Sequencing, expression and biochemical characterization of the *Porphyromonas gingivalis pepO* gene encoding a protein homologous to human endothelin-converting enzyme

Shuji Awano[a], Toshihiro Ansai[a,*], Hajime Mochizuki[a], Weixian Yu[a], Kazuhiko Tanzawa[b], Anthony J. Turner[c], Tadamichi Takehara[a]

[a] *Department of Preventive Dentistry, Kyushu Dental College, Kokurakita-ku, Kitakyushu 803-8580, Japan*
[b] *Biological Research Laboratories, Sankyo Co. Ltd., Shinagawa-ku, Tokyo 140-8710, Japan*
[c] *School of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT, UK*

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Abstract We have determined the nucleotide sequence of the clone pAL2 obtained from *Porphyromonas gingivalis* 381 in the previous study [Ansai et al. (1995) Microbiology 141, 2047–2052]. The DNA sequence analysis of this fragment revealed one complete ORF and one incomplete ORF. The ORF encoded a protein (PgPepO) of 690 amino acids with a calculated molecular weight of 78 796. The deduced amino acid sequence exhibited a significant homology with human endothelin-converting enzyme (ECE)-1. Recombinant PgPepO was purified to homogeneity and characterized. The purified enzyme was strongly inhibited by phosphoramidon, and converted big endothelin-1 to endothelin-1. Furthermore, the purified PgPepO strongly cross-reacted with a monoclonal antibody against rat ECE-1. These results indicate that PgPepO has striking similarity to mammalian ECE in structure and function.

Key words: Protease; Neutral endopeptidase; Endothelin; Endothelin-converting enzyme; *Porphyromonas gingivalis*

1. Introduction

Adult periodontitis is a chronic inflammatory disease induced by a microbial infection that is the major cause of tooth loss in the adult population [1]. Although a variety of bacterial species are associated with the initiation and progression of periodontitis, accumulated evidence suggests that *Porphyromonas gingivalis*, a Gram-negative anaerobe, is an important pathogen in severe manifestation of the disease [1,2]. *P. gingivalis* has been shown to express a variety of factors which may play significant roles in pathogenicity, including adhesins, proteases, endotoxins, and cytotoxins [3]. These proteases are considered to be involved directly in tissue invasion and destruction by bacteria and in evasion and modulation of host immune defences, though the primary function of proteases is to provide peptides for growth [4].

Recently, some reports have described the association between periodontal diseases and coronary artery diseases [5]. Also, several studies suggest that some bacteria and viruses contribute to the development of atherosclerosis and coronary artery diseases [6,7]. Endothelin-1 (ET-1) has been identified as the most potent vasoconstrictor peptide produced by endothelial cells [8]. ET-1 is generated by a unique proteolytic cleavage of big endothelin-1 (bigET-1), which is catalyzed by endothelin-converting enzyme (ECE)-1 [9]. Elevation of ET-1 levels has been implicated in the pathogenesis of several diseases, including atherosclerosis [10] and heart failure [11].

While studying an approximately 3.8 kb DNA fragment (pAL2) which we have previously cloned [12], we found that it contained a DNA fragment encoding a novel protease. Here, we report the identification and characterization of the protease homologous to human ECE-1, designated PgPepO, from *P. gingivalis* 381.

2. Materials and methods

2.1. Materials

The endopeptidase 24.11 (NEP) substrate [Met]-enkephalin (YGGFM), bradykinin (RPPGFSPFR), substance P (RPKPQQFFGLM), and succinyl-RPFHLLVY-7-amino-4-methylcoumarin were obtained from Sigma (St. Louis, MO, USA). A precoated 0.25 cm thick silica gel 60 plate was purchased from Merck (Darmstadt, Germany). The peptides human bigET-1, bigET-2 and bigET-3 were obtained from Peptide Institute Inc. (Osaka, Japan).

