Assessment of Substrate Inhibition of Bacterial Oligopeptidase B

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Oligopeptidase B (OPB; EC 3.4.21.83) from 2 Gram-negative bacteria, *Stenotrophomonas maltophilia* (Stm) and *Serratia marcescens* (Sem), and the Gram-positive bacterium *Rhodococcus erythropolis* (Re) were cloned and characterized to clarify their activities and substrate specificities using peptidyl-MCA substrates containing Arg or Lys. The cloned enzymes, Stm, Sem and ReOPBs, in addition to *Escherichia coli* OPB (EcOPB) were expressed using a pET expression system. Although the Stm and SemOPBs share 45% sequence identity to each other and up to 60% identity with respect to their catalytic domains, their activities towards MCA substrates were quite different. StmOPB is approximately 100–500 times more active than SemOPB and 3–30 times more active than EcOPB. The activity of ReOPB is comparable to that of StmOPB and it shares 40% and 36% identity to StmOPB and SemOPB, respectively. Some features of Stm, Re and EcOPBs are similar to those of previously cloned OPBs, which were also strongly inhibited by substrates, but SemOPB differs from all other OPBs in that it is not inhibited by substrates; even substrates containing double arginine at 35 μM did not inhibit SemOPB. On the other hand, the same substrates at only 5 μM inhibited the activity of the Stm, Re, and EcOPB. This phenomenon was not observed with substrates containing single or double lysine.

Key words oligopeptidase B; prolyl oligopeptidase family; substrate inhibition; opportunistic bacteria; substrate specificity

The oligopeptidase B (OPB, EC 3.4.21.83) subfamily represents one of two branches of the prolyl oligopeptidase family of serine peptidases (clan SC, family S9).[1)] The substrate specificities of the subfamily enzymes are different, with prolyl oligopeptidase exclusively hydrolyzing peptide bonds at the C-terminal to proline residues in peptides,[2,3)] while OPB demonstrates a trypsin-like substrate specificity, hydrolyzing peptide bonds on the C-terminal side of basic amino acid residues. Arginine- or lysine-containing peptides of no more than about 30 residues can be hydrolyzed,[4–6)] since the N-terminal β-propeller domain of the enzymes blocks access of large globular proteins to the catalytic machinery.[3)] However, it has been demonstrated that OPB can cleave, in addition to low-molecular-mass peptides, several basic proteins in a restricted fashion, including human histones H1, H2A, H3, and H4.[7)] Peptides with Arg residues in both P1 and P2 are hydrolyzed at a much faster rate by OPB than peptides with only one Arg residue at the P1 site.[7,8)]

OPB is found in bacteria, plants and trypanosomatid pathogens, where it has been identified as a virulence factor and a potential drug target, since the genes coding for this enzyme are absent from mammals. The exact physiological function(s) and the physiological substrates of the OPB are unknown. Although the OPB was first cloned and characterized from *Escherichia coli*[4,9)] and *Moraxella lacunata*,[6)] bacterial OPBs have received much less attention than their homologues from protozoa. Thus their 3D structures and roles in bacterial virulence have not been clarified. In protozoa, on the other hand, *Trypanosoma cruzi* oligopeptidase B has been implicated in the pathogenesis of Chagas' disease, and it participates in processing events in the cytoplasm of the parasites, generating a factor with Ca^{2+} signaling activity for mammalian cells.[10)] Targeted deletion of the OPB gene in *Trypanosoma cruzi* resulted in trypanosomes that were unable to elicit the OPB-mediated Ca^{2+} response in mammalian cells and were severely impaired with respect to host cell invasion and the establishment of infections in mice.[11)] OPB may also be involved in the pathogenesis of African trypanosomiasis. In fact, it has been identified as a target of several drugs (pentamidine, diminazene and suramin) used in the treatment of African trypanosomiasis.[12)] Administration of irreversible OPB inhibitors to trypanosome-infected mice has been shown to significantly impair disease progression, and irreversible inhibitors of the enzyme exhibit antitrypanosomal activity *in vitro* and *in vivo*.[13)] The mechanism by which *Trypanosoma brucei* and *T. evanci* OPBs cause African sleeping sickness and surra may involve the release of these pathogens from dying parasites into the host bloodstream, where they remains stable and catalytically active and degrade biologically active peptides.[14,15)] It has also been shown the *T. evanci* OPB inactivates atrial natriuretic factor in the bloodstream of infected hosts; it cleaves the hormone at the carboxyl side of four Arg residues to reduce its circulating levels 16-fold.[15)]

