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Dipeptidyl peptidase with strict substrate specificity of an anaerobic periodontopathogen *Porphyromonas gingivalis*

Setsuo Fujimura *, Kaname Hirai, Yukinaga Shibata

Department of Oral Microbiology, Matsumoto Dental University, Shiojiri-Shi, Nagano-Ken 399-0781, Japan

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Abstract

A dipeptidyl peptidase which hydrolyzed Xaa-Ala-*p*-nitroanilide was purified to homogeneity by sequential procedures including ammonium sulfate precipitation, ion-exchange chromatography, hydrophobic interaction chromatography, gel filtration and isoelectric focusing from the cell extract of *Porphyromonas gingivalis*. The purified enzyme hydrolyzed *p*-nitroanilide derivatives of Lys-Ala, Ala-Ala, and Val-Ala, but not Xaa-Pro. Enzyme activity was maximum at neutral pHs. Its molecular mass was 64 kDa with an isoelectric point of 5.7. The enzyme belonged to the family of serine peptidases. © 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

1. Introduction

Porphyromonas gingivalis is an asaccharolytic putative pathogen of human adult periodontitis [1–4]. It is known that this anaerobic organism utilizes peptides but not free amino acids as its energy source [5,6]. *P. gingivalis* elaborates extracellular and cell-associated proteinases such as arginine- and lysine-specific proteinases [7–12] which degrade proteins surrounding the organisms to peptide fragments. Then, these fragments must be further split into smaller peptides (dipeptides) by peptidases to provide adequate nutrients. Therefore, both endo- and aminopeptidases may be important for nutritional requirement of *P. gingivalis*. Nonetheless, those enzymes have not been sufficiently studied except for dipeptidyl peptidase (DPP) IV which cleaves primarily Gly-Pro [13–19]. In this report we describe the isolation and characterization of a DPP that hydrolyzes Xaa-Ala-*p*-nitroanilide.

2. Materials and methods

2.1. Bacterial strains and cultivation methods

P. gingivalis ATCC 332777 was maintained anaerobically at 37°C on blood agar plates containing hemin (5 μg ml^{-1}) and menadione (0.5 μg ml^{-1}). This strain was grown in Trypticase peptone (BBL)-base medium [20] anaerobically at 37°C in a glove box filled with a mixture of gases (N_2+H_2+CO_2; 85:10:5) for 3 days. *Prevotella intermedia* ATCC 25611 and *Prevotella nigrescens* ATCC 33563 were cultured by the same methods.

2.2. Preparation of cell extract

The cells, collected and washed by centrifugation at 10 000×*g* for 15 min, were suspended in 50 mM Tris–HCl buffer (pH 7.4) and disrupted by sonication at 150 W for 20 min. The sonicate was centrifuged at 5000×*g* for 10 min to remove unbroken cells and the supernatant was centrifuged at 120 000×*g* for 1 h. The clear supernatant obtained by this centrifugation was designated cell extract.

2.3. Purification of DPP

All steps of the purification were conducted at 4°C. Ammonium sulfate was added to the cell extract prepared from 18 g cell (wet weight) to 70% saturation of this agent and stirred for 2 h. The precipitate was collected by cen-

* Corresponding author Tel: +81 (263) 51 2082; Fax: +81 (263) 51 2084. *E-mail address:* fujimura@po.mdu.ac.jp (S. Fujimura).

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trifugation at 10 000×*g* for 15 min, dissolved in 50 mM Tris–HCl buffer (pH 8.2), and dialyzed against the same buffer overnight. The dialyzed material was clarified by centrifugation at 10 000×*g* for 10 min before application to a column (1.5×30 cm) of Q-Sepharose fast flow (Amersham Pharmacia Biotech AB, Uppsala, Sweden) equilibrated with 50 mM Tris–HCl buffer (pH 8.2) and washed with 280 ml of this buffer. The column was eluted with a linear concentration gradient of NaCl, which was generated by mixing 150 ml of Tris–HCl buffer (pH 8.2) containing 600 mM NaCl into an equal volume of Tris–HCl buffer (pH 8.2). The active fractions were collected and dialyzed against 50 mM Tris–HCl buffer containing 1.5 M ammonium sulfate and applied to a column (0.9×2 cm) of Phenyl Sepharose CL-4B (Amersham Pharmacia Biotech) equilibrated with 50 mM Tris–HCl (pH 8.2) containing 1.5 M ammonium sulfate. The column was eluted with a descending gradient of ammonium sulfate from 1.5 to 0 M (100 ml each side)(Fig. 1). Fractions containing DPP activity were collected and concentrated in vacuo, dialyzed against 50 mM Tris–HCl buffer (pH 7.4) containing 200 mM NaCl and applied to a column (2.6×96 cm) of Sephacryl S-300 (Amersham Pharmacia Biotech) equilibrated with the same buffer saline. They were eluted with this buffer saline at flow rate of 40 ml/h. The active fractions were dialyzed against 1% glycine solution extensively and further purified by isoelectric focusing with 1% (v/v) ampholine (pH 4.0–6.5) at 600 V for 24 h. DPP was detected at the zone of pH 5.7.