2.2. Bacterial strains and plasmids

P. gingivalis 381, *P. gingivalis* ATCC 33277, *P. gingivalis* W83, *Actinobacillus actinomycetemcomitans* Y4, and *Prevotella intermedia* ATCC 25611 were kindly provided by K. Ishihara, Tokyo Dental College, Japan and grown anaerobically as described previously [12]. *Escherichia coli* JM109 and BL21(DE3)pLysS (Novagen) were used in subcloning and expression experiments. All *E. coli* strains were grown on LB agar plates or LB broth (Difco Laboratories, Detroit, MI, USA) in the presence of appropriate antibiotics (ampicillin, 50 μg/ml; chloramphenicol, 34 μg/ml).

2.3. DNA sequence analysis

Plasmid pAL2 [12] was used as a template in sequencing reactions and sequenced by the dideoxy method of Sanger et al. [13] with a dye terminator sequencing kit (Applied Biosystems) together with a synthetic oligonucleotide primer. The sequence was determined with an Applied Biosystems model 373S automated DNA sequencer. The coding region was sequenced and confirmed on both strands by using overlapping fragments. The nucleotide sequences were analyzed with computer software package 'DNA strider v. 1.2' [14]. Amino acid homology and comparisons were done with GENETYX-Mac software (Software Development Co., Ltd., Japan) and BLAST network services of DDBJ.

2.4. Southern blot analysis

Genomic DNAs from *P. gingivalis* 381, *P. gingivalis* ATCC 33277, *P. gingivalis* W83, *A. actinomycetemcomitans* Y4, and *P. intermedia* ATCC 25611 were isolated as previously described [12]. Southern blot analysis was performed as previously described [12] using digoxigenin-labelled probes according to the instructions of the supplier (Boehringer Mannheim).

*Corresponding author. Fax: (81) (93) 591-7736.
E-mail: ansai@kyu-dent.ac.jp

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2.5. Enzyme assay

Endopeptidase activity was measured using succinyl-RPFHLLVY-7-amino-4-methylcoumarin as a substrate. Reaction mixtures contained 20 mM Tris-HCl buffer, 25 μM substrate and enzyme in a final volume of 0.1 ml. Enzyme assays were performed as described [15].

The optimal pH was determined using 20 mM acetate buffer (pH 3.5–6.5) and 20 mM Tris-HCl buffer (pH 6.5–9.5).

The measurements of endopeptidase activity were also carried out using thin-layer chromatography (TLC) as follows: the reaction mixture containing 1.25 mM [Met]-enkephalin in 20 mM Tris-HCl buffer (pH 7.0) was incubated with an appropriate amount of enzyme for 10 min at 37°C. The reaction was terminated on ice, and the mixture (5 μl) was then spotted onto a precoated 0.25 cm thick silica gel 60 plate, and TLC was performed as described [16]. Essentially similar methods were carried out on the other substrates such as bradykinin and substance P.

2.6. Expression and purification of PgPepO gene in E. coli

The open reading frame (ORF) in pAL2 was subcloned in the T7-based bacterial expression plasmid pET3a (Novagen) as follows. The gene was amplified by the following primers (corresponding to the 5′- and 3′-ends of the gene, respectively): 5′-TATGCTTTACATATGAACAAGACAATCAAG-3′ and 5′-AGAGCGAGGATCCTTACCAAACGACTACAC-3′ (the *Nde*I and *Bam*HI sites are underlined). The PCR product was restricted with *Nde*I and *Bam*HI and cloned into the same sites of pET3a. The resulting clone, pET3a-PgPepO, was confirmed by DNA sequencing, and introduced into *E. coli* BL21(DE3)pLysS. Growth of the transformant, induction with isopropyl-1-thio-β-D-galactopyranoside (IPTG), and lysis with lysosome were carried out essentially as described previously [17]. The crude enzyme extract from sonicate of induced cells was applied to a column of Resource Q equilibrated with 25 mM Tris-HCl buffer (pH 7.5) connected to a FPLC system (Amersham Pharmacia Biotech, Sweden). The column was washed with the equilibration buffer, and then developed with a linear gradient of NaCl (0–1 M) in the buffer. The active fraction was subjected to HPLC using TSK-gel G3000SWXL (Tosoh, Japan) pre-equilibrated with 25 mM Tris-HCl buffer (pH 7.5), which was also used for determination of the molecular mass of PgPepO. For molecular weight standards, the MW-Marker kit was used (Oriental Yeast, Japan). The purified enzyme was frozen and stored at −80°C.