Oligopeptidase B from *T. brucei* has been crystallized,[16)] but the crystal structure has not been reported yet. OPB has been described in *Leishmania* species.[17,18)] Genes coding for OPB from *Leishmania* were cloned and expressed, and the three-dimensional model of *Leishmania amazonensis* OPB was obtained by homology modeling.[19–21)] The first crystal structure of OPB was determined for *L. major* OPB as a complex with antipain.[22)] Although *Trypanosoma* OPB is a virulence factor and represents potential chemotherapeutic targets,[23)] *L. major* OPB may not be an important virulence

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factor, indicating functional differences between trypanosomes and *Leishmania* in their interaction with the mammalian host.[24] However, *Leishmania donovani* OPB may play a specific role in parasite physiology; it is responsible for regulating levels of enolase on the parasite cell surface.[25] OPB is also present in plants; it has been isolated from pollen from mesquite (*Prosopis velutina*),[26] ragweed (*Ambrosia artemisiifolia*),[27] and soybean (*Glycine max*),[28] and purified from wheat germ (*Triticum aestivum*).[29]

We focused on four bacterial OPBs, for which there is limited structural information. Previously, we cloned the OPB genes from *Escherichia coli* (Ec)[4,9,30] and *Moraxella lacunata*.[6] OPBs from *Salmonella enteric*,[7] *Treponema denticola*,[31–33] *Serratia proteamaculans* II[34] and *Streptomyces griseus*[35] have also been cloned. However, the structural details are yet to be solved. In the current work, OPBs were cloned from *Serratia marcescens* (Sem), *Rhodococcus erythropolis* (Re) and *Stenotrophomonas maltophilia* (Stm), and the expressed enzymes were purified. The substrate specificity of the OPBs and some physicochemical properties were determined. Although some of their features were similar to those of previously characterized OPBs *Serratia marcescens* OPB does not show any inhibition by substrate, a common feature of OPBs. The absence of this effect may be a key to elucidating the mechanism of the strong inhibition of the OPBs by substrates.

MATERIALS AND METHODS

Materials Peptidyl-MCA (4-methylcoumaryl 7-amide) substrates were purchased from Bachem AG (Bubendorf, Switzerland). Restriction endonucleases and other DNA modification enzymes were purchased from Toyobo Biochemicals, New England Biolabs or TaKaRa Bio Inc. The oligonucleotide primers were synthesized by Genenet Co., Ltd. (Fukuoka, Japan). The BigDye Terminator v1.1 cycle sequencing kit and other reagents used for sequencing were obtained from Life Technologies Co. Isopropyl β-D-1-thiogalactopyranoside (IPTG), ammonium sulfate, Tris-(hydroxymethyl)-aminomethane and all other chemicals were purchased from Nacalai Tesque Inc. (Kyoto, Japan). Centriprep and Centricon were from Amicon (Millipore Corporation). The DEAE-Toyopearl was from Tosoh (Japan) and the histidine-tag affinity column was purchased from Qiagen (Hilden).

Bacterial Strains, Plasmids and Media *Escherichia coli* XL1Blue (*endA1 gyrA96 hsdR17 lac recA1 relA1 supE44 thi-1 F′* [*proABlacI*^{q}*ZΔM15* Tn*10*]) (Stratagene, La Jolla, CA, U.S.A.) was used for DNA manipulation. Plasmid pGEM-T Easy (Promega) was used for cloning the PCR products. *E. coli* BL21 (DE3) [B F$^-$*ompThsdS*$_B$ ($r_B^-m_B^-$) *gal dcm* (DE3)] (Novagene) was used as a host for expression. Plasmids pET-22b(+) and pET-28a(+) (Novagene) were used as vectors for expression. The genomic DNA clone from *Rhodococcus erythropolis* PR4 (NBRC G12-001-257) was purchased from NBRC (Chiba, Japan). Bacteria were grown in LB broth (1% tryptone, 1% NaCl, and 0.5% yeast extract). The concentrations of antibiotics used in this study were as follows: 50–100 μg/mL ampicillin and 30 μg/mL kanamycin.