2.4. Assay of enzyme activity

DPP activity was assayed routinely using Lys-Ala-*p*-nitroanilide as the methods described earlier [21]. The activity against other *p*-nitroanilide derivatives of amino acids or peptides was examined by the same methods as described above. 1 U of activity was defined as the liberation of 1 μmol of *p*-nitroaniline min^{-1}.

Hydrolysis of azocasein and azocoll were examined by the methods described elsewhere [21].

2.5. Estimation of optimum pH for the enzyme activity

The relation between activity and pHs was examined from pH 4.5 to 10.0 using the following buffers: acetate buffer (pH 4.5–6.0), Na–phosphate buffer (pH 6.9–7.5), Tris–HCl buffer (pH 7.5–9.0), and Na–bicarbonate buffer (pH 9.0–10.).

2.6. Protein determination

Protein concentration was estimated with the DC protein assay kit (Bio-Rad, Hercules, CA, USA), using bovine serum albumin as a standard.

2.7. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE (12.5% polyacrylamide) was carried out according to the methods of Laemmli [22]. The molecular mass markers used were phosphorylase *b* (94 kDa)

3. Results and discussion

3.1. Production of DPP among periodontopathogens

First, we compared the production of DPP among three species of periodontopathogens using *P. gingivalis* ATCC 33277, *P. intermedia* ATCC 25611 and *P. nigrescens*

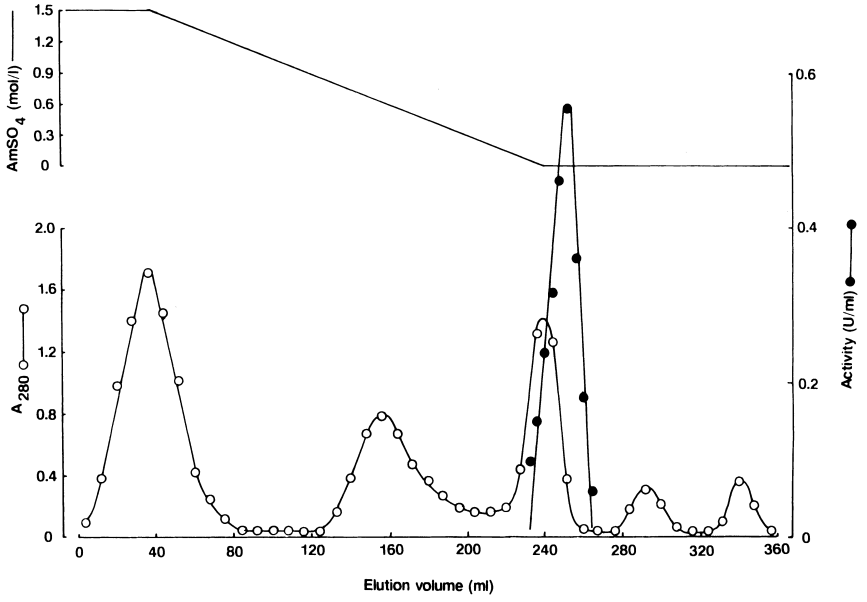

Fig. 1. Hydrophobic interaction chromatography on Phenyl Sepharose CL-4B. Symbols: ○–○, OD_{280}; ●–●, enzyme activity against Lys-Ala-pNA.

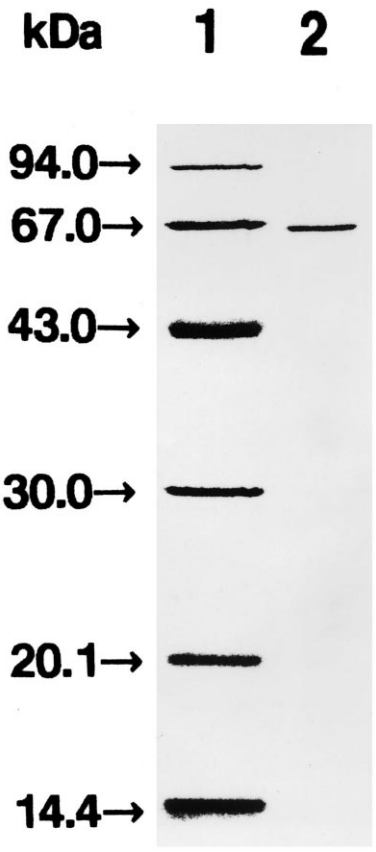

Fig. 2. SDS–PAGE of the purified DPP. Lane 1, marker proteins; lane 2, purified DPP.