2.7. SDS-PAGE and immunoblotting

SDS-PAGE was performed with a 12% gel, according to the method of Laemmli [18], and the gel was stained with Coomassie blue R250. Proteins were subjected to 12% polyacrylamide gels containing SDS followed by transfer to a PVDF membrane for immunoblot (Western) analysis using monoclonal antibody AEC27-121 (1:2000) [19] and the ECL method (Amersham).

2.8. Effect of various chemicals on enzyme activity

The purified enzymes were preincubated for 30 min at 37°C with phosphoramidon, thiorphan, EDTA, $ZnCl_2$, *p*CMB, DEP, PAO, *o*-phenanthroline, phenylglyoxal, antipain or leupeptin in 50 mM Tris-HCl buffer (pH 7.0), 150 mM NaCl, 0.1 μM $ZnCl_2$ and 0.1% BSA (buffer A). The reaction mixture was incubated for 15 min at 37°C with 0.1 μM bigET-1 and terminated by the addition of 100 μl of 5 mM EDTA. The reaction products were analyzed using EIA kit (Cayman Chemical Co., Ann Arbor, MI, USA).

2.9. Determination of K_m and V_{max} for bigET-1, bigET-2, and bigET-3

The enzymatic conversions of bigETs to ETs were carried out in 100 μl buffer A including 0.1 μM bigET-1, bigET-2 or bigET-3. The reaction mixtures were incubated for 15 min at 37°C and the reactions were terminated by adding 100 μl of 5 mM EDTA. The amount of the generated ET-1, ET-2 or ET-3 was measured by the EIA kit as described above. For the determination of K_m and V_{max}, bigET-1, bigET-2 and bigET-3 were used at concentrations in the range 0.05–5 μM. Reaction condition was maintained such that hydrolysis was linear with respect to time and protein concentration.

2.10. Nucleotide sequence accession number

The GenBank accession number for the nucleotide sequence of PgPepO reported in this paper is AB010440.

3. Results

3.1. Nucleotide and amino acid sequence analysis

We have determined the nucleotide sequence of a 3804 bp DNA fragment (pAL2) of *P. gingivalis* 381. The total sequence contained one complete ORF and one incomplete ORF. The nucleotide sequence and deduced amino acid sequence of the ORF are shown in Fig. 1. The ORF contained 2070 bp encoding a putative polypeptide of 690 amino acids with a calculated molecular mass of 78 796 Da and an estimated p*I* of 5.33. A potential ribosome binding site was not identified (data not shown).

The first 25 amino acid residues appeared to have the putative signal sequence as defined by the criteria of von Heijne [20] (Fig. 1). The deduced amino acid sequence corresponding to the ORF was compared to all proteins in the Swiss-Prot database with the GENETYX-Mac program (Software Development). A significant degree of sequence homology was found between the ORF and ECE-1 of human (31.1% identical residues) [21], NEP of human (29.9% identical residues) [22], endopeptidase (PepO) from *Lactococcus lactis* (formerly *Streptococcus lactis*) (*L. lactis* PepO) (28.3% identical residues) [23] and endopeptidase from *Mycobacterium leprae* (38.0% identical residues) (unpublished data, accession number Z95398), which all belong to the NEP family. Therefore, the ORF was named PgPepO. The zinc binding motif (HEXXH) among zinc metalloproteases including the NEP family [24] was conserved from position 526 to 530 of PgPepO (Fig. 1). The PgPepO also had the two consensus sequences of the zinc binding domains found among the ECE and NEP family from position 523 to 532, and from position 585 to 594, and contained the glutamic acid (Glu-587) at position 62 downstream of the N-terminal side (i.e. His-526) of the motif HEXXH (Fig. 1). The alignment of the amino acid sequences of PgPepO and the human ECE-1 are shown in Fig. 2. The putative active sites in human ECE-1 were conserved in PgPepO (Arg-78, Asn-485, Ala-486, Val-523, Glu-527, Glu-587, Asp-591, His-649 and Arg-655) (Fig. 2, box). On the other hand, all 10 cysteine residues (Fig. 2, arrow) conserved in the ECE and NEP family [24] were replaced by other amino acid residues.