Cloning, Expression and Purification Chromosomal DNA was prepared by phenol extraction method.[36] OPB genes were amplified by polymerase chain reaction (PCR) from the respective genomic DNAs with Ex Taq polymerase using StmOPB1s*Nde*I (5-GGG**CATATG**AAA TCC ACC CTC TGC C-3) and StmOPB3a*Hin*d3 (5-GGG**AAGCTT**CGG CTG GGC GAT GCC -3) for StmOPB, ReryOPB2sNde (5-GGG **CATATG**AGC CTG ATC CCG CCG GT-3) and ReryOPB3aH3 (5-GGG**AAGCTT**CTA GTT TGC GCC AAC CGT GTC -3) for ReOPB and SerOPB*Nde*1s-V3 (5-GGG**CATATG**AAT CCG CTG TTA TTC ACC -3) and SerOPBXH5a (5-CGC **CTCGAG**TTT CTG CGC CTG CCG ATC -3) for SemOPB (the restriction sites are shown in bold type and the initiation codon of the forward primer is underlined). The whole structural gene for ReOPB was amplified by PCR using the genomic clone (NBRC G12-001-257) harboring the OPB gene as a template. The PCR products were cloned into pGEM-T Easy vector and sub-cloned into pET-28a(+) and pET-22b(+) expression vectors using *Nde*I-*Hin*dIII/*Xho*I restriction sites, to give pET28OPB-NTHS, pET22OPBCTHS, and pET22OPBWT for the production of recombinant proteins with an N-terminal 6His-tag (NTHS), C-terminal 6His-tag (CTHS), or no tags (WT), respectively.

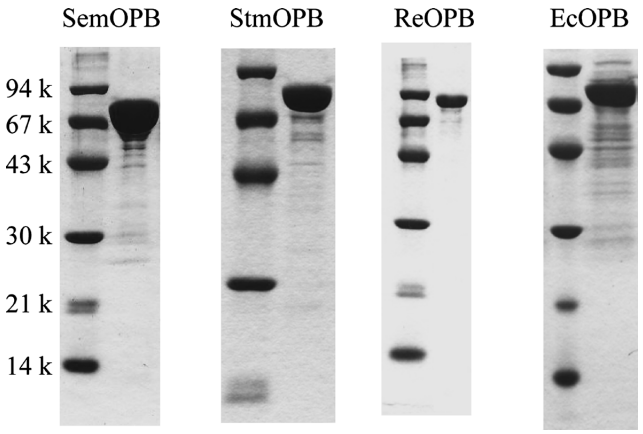

Fig. 1. SDS-PAGE of the Purified Bacterial Oligopeptidase B

Molecular weight marker proteins used were 94k, phosphorylase b; 67k, bovine serum albumin; 43k, ovalbumin; 30k, carbonic anhydrase; 21k, soybean trypsin inhibitor; and 14k, α-lactoglobulin.

For expression, all the expression vectors for wild-type (WT) and both the N- and C-terminal His-tagged OPBs were transformed into *E. coli* BL21 (DE3). OPB expression was induced in a log-phase culture by 1 mM IPTG for *ca.* 22 h at 18°C with continuous shaking in the presence of appropriate antibiotic. Transformants from a 1200 mL culture were harvested by centrifugation (12000 rpm, 15 min, 4°C) and then homogenized with 300 mL of 20 mM Tris–HCl buffer (pH 8.0). The homogenate was thawed and sonicated (output 7, 1 min, 40 cycles) on ice. Following centrifugation (12000 rpm, 15 min, 4°C), the supernatant was applied to a metal-chelating column, charged with Ni^{2+} and washed sequentially with 20 mM Tris–HCl/500 mM NaCl and the same buffer containing 10 mM imidazole. The recombinant proteins were eluted with a linear gradient of 10 to 500 mM imidazole. Relatively pure fractions were combined and desalted by dialysis against 20 mM Tris–HCl buffer (pH 8.0), and further purified using an ion exchange chromatographic column of DEAE-Toyopearl, and the protein was eluted with a linear gradient of 0–500 mM NaCl. Active fractions were combined and desalted by dialysis against 20 mM Tris–HCl buffer (pH 8.0). The purified protein

