The acidic residues in OPB are likely to be determinants of protamine inhibition because they may interact electrostatically with the basic side chains of arginine residues in protamine. However, the binding of protamine to OPB does not result from a nonspecific electrostatic effect, because a high concentration of NaCl did not affect the binding of protamine to OPB and the binding was not inhibited completely by 0.1% SDS. Thus, it seems most likely that negatively charged residues in substrate recognition sites are involved mainly in the inhibition by protamine.

Interaction of OPB with Protamine

The sensitivity to protamine of OPB and POP differed significantly. Acidic residues conserved in OPB but not in POP might be responsible for protamine inhibition. An alignment of the OPB and POP family revealed that 16 acidic residues are conserved in OPBs from *E. coli* [2], *Trypanosoma brucei* [13] and *Arabidopsis thaliana* (F14I3.4 protein) [26]. These acidic residues are not conserved in POPs from humans [27] and *Flavobacterium meningosepticum* [28]. Glu^{43}, Glu^{64}, Glu^{73}, Asp^{146}, Asp^{253} and Glu^{390} are conserved in the *N*-terminal β-propeller domain of OPB. Asp^{460}, Asp^{472} Asp^{567}, Glu^{576}, Glu^{578}, Glu^{579}, Asp^{599}, Glu^{624}, Glu^{634} and Glu^{638} are conserved in the *C*-terminal catalytic domain of OPB. The three-dimensional model of *E. coli* OPB based on the crystal structure of the closely related POP and mutation analysis of Salmonella OPB indicated that Glu^{576} and Glu^{578} are an S1 subsite and that Asp^{460} and Asp^{462} are an S2 subsite [6,10]. To identify the binding region of OPB to protamine, *E. coli* OPB (0.5 mg) immobilized to protamine–Sepharose (0.2 ml) was digested with lysyl endopeptidase (5 μg) at 37 °C for 90 min. After washing the gel with 50 mM Tris-HCl, pH 7.5, containing 0.1 M NaCl, the fragment bound to the gel was eluted with 0.1% trifluoroacetic acid and its sequence was determined after purification by RP-HPLC. The sequence did not contain lysine residues indicating that protamine was not cleaved by lysyl endopeptidase. One major fragment was isolated and its sequence was determined as QTEVPGFYAANYRSEHLWIVARDGVEVPVSLVYHRK, which corresponds to amino acids 403–438 of *E. coli* OPB. This fragment is located in the boundary between the β-propeller domain and the catalytic domain of OPB. The region Glu^{424}–Gly^{459} of pig POP corresponding to Q^{403}–K^{438} of *E. coli* OPB is located on the surface of the POP molecule along the boundaries of the two domains [6]. This fragment does not contain the S1 and S2 substrate binding sites identified previously [10].

Importance of the Length of Protamines to OPB Inhibition

To examine the inhibitory activity of shorter protamines, protamine was digested with trypsin or prolyl endopeptidase and the inhibitory activity of the shorter fractions assessed. Protamine (1 mg) was partially digested with trypsin under mild condition (1 μg at 15 °C for 1 h) and fractionated by RP-HPLC, and the inhibitory activity of the resultant fractions was measured. As shown in Figure 6A, the inhibitory activity was detected only in the uncleaved protamine fraction. The digested protamine fragments eluted faster than native protamines and had no inhibitory activity. Similarly, the inhibitory activity of protamine was lost completely by digestion with prolyl endopeptidase. Because all protamines have one or two proline residues in addition to the *N*-terminal proline (Figure 2), this digestion generates various shorter peptides containing polyarginine. The longest peptide Arg18–Arg32 was produced from protamine TP17 and 2C. However, the protamines completely lost their inhibitory activity against OPB