3.2. Purification of recombinant PgPepO and immunoblotting

The purification of recombinant PgPepO is summarized in Table 1. The enzyme was purified 41-fold with a final specific activity of 24.6 U/mg, and the overall yield of the activity was 0.7%. Upon induction of *E. coli* BL21(DE3)pLysS containing pET3a-PgPepO with IPTG, a polypeptide of M_r ∼78 kDa was produced (Fig. 3A), which is in good agreement with the calculated molecular mass of the predicted amino acid sequence (78 796 Da). The molecular mass of PgPepO was estimated by gel filtration to be about 78 kDa. These results suggested that PgPepO was a monomer of ∼78 kDa polypeptide.

The 78 kDa protein corresponding to PgPepO cross-reacted effectively against the anti-rat ECE monoclonal antibody, AEC27-121, as shown in Fig. 3B.

3.3. Characterization of PgPepO

The optimal pH for PgPepO was determined in vitro by using succinyl-RPFHLLVY-7-amino-4-methylcoumarin as a substrate. The optimal pH value for the activity was in a narrow neutral range between 6.8 and 7.0, similar to ECE-1

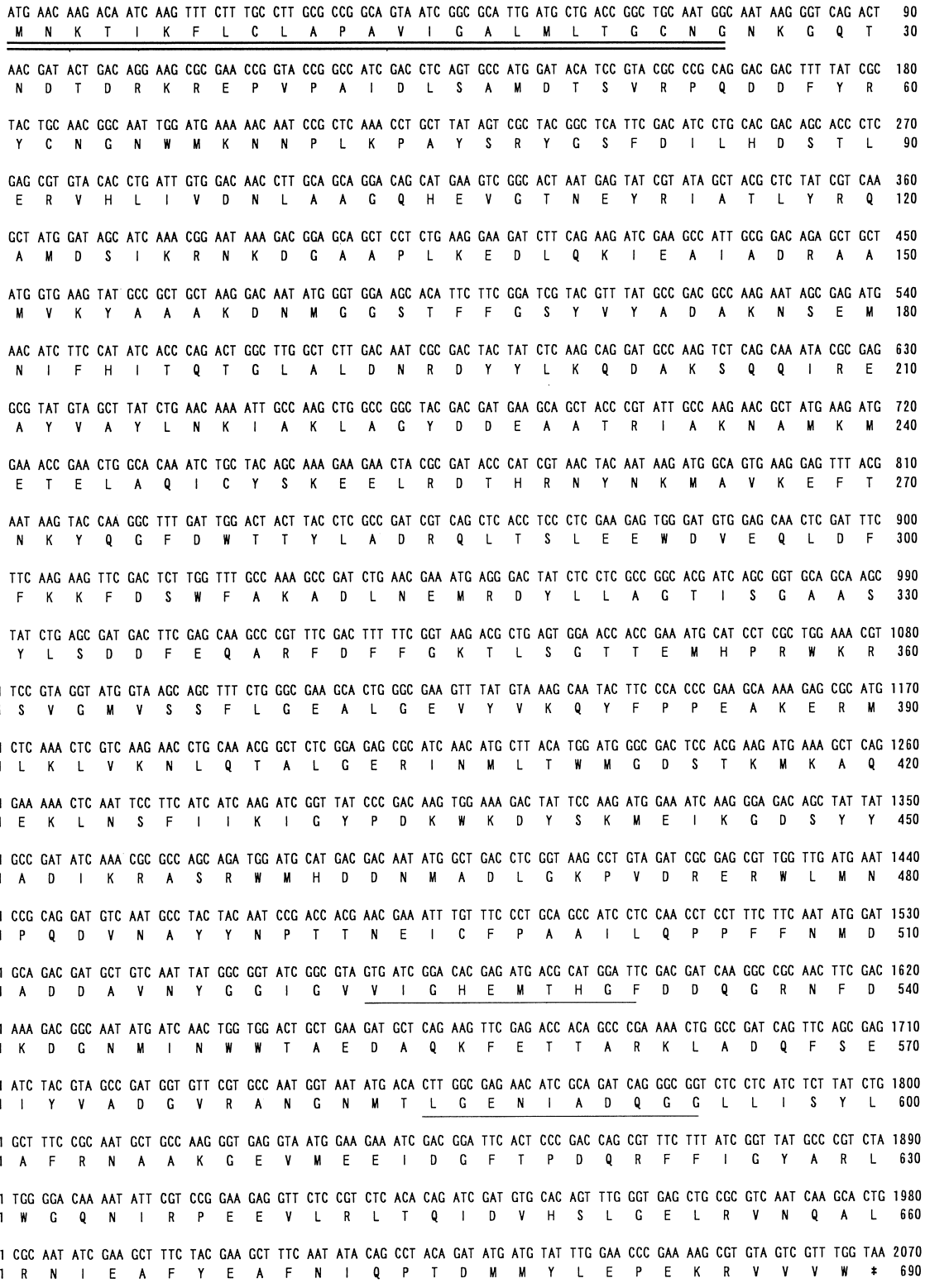

Fig. 1. Nucleotide and deduced amino acid sequence of PgPepO. The putative signal sequence is doubly underlined. The consensus sequences including zinc binding motif conserved in NEP family are underlined.

(pH 6.6–6.8) [25]. To characterize PgPepO further, the effect of various chemicals on the enzymatic activity was investigated by using bigET-1 as a substrate, which was a specific substrate of ECE in vivo, as shown in Table 2. In this experiment, we did not use the assay system using succinyl-RPFHLLVY-7-amino-4-methylcoumarin as substrate since some chemicals were found to inhibit the activity of aminopeptidase M (data not shown). In the bigET-1 conversion