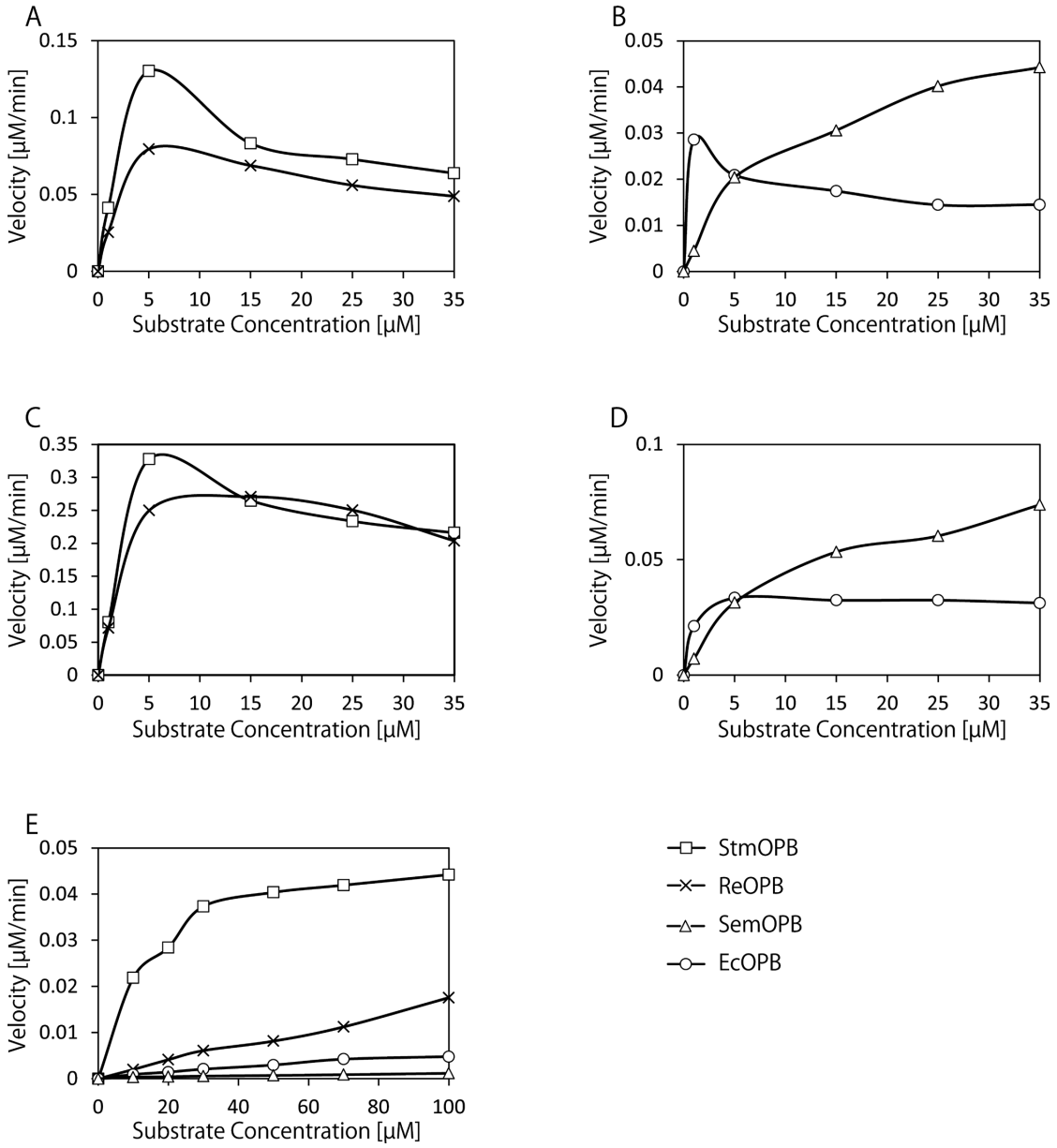

Fig. 2. Substrate Inhibition of Bacterial OPBs

The amount of enzymes used was 10 ng in 0.02 M Tris–HCl buffer (pH 8.0), with the exception that SemOPB was prepared in 0.02 M potassium phosphate (pH 7.8) to avoid the activity decrease. Substrates used were Boc-Gly-Arg-Arg-MCA (A and B), Boc-Gln-Arg-Arg-MCA (C and D), and Boc-Lys-MCA (E).

was concentrated to 15, 20 and 30 mg/mL using Centriprep, as estimated on the basis of absorbance at 280 nm. The CTHS clones (C-terminal His-tag) for Stm, and SemOPBs and the NTHS clone for ReOPB were used mainly because the yield was higher than that of the others.