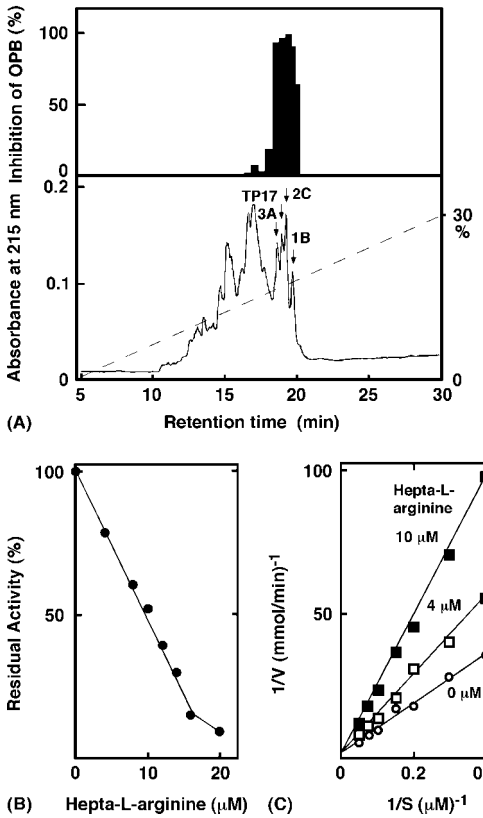


Figure 6 Importance of the length of protamines to OPB inhibition. (A) Effects of partial digestion with trypsin on the inhibitory activity of protamines. Partial digests of protamine with trypsin were separated by RP-HPLC (acetonitrile 0%–30% linear gradient in 0.1% trifluoroacetic acid) and the inhibitory activity was assayed. The eluate was dried and dissolved in 200 μl of water and 1 μl of the dissolved digest was used to assess the inhibitory activity. (B) Inhibition of *E. coli* OPB by hepta-L-arginine. (C) Kinetic analysis of the inhibitory effects of hepta-L-arginine on *E. coli* OPB. OPB activity was measured in the absence (open circles) and presence (4 μM; opened square, 10 μM; closed square) of hepta-L-arginine using Z–Phe–Arg–MCA.

after prolyl endopeptidase treatment (data not shown). The effect of hepta-L-arginine on OPB activity was also examined (Figure 6B, C). Although hepta-L-arginine is a competitive inhibitor like protamine, its K_i value (5 μM) for *E. coli* OPB was 5×10^2 fold higher than K_i value of protamine. These results suggest that the length of polyarginine is critical to the inhibition by OPB and that inhibition of OPB by protamine cannot be explained by the simple docking of the inhibitor with the active site. Further analysis is necessary to clarify the structural basis of the interaction of protamine with OPB.

Inhibitory Activity of Protamine in Serum

Trypanosoma OPB is widely implicated as an important virulence factor and chemotheraupetic target in the prevention and treatment of trypanosomiasis. Parasite

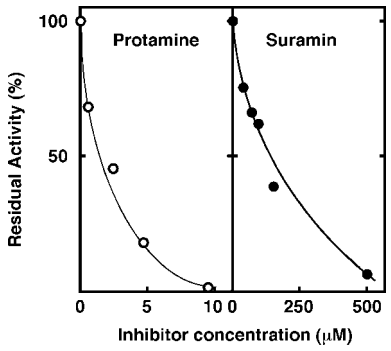


Figure 7 Inhibition of *E. coli* OPB in serum by protamine and suramin. *E. coli* OPB was reacted with 1.0 ml of 100% fetal calf serum containing 50 microM Z–Phe–Arg–MCA in the presence of various concentrations of protamine and suramin.

OPB is released as an active enzyme into the bloodstream. OPB activity is not affected by serum protease inhibitors such as α2-macroglobulin [29]. The released enzyme is purported to contribute to the pathogenesis of trypanosomiasis through the degradation of peptide hormones such as atrial natriuretic peptide [29,30]. To understand the inhibitory activity of protamines toward OPB in the serum, the effect of protamine on the activity of *E. coli* OPB in bovine serum was examined. As shown in Figure 7, OPB was sensitive to protamine in serum. The IC_{50} of protamine (1.1 μM) was 120-fold lower than that of the trypanocidal drug, suramin (130 μM).

Taken together, our results clearly indicated that protamine is a potent inhibitor of OPB. Protamine showed highly selective inhibition against OPB compared with prolyl oligopeptidase B, trypsin and furin, and was a stronger inhibitor of OPB than the trypanocidal drug, suramin. Protamine is commonly administered in cardiovascular surgery procedures to reverse the anticoagulant effect of heparin and to alleviate the risk of heparin-induced bleeding. Protamine might be useful in inhibiting OPB released from trypanosomes in the blood. Furin was inhibited by protamines (shown in Figure 1), whereas polyarginines (6–9 residues) are also potent inhibitors of furin [31]. Kacprzak *et al.* reported recently that nona-D-arginine amide is the strongest inhibitor of furin [32]. Although nona-L-arginine is also a strong inhibitor, it can be cleaved by furin. Nona-D-arginine and its amide form are not cleaved by furin and possess strong inhibitory potency. In contrast, we found that shorter protamine peptides have no inhibitory activity against OPB. Further it was shown that the K_i value of hepta-L-arginine for OPB is 5×10^2 fold higher than the K_i value of native protamine. For effective therapeutic use in the treatment of trypanosomiasis, it will be necessary to develop OPB inhibitors that can cross the parasite plasma membrane and the blood–brain barrier in sufficient quantities to eliminate the trypanosomes present within the central nervous system. It would be of interest to determine whether polyarginine-containing D-arginine can inhibit OPB.