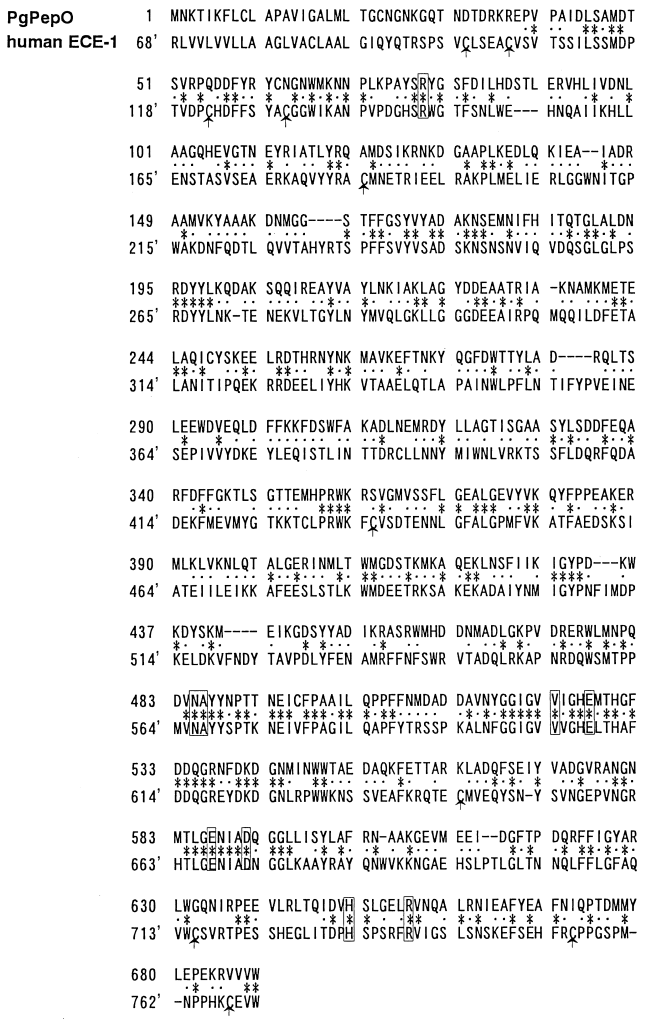

Fig. 2. Alignment of amino acid sequences of PgPepO and human ECE-1. The respective amino acid numbers starting from the N-terminus are listed at the left margin. Asterisks and dots indicate identical amino acids and conservative amino acid substitutions, respectively. The putative active site amino acids are boxed. Arrows show cysteine residues conserved in the ECE and NEP family, which are replaced with other amino acid residues in PgPepO.

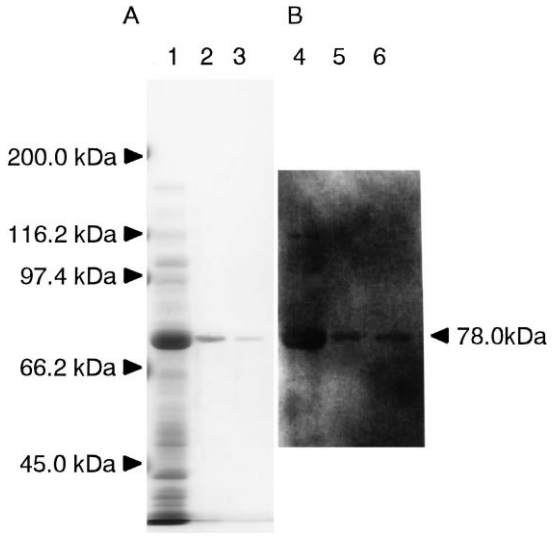

Fig. 3. SDS-PAGE and immunoblotting of PgPepO. A: Samples from each purification step were resolved by SDS-PAGE in a 12% gel and then stained with Coomassie blue R250. B: Samples for immunoblots were subjected to SDS-PAGE on a 12% gel, transferred to a PVDF membrane and analyzed using monoclonal antibody AEC27-121. The immunoreactive bands were visualized with the ECL detection kit. Lane 1, the crude enzyme extract; lane 2, the enzyme preparation eluted from Resource Q; lane 3, the purified enzyme. The positions and molecular weights of standard proteins are indicated in the left lane. The position and molecular weight of PgPepO is indicated by the arrow in the right lane.

assay, the enzyme was strongly inhibited by the NEP inhibitor, phosphoramidon, as well as by *o*-phenanthroline, EDTA, *p*CMB and PAO, and moderately inhibited by DEP, phenylglyoxal, leupeptin and antipain. However, the enzyme was resistant to another potent NEP inhibitor, thiorphan, as is the case for ECE-1. Also, the enzyme activity was activated by 10 mM Zn^{2+}.

Kinetic parameters of PgPepO for bigET-1, bigET-2, and bigET-3 are shown in Table 3. The K_m and V_{max} values for bigET-1 of PgPepO were 12.78 ± 3.24 nM and 3.33 ± 0.32 μmol/min·mg, respectively, which indicated that bigET-1 was converted most efficiently, compared with bigET-2 and bigET-3.

3.4. Distribution of genes homologous to the PgpepO gene

As shown in Fig. 4, specific signals for the PgPepO gene were found in *P. gingivalis* strains including 381, ATCC 33277 and W83, but not detected in other periodontopathogenic bacteria such as *P. intermedia* ATCC 25611 and *A. actinomycetemcomitans* Y4 strains.