The nucleotide sequence data are available in the DDBJ/EMBL/GenBank databases under the accession numbers AB727994 (*Serratia marcescens*), AB727995 (*Stenotrophomonas maltophilia*), and AB727996 (*Rhodococcus erythropolis* PR4).

Enzyme Activity Assay and Kinetic Studies The enzyme activity was assayed using (X)-Arg-MCA substrate. The reaction mixture (total 100 μL) contained 0.02 M Tris–HCl (pH 8.0), 1–25 μM (X)-Arg-MCA substrate and 10 ng of an enzyme. The reaction was initiated by the addition of the enzyme solution. Following incubation at 30°C for 10–30 min in a 96-well plate inside a Tecan plate reader (Infinite M1000), the amount of MCA liberated was determined fluorometrically (excitation wavelength at 380 nm and emission wavelength at 460 nm). One unit (U) was defined as the amount of enzyme that produced one nmol of 7-amino-4-methylcoumarin per min at 30°C.

Kinetic parameters (K_m and V_{max} values) were determined by the nonlinear fitting method using the SigmaPlot software package (Systat Software) and by the Lineweaver–Burk plots with various substrate concentrations. The amount of enzyme used was approximately 2 ng, 5 ng, and 10 ng for double Arg-, single Arg- and single or double Lys-containing substrates, respectively. The enzyme concentrations of Stm, Re, Ec and SemOPBs were estimated from $E_{1\%,\ 280}$ nm values of 1.87, 1.88, 1.92 and 1.93, respectively. For the k_{cat} calculations, 79160, 78380, 79480 and 80740 were used as the molecular

Table 1. Specific Activities of Bacterial Oligopeptidase B Measured at Various Substrate Concentrations

Substrates	StmOPB		ReOPB		SemOPB		EcOPB	
	1 μM	5 μM	1 μM	5 μM	1 μM	5 μM	1 μM	5 μM
Bz-Arg-MCA	711	3130	400	908	5.9	18	25	105
Z-Leu-Arg-MCA	893	3440	982	3500	13.2	53	162	570
Boc-Gln-Gly-Arg-MCA	575	3500	1689	4260	3.4	13	91	408
Boc-Gln-Ala-Arg-MCA	1420	2470	1615	3000	2.3	6.0	42	126
Boc-Ala-Gly-Pro-Arg-MCA	2180	3055	700	3690	3.0	6.0	78	382
Boc-Val-Leu-Lys-MCA	640	1330	1635	1850	1.7	5.0	67	243
Z-Arg-Arg-MCA	4740	1810	10100	1430	225	340	460	328
Boc-Gly-Arg-Arg-MCA	4250	1300	11400	663	85	100	162	124
Boc-Gln-Arg-Arg-MCA	13270	3275	8350	2230	90	158	175	220
Boc-Arg-Val-Arg-Arg-MCA	7330	1310	9500	645	55	86	320	170
Boc-Gly-Lys-Arg-MCA	8910	2380	9130	1230	35	86	80	370
Boc-Glu-Lys-Lys-MCA	270	457	322	519	2.0	6.0	23	90

Values are expressed in nmol/min/mg protein. Enzyme activity of Stm, Re and EcOPB was determined in 0.02 M Tris–HCl buffer (pH 8.0) and SemOPB in 0.02 M potassium phosphate (pH 7.8) with various substrates at 1 and 5 μM. The amount of enzyme used was approximately 10 ng.

weights of the Stm, Re, Ec and Sem, respectively.