ATCC 33563. The concentrations of bacterial suspensions of these strains were adjusted to the same level before preparation of cell extract. DPP activity was detected only in cell extract of *P. gingivalis* and *P. intermedia*, but the amount of the activity of the latter strain produced only 20% of that of the former strain. Both organisms did not liberate the enzyme to extracellular medium. *P. nigrescens* was found not to produce DPP at all in either fraction.

3.2. Purification of DPP and molecular mass

The enzyme was purified from the cell extract by the sequential procedures to homogeneity as shown in Fig. 2. Molecular mass of DPP was estimated as 64 kDa. The enzyme was purified 874-fold over cell extract with a recovery of 12%. Typical purification of DPP is shown in Table 1. In all procedures of chromatography and electric focusing, the activity emerged as single peaks. Hydrophobic interaction chromatography on Phenyl Sepharose CL-4B was obviously effective at removing the impurities with below about 36 kDa in the purification step. Molecular mass was calculated as 64 kDa from the electrophoretogram.

3.3. Optimum pH

When the enzyme activity measured in different pHs ranging from 4.5 to 10.0, DPP was most active at pH 7.5. Only low activity was observed at pHs below 6.0 or over 8.0.

3.4. Substrate specificity

It was clear that the specificity of this enzyme is strict. Only dipeptidyl-pNA with alanine residue at the second position was cleaved by the enzyme (Table 2) but not totally; Lys-Ala-pNA was most rapidly hydrolyzed. Though Ala-Ala-pNA was significantly hydrolyzed, neither Ala-pNA nor Ala-Ala-Ala-pNA was split. Weak activity was found in Val-Ala-pNA. The substrates with blocked amino termini were not split. The following substrates tested were not hydrolyzed at all or were only negligibly hydrolyzed: Glu-Ala-pNA, Ala-Phe-pNA, glutaryl-Ala-Ala-Pro-Leu-pNA, Arg-Pro-pNA, Lys-Pro-pNA, His-Pro-pNA, Glu-Pro-pNA, Ser-Pro-pNA, Met-Pro-pNA, Phe-Pro-pNA, Gly-Pro-pNA, Ala-Pro-pNA, Arg-pNA, Leu-pNA, succinyl-Ala-pNA, succinyl-Ala-Ala-Pro-Phe-pNA, succinyl-Ala-Ala-pNA, succinyl-Ala-Ala-Ala-pNA, benzoyl-Arg-pNA, and tosyl-Gly-Pro-Lys-pNA.

Activity against azocoll, azocasein and remazol brilliant blue conjugated hyde powder, and elastin Congo red could not detected.

3.5. Effect of reagents on DPP activity

DPP was completely and partially inhibited by a serine enzyme inhibitor 3,4-dichloroisocoumarin and diisopropylfluorophosphate, respectively, indicating it is classified as a serine enzyme. Bestatin, an aminopeptidase inhibitor did not influence the activity of DPP. The proteinase inhibitors, as well as metal-chelating reagents and sulfhy-

Table 1
Purification of DPP of *P. gingivalis*

Step	Protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Purification (fold)	Yield (%)
Crude extract	8213	21.0	0.0026	1	100
Ammonium sulfate	3432	18.0	0.0052	2	86
Q-Sepharose	79.5	9.7	0.1220	47	46
Phenyl Sepharose	54.2	9.6	0.1771	68	46
Sephacryl S-300	5.4	4.7	0.8704	335	22
Isoelectric focusing	1.1	2.5	2.2727	874	12

Table 2
Specificity and kinetics of DPP of *P. gingivalis*

Substrate	Relative activity (%)	K_m (mM)	V_{max} (U mg^{-1} min^{-1})	V_{max}/K_m
Lys-Ala-pNA	100	0.42	14.68	34.95
Ala-Ala-pNA	36	0.43	2.52	5.86
Val-Ala-pNA	21	0.37	2.37	6.41

dryl-group reagents did not inhibit activity. However, it was significantly inhibited by tosyl-L-lysine chloromethyl ketone. Heavy metal ions including Ca^{2+}, Mg^{2+} and Zn^{2+} did not influence the activity (Table 3).

We isolated DPP which hydrolyzed primarily Lys-Ala-pNA, which is a standard substrate of DPP II. Mammalian DPP II is generally active to Xaa-Pro-pNA, especially Lys-Pro-pNA, Phe-Pro-pNA, Ala-Pro-pNA, and Gly-Pro-pNA [23]. However, DPP of *P. gingivalis* did not demonstrate the activity against these substrates. Therefore, the purified DPP does not appear to be typical DPP II.

Recently, DPP VII which hydrolyzed Ala-Phe-pNA, Ala-Ala-pNA, and Gly-Phe-pNA (hydrolysis of Lys-Ala-pNA and Val-Ala-pNA was not described) was isolated from *P. gingivalis* [24]. Although both DPP VII and DPP hydrolyzed Ala-Ala-pNA, common properties in hydrolytic