4. Discussion

In the present study, we have identified and characterized a novel endopeptidase gene, PgPepO gene, from *P. gingivalis* 381. PgPepO had significant homology to the NEP family including human ECE-1. ECE-1, like mammalian NEP, is a class II integral membrane protein with a short N-terminal cytoplasmic tail, a transmembrane domain that represents the uncleaved signal peptide, and a large putative extracellular domain [24]. In contrast, PgPepO had a putative signal se-

Table 1
Purification of recombinant PgPepO

Fraction	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	80.3	49.2	0.6	100	1
Resource Q	2.3	48.3	21.0	2.8	35
TSK-gel G3000SWXL	0.6	14.8	24.6	0.7	41

One unit corresponds to the cleavage of 1 μmol of succinyl-RPFHLLVY-7-amino-4-methylcoumarin per minute.

Table 2
Effect of various chemicals on enzyme activity of PgPepO

Chemical (conc.)	Activity (%)
None	100
Phosphoramidon (10 μM)	25
Thiorphan (10 μM)	93
EDTA (10 mM)	4
o-Phenanthroline (10 mM)	3
*p*CMB (10 mM)	3
PAO (10 mM)	22
DEP (10 mM)	58
Phenylglyoxal (10 mM)	41
Leupeptin (10 mM)	30
Antipain (10 mM)	65
$ZnCl_2$ (10 mM)	173

The indicated amounts of inhibitor were preincubated for 30 min at 37°C in buffer A with the purified enzyme (PgPepO) before the addition of 0.1 μM bigET-1. The reaction mixture was incubated for 15 min at 37°C, stopped by adding 100 μl of 5 mM EDTA and tested for ET-1 concentration in EIA. Control activity (100%) was determined in the absence of inhibitors. Data are the means of three independent experiments.

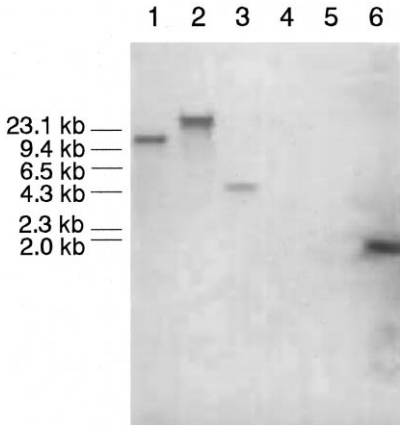

Fig. 4. Southern blot hybridization of chromosomal DNA from periodontopathogenic bacteria with the PgpepO gene. Lanes: 1, *P. gingivalis* 381; 2, *P. gingivalis* ATCC 33277; 3, *P. gingivalis* W83; 4, *A. actinomycetemcomitans* Y4; 5, *P. intermedia* ATCC 25611; 6, PgPepO gene probe; 1–5, *Eco*RI digests. The numbers on the left are size markers.

quence in the N-terminal region, but it did not appear to include any membrane-spanning domains. This indicated that PgPepO may be secreted across the cytoplasmic membrane by using the signal peptide. This finding differs from *L. lactis*PepO, which is one of the prokaryotic endopeptidases reported to date. In *L. lactis* PepO, the possible membrane-spanning domains and typical signal sequence were not found. In practice, *L. lactis* PepO was shown to be localized on the inner side of the cell membrane [23].

PgPepO had the consensus sequences including zinc binding sites (His-526, His-530, and Glu-587) and all the conserved active sites in the NEP family [24], although Arg-747 of human NEP was replaced by Glu-751 in human ECE-1 and Val-687 in PgPepO. These findings support well the results that the arginine modifier, phenylglyoxal, and the histidine modifier, DEP, inhibited the enzyme activity (Table 2). Furthermore, interestingly, all 10 cysteine residues conserved in ECE and NEP family were not conserved in PgPepO. However, considering that PgPepO was inhibited by *p*CMB, PAO, leupeptin and antipain, which are known cysteine protease inhibitors, five cysteine residues in PgPepO might have important roles in enzyme activity. On the other hand, it has been reported that rat ECE-1 exists as a disulfide-linked dimer, and Cys-412 is responsible for the dimerization of rat ECE-1 [26]. In PgPepO, the amino acid residue corresponding to the Cys-412 was replaced by Met-354. This observation would be reasonable for the finding that PgPepO is a monomer. Mutational analysis is needed to clarify the exact roles of these amino acid residues.