RESULTS

Cloning and Expression OPBs from *S. maltophilia*, *R. erythropolis* and *S. marcescens* were successfully cloned, expressed and purified (Fig. 1). EcOPB, which has been cloned previously,[4] was also expressed and purified to compare its activity with the enzymes newly cloned in this study. The Stm, Re and SemOPBs consisted of open reading frames of 2112, 2115 and 2127 bp encoding a polypeptide of 704, 705 and 709 amino acids, respectively. The enzymes from the 2 Gram-negative bacteria Stm and SemOPBs share 45% identity with each other, while ReOPB shares 40% and 36% identity with Stm and SemOPBs, respectively (Supplementary Material, Table S1). All these bacterial OPBs contained a serine active site signature sequence -G-X-S-X-G-G-, which is common in the prolyl endopeptidase family, in addition to the Asp and His, which form the catalytic triad along with the active site Ser (Supplementary Material, Fig. S1).

Most of the OPBs expressed at a temperature above 20°C were aggregated to form inclusion bodies. A high yield of purified Stm and ReOPBs (*ca.* 100 mg) from 3 L *E. coli* culture could be obtained using the pET expression system at 18°C following the 1 mM IPTG induction. The soluble form of SemOPB was obtained in a considerably low yield even at 16°C.

Substrate Specificity, Substrate Inhibition and Kinetic Studies All the purified OPBs exhibited hydrolytic activity toward the fluorogenic substrates used in this study, and the Michaelis–Menten kinetic parameters were determined (Supplementary Material Table S2). Since substrate inhibition was observed with substrates containing double arginine, estimation of kinetic parameters was difficult and the values in Table S2 may contain significant errors. In order to assess the inhibitory effect of substrates, specific activity was measured with different substrate concentrations. As shown in Table 1, substrates with Arg residues at both P1 and P2 are hydrolyzed at a much faster rate by all OPBs than substrates with only single Arg at the P1 site when used at 1 μM. Such a difference was not observed with substrates having Lys at the P1 position. If the substrate concentration was increased, strong substrate inhibition was observed with double arginine-containing substrates. Although the OPBs from Gram-negative bacteria (Stm and SemOPBs) share up to 60% sequence identity with respect to their catalytic domains, their activities towards MCA substrates were quite different. StmOPB is the most efficient enzyme in the processing of most substrates; its specific activity towards Boc-Gln-Gly-Arg-MCA was 3500 u/mg, in comparison to 13 and 408 u/mg for Sem and EcOPBs, respectively (Table 1). The activity of the OPB from Gram-positive *R. erythropolis* (ReOPB) is comparable to that of StmOPB. Strong substrate inhibition was observed for Stm, Re and EcOPBs but not SemOPB (Table 1, Fig. 2). Substrates containing double arginine at 35 μM did not inhibit the SemOPB, which showed low-level activity (Figs. 2B, D). On the other hand, the same substrates at only 5 μM inhibited the activity of the highly active Stm, Re, and EcOPB. Such a phenomenon was not observed with substrates containing single lysine at 100 μM (Fig. 2E) or double lysine (data not shown).

Thermal Stability and Activity The activity of the OPBs was lost completely when they were incubated at 50°C for 15 min, and they lost 75% of their activity when incubated at 40°C for 30 min; however, EcOPB lost only 35% of its activity under the latter condition. The enzymes were relatively stable under incubation at 30°C: they retained more than 75% of their activity after incubation at 30°C for 30 min (Fig. 3A).

Sem, Re, and EcOPBs were most active at 20°C, while StmOPB showed the maximal activity at 30°C (Fig. 3B).

DISCUSSION

The genes encoding OPBs have been found in bacteria, plants and trypanosomatid pathogens, but are absent from mammals. In protozoa, OPB may be involved in the pathogenesis of several diseases, and it has been identified as a virulence factor and potential drug target.[11–15] OPB was originally found in bacteria and it was purified from *E. coli*.[9] Although the involvement of OPB in the infection of bacteria was not established, active OPB enzymes may play a role as observed for the pathogenesis of trypanosomes. We have cloned the genes from *E. coli* and *Moraxella lacunata* and characterized the expressed enzymes.[4,6] Some properties of the OPBs from other bacterial origins have also been studied.[7,30–35] However, the three-dimensional structure of OPB was unknown until