CONCLUSIONS AND FUTURE PROSPECTS

Although trypanosoma oligopeptidase B is involved in the pathogenesis of trypanosomiasis, the highly potent and selective inhibitors of this enzyme have not been found. Our data indicate that a protamine is a potent and selective inhibitor of oligopeptidase B. Based on these results, modification of protamine and polyarginine are underway to develop potential trypanocidal drugs.

Acknowledgements

We wish to thank Messrs Takehiro Suzuki and Tadashi Miyashita for technical assistance, and Ms Yuka Sasaki for determining the amino acid sequences.

REFERENCES

1. Rawlings ND, Polgar L, Barrett AJ. A new family of serine-type peptidase related to prolyl oligopeptidase. *Biochem. J.* 1991; **279**: 907–911.
2. Kanatani A, Matsuda T, Shimada T, Misoka F, Lin XS, Yoshimoto T, Tsuru D. Protease II from *Escherichia coli*: sequencing and expression of the enzyme gene and characterization of the expressed enzyme. *J. Biochem. (Tokyo)* 1991; **110**: 315–320.
3. Venäläinen JI, Juvonen RO, Männistö PT. Evolutionary relationships of the prolyl oligopeptidase family enzymes. *Eur. J. Biochem.* 2004; **271**: 2705–2715.
4. Yoshimoto T, Ogita K, Walter R, Koida M, Tsuru D. Post-proline cleaving enzyme. Synthesis of a new fluorogenic substrate and distribution of the endopeptidase in rat tissue and body fluids of man. *Biochim. Biophys. Acta* 1979; **569**: 184–192.
5. Bõcskei Zs, Fuxreiter M, Náray-Szabó G, Szabó E, Polgár L. Crystallization and preliminary x-ray analysis of porcine muscle prolyl oligopeptidase. *Acta Crystallogr. Sect. D: Biol. Crystallogr.* 1998; **D54**: 1414–1415.
6. Gérczei T, Kesurü GM, Náray-Szabó G. Construction of a 3D model of oligopeptidase B, a potential processing enzyme in prokaryotes. *J. Mol. Graphics Modelling* 2000; **18**: 7–17.
7. Polgár L. A potential processing enzyme in prokaryotes: oligopeptidase B, a new type of serine peptidase. *Proteins* 1979; **28**: 375–379.
8. Fülöp V, Böcskei Z, Polgár L. Prolyl oligopeptidase: an unusual β-propeller domain regulates proteolysis. *Cell* 1998; **94**: 161–170.
9. Pacaud M, Richard C. Protease II from *Escherichia coli*. Purification and characterization. *J. Biol. Chem.* 1975; **250**: 7771–7779.
10. Morty RE, Fülöp V, Andrews NW. Substrate recognition properties of oligopeptidase B from salmonella enterica Serovar Typhimurium. *J. Bacteriol.* 2002; **184**: 3329–3337.
11. Kornblatt MJ, Mpimbaza GWN, Lonsdale-Eccles JD. Characterization of an endopeptidase of *Trypanosoma brucei*. *Arch. Biochem. Biophys.* 1992; **293**: 25–31.
12. Morty RE, Authié E, Troeberg L, Lonsdale-Eccles JD, Coetzer THT. Purification and characterization of a trypsin-like serine oligopeptidase from *Trypanosoma congolense*. *Mol. Biochem. Parasitol.* 1999; **102**: 145–155.
13. Morty RE, Lonsdale-Eccles JD, Morehead J, Caler EV, Mentele R, Auerswald EA, Coetzer THT, Andrews NW, Burleigh BA. Oligopeptidase B from *Trypanosoma brucei*, a new member of an emerging subgroup of serine oligopeptidases. *J. Biol. Chem.* 1999; **274**: 26 149–26 156.
14. Morty RE, Lonsdale-Eccles JD, Mentele R, Auerswald EA, Coetzer THT. Trypanosoma-derived oligopeptidase B is released into the plasma of infected rodents, where its persists and retains full catalytic activity. *Infect. Immun.* 2001; **69**: 2757–2761.
15. Tsuji A, Yuasa K, Matsuda Y. Identification of oligopeptidase B in higher plants. Purification and characterization of oligopeptidase B from quiescent wheat embryo, *Triticum aestivum*. *J. Biochem (Tokyo)* 2004; **136**: 673–681.