In our study, purified PgPepO was able to convert bigET-2 and bigET-3, although the enzyme preferred bigET-1 to bigET-2 or bigET-3, as does ECE-1. The K_m values of PgPepO for bigET-1, bigET-2 and bigET-3 was lower than ECE-1 ($K_m = 200$, 460 and 270 nM, respectively) [25]. Therefore, it was suggested that PgPepO might be able to convert bigET-1, bigET-2 and bigET-3 to ET-1, ET-2 and ET-3 more efficiently than ECE-1. Furthermore, the purified enzyme also cleaved [Met]-enkephalin as well as bradykinin and substance P (our unpublished data), and succinyl-RPFHLLVY-7-amino-4-methylcoumarin, which are known substrates of NEP [15]. In general, it has been considered that NEP has a much broader substrate specificity compared with ECE-1. However, recently ECE-1 has been shown to hydrolyze bradykinin efficiently [27] and this study has been extended in [28] to show that ECE-1 can also hydrolyze some other NEP substrates such as substance P, neurotensin and insulin B chain but [Leu]-enkephalin was hydrolyzed extremely poorly. These results suggest that PgPepO exhibits a similar substrate specificity to ECE-1 and NEP although further detailed kinetic studies will be required to compare the specificity of PgPepO with its mammalian homologues more quantitatively. The sensitivity of PgPepO to phosphoramidon but not thiorphan also emphasizes the greater similarity with ECE-1 than with NEP.

Thus, PgPepO has a striking similarity with human ECE-1 in structure and function. ET-1 acts as a potent mitogen on various kinds of cells, including vascular smooth muscle cells [29,30]. The mitogenic action is synergistic with the effects of other mitogens such as epidermal growth factor [31]. Most

Table 3
Kinetic parameters of PgPepO for bigET-1, bigET-2 and bigET-3

Substrate	K_m (nM)	V_{max} (μmol/min·mg)	K_m/V_{max} (min·mg/ml)
Big endothelin-1 (human, 1–38)	12.78 ± 3.24	3.33 ± 0.32	0.0037 ± 0.0005
Big endothelin-2 (human, 1–37)	38.43 ± 7.55	2.89 ± 0.23	0.0131 ± 0.0015
Big endothelin-3 (human, 1–41 amide)	49.00 ± 13.75	1.39 ± 0.02	0.0348 ± 0.0092

Results from three independent sets of experiments are shown as mean ± S.D. BigET-1, bigET-2 and bigET-3 were used at concentrations in the range 0.05–5 μM. The amount of generated ET-1, ET-2 or ET-3 was measured by EIA.

recently, common bacteria and viruses, such as *Helicobacter pylori*, *Chlamydia pneumoniae* and cytomegalovirus, have been reported to contribute to the development of atherosclerosis, perhaps by triggering inflammation [6,7]. Furthermore, several studies have shown that periodontopathogenic bacteria may have important roles in atherosclerosis [5]. *P. gingivalis* was found to invade aortic and heart endothelial cells in vitro [32]. Also, it is reported that ET-1 production increases in vascular smooth muscle, gastric epithelium and gastric smooth muscle cells with *H. pylori* [33]. In the present study, Southern blot analysis revealed that genes homologous to the PgPepO gene were distributed in only *P. gingivalis* strains such as 381, ATCC 25611 and W83 among periodontopathogenic bacteria. Therefore, it is of interest whether *P. gingivalis* influences the expression of ET-1 in endothelial cells and other cells. Additional studies are needed to investigate the exact nature and function of PgPepO to the host.

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