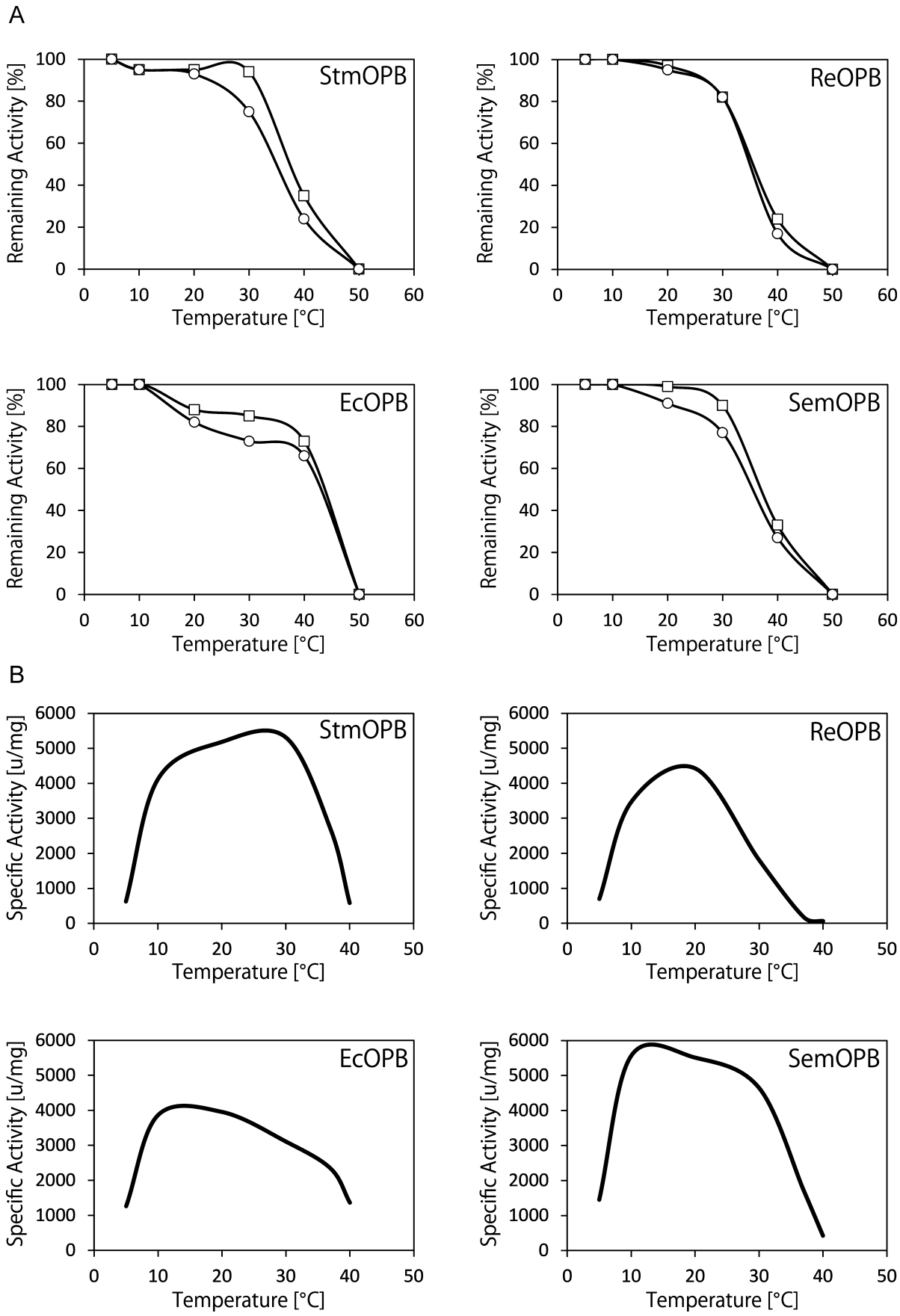

Fig. 3. Thermal Stability and Activity

(A) Thermal stability was determined by measuring the remaining activity after the incubation of the enzymes for 15 min (square) or 30 min (circle) at various temperatures using Z-Arg-Arg-MCA as a substrate. The amount of enzyme used was 100 ng. (B) Thermal activity was measured using Z-Arg-Arg-MCA as a substrate at various temperatures.