16. Burleigh BA, Caler EV, Webster P, Andrews NW. A cytosolic serine endopeptidase from *Trypanosoma cruzi* is required for the generation of Ca^{2+} signaling in mammalian cells. *J. Cell. Biol.* 1997; **136**: 609–620.
17. Burleigh BA, Woolsey AM. Cell signaling and *Trypanosoma cruzi* invasion. *Cell. Microbiol.* 2002; **4**: 701–711.
18. Morty RE, Troeberg L, Pike RN, Jones R, Nickel P, Lonsdale-Eccles JD, Coetzer THT. A trypanosome oligopeptidase as a target for the trypanocidal agents pentamidine, diminazone and suramin. *FEBS Lett.* 1998; **433**: 251–256.
19. Morty RE, Troeberg L, Powers JC, Ono S, Lonsdale-Eccles JD, Coetzer THT. Characterization of the antitrypanosomal activity of peptidyl α-aminoalkyl phosphate diphenyl esters. *Biochem. Pharmcol.* 2000; **60**: 1497–1504.
20. Tsuji A, Ikoma T, Hashimoto E, Matsuda Y. Development of selectivity of a1-antitrypsin variant by mutagenesis in its reactive site loop against proprotein convertase. A crucial role of the P4 arginine in PACE4 inhibition. *Prot. Eng.* 2002; **15**: 123–130.
21. Tsuji A, Kurachi K. Isolation and characterization of a novel large protease accumulated in mammalian cells in the presence of inhibitors. *J. Biol. Chem.* 1989; **264**: 16 093–16 099.
22. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 1976; **72**: 248–254.
23. Laemmli UK. Cleavage of structure proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; **227**: 680–685.
24. Hemerly JP, Oliveira V, Nery ED, Morty RE, Andrews NW, Juliano MA, Juliano L. Subsite specificity (S3, S2, S1′, S 2′ and S3′) of oligopeptidase B from *Trypanosoma cruzi* and *Trypanosoma brucei* using fluorescent quenched peptides: comparative study and identification of specific carboxypeptidase activity. *Biochem. J.* 2003; **373**: 933–939.
25. McKay DJ, Renaux BS, Dixon GH. Rainbow trout protamines. Amino acid sequences of six distinct proteins from a single testis. *Eur. J. Biochem.* 1986; **158**: 361–366.
26. Seki M, Narusaka M, Kamiya A, Ishida J, Satou M, Sakurai T, Nakajima M, Enju A, Akiyama K, Oono Y, Muramatsu M, Hayashizaki Y, Kawai J, Carninci P, Itoh M, Ishi Y, Arakawa T, Shibata K, Shinagawa A, Shinozaki K. Functional annotation of a full-length Arabidopsis cDNA collection. *Science* 2002; **296**: 141–145.
27. Yoshimoto T, Miyazaki K, Haraguchi N, Kitazono A, Kabashima T, Ito K. Cloning and expression of the cDNA encoding prolyl oligopeptidase (prolyl endopeptidase) from bovine brain. *Biol. Pharm. Bull.* 1997; **20**: 1047–1050.
28. Yoshimoto T, Kanatani A, Shimoda T, Inaoka T, Kokubo T, Tsuru D. Prolyl endopeptidase from *Flavobacterium meningosepticum*: cloning and sequencing of the enzyme gene. *J. Biochem. (Tokyo)* 1991; **110**: 873–878.
29. Troeberg L, Pike RN, Morty RE, Berry RK, Coetzer TH, Lonsdale-Eccles JD. Proteases from *Trypanosoma brucei*. Purification, characterization and interactions with host regulatory molecules. *Eur. J. Biochem.* 1996; **238**: 728–736.
30. Morty RE, Pellé R, Vadász I, Uzcanga GL, Seeger W, Bubis J. Oligopeptidase B from *Trypanosoma evansi*: A parasite peptidase that inactivates atrial natriuretic factor in the bloodstream of infected hosts. *J. Biol. Chem.* 2005; **280**: 10 925–10 937.
31. Cameron A, Appel J, Houghten RA, Lindberg I. Polyarginines are potent furin inhibitors. *J. Biol. Chem.* 2000; **275**: 36 741–36 749.
32. Kacprzak MM, Peinado JR, Than ME, Appel J, Henrich S, Lipkind G, Houghten RA, Bode W, Lindberg I. Inhibition of furin by polyarginine-containing peptides. Nanomolar inhibition by nona-D-arginine. *J. Biol. Chem.* 2004; **279**: 36 788–36 794.