the recent determination of the crystal structure of *Leishmania major* OPB. In this study, we have cloned additional OPB genes from 2 Gram-negative bacteria, *Stenotrophomonas maltophilia* and *Serratia marcescens*, and one Gram-positive bacterium, *Rhodococcus erythropolis*, to study some of their properties and to determine the crystal structure. EcOPB could be expressed at 37°C under control of the *tac* promoter, but high-level expression of Stm, Re, and SemOPBs was achieved only by using the pET system. Stm and ReOPBs were obtained in soluble form with a relatively high yield if the expression temperature was maintained at 18°C or less. The soluble form of SemOPB was obtained in a considerably low yield even at 16°C. EcOPB was also produced in a reasonable yield using the pET expression system.[30)]

It should be noted that the SemOPB lost its activity completely if the Tris–HCl buffer was used for purification. The same buffer did not affect the activity of Stm, Re and EcOPBs. Potassium phosphate buffer (pH 7.8) was used for the purification and kinetic study of SemOPB and no decrease in activity was observed in this buffer system. The reason was unclear but there may be a site that interacts with an amine such as Tris. This might be related to a unique characteristic of SemOPB—namely, that this enzyme was not potently inhibited by substrate.

The cleavage specificity and hydrolyzing activity of Stm and ReOPBs on various fluorogenic substrates were approximately the same (Table 1); they were more active than Ec and SemOPBs by 3–30 and 100–500 times, respectively. OPB hydrolyzes short peptides and substrates with Arg residues at both P1 and P2 at a much faster rate than substrates with only one Arg residue at the P1 site.[7,8,30)] OPB activity has routinely been assayed using Z-Arg-Arg-MCA.[37)] We found that Z-Arg-Arg-MCA was the best substrate for Ec and SemOPBs, Boc-Gln-Arg-Arg-MCA was best for StmOPB, and Boc-Gly-Arg-Arg-MCA was best for ReOPB (Table 1). The efficiency of all OPBs was very low in the processing of Boc-Ala-Gly-Pro-Arg-MCA and Boc-Glu-Lys-Lys-MCA (Table 1). A substrate containing an Arg residue at the P1 site and Pro at the P2 site was used to differentiate between OPB and oligopeptidase A (OPA): Boc-Val-Pro-Arg-MCA is a specific substrate for OPA which does not cleave Boc-Glu-Lys-Lys-MCA and it is very weak in the processing of Z-Arg-Arg-MCA.[30)]

E. coli and wheat OPB are very sensitive to basic peptides rich in arginine, such as protamine, and in fact are inhibited even by nanomolar levels of protamine.[38)] The OPB from *Serratia proteamaculans* was inhibited by substrate peptides with hydrophobic residues at the P2 position.[34)] We observed a strong substrate inhibition effect on Stm, Re, and Ec OPBs. The SemOPB differs from all other OPBs, showing no substrate inhibition, even substrates containing double arginine at 35 μM did not inhibit the SemOPB. On the other hand, the same substrates at only 5 μM inhibit the activity of the highly active Stm and Re in addition to EcOPB. Such a phenomenon was not observed with substrates containing lysine at P1 site.

The determination of the crystal structure of *L. major* OPB provided good definition of the S1 site. However, information about the S2 and S3 sites is still poor. No acidic residues could be found to interact with the arginine residue at the P2 site. In the *L. major* OPB structure, Arg664 forms a hydrogen bond with the P2 carbonyl oxygen of the inhibitor. This Arg can also be found in prolyl oligopeptidase, indicating that it is not involved in the substrate specificity. ReOPB has Arg at this position, but the other 4 bacterial OPBs have Gln instead. It is unlikely that Arg664 directly contributes to the strong substrate inhibition phenomenon. Based on the modeling of the tri-Arg peptide to the *L. major* OPB structure, Tyr499 appears to stabilize the substrate at P2 through stacking and a hydrogen bond. Since this Tyr499 is completely conserved among all the bacterial OPBs studied in this work, it is undoubtedly involved in the substrate interaction. However, this Tyr is also conserved in the SemOPB. Further experiments will be needed to clarify the mechanism of the strong inhibitory effect of substrate on OPBs. SemOPB will be a good model enzyme to study this phenomenon. In addition, the mechanism of substrate inhibition will be important for development of an effective inhibitor against OPB, which might be involved in the infection of opportunistic pathogens including *Serratia marcescens*. Since we have obtained some crystals using the recombinant enzymes described in this work, X-ray crystallographic analysis is under way to solve the three-dimensional structure of this unique OPB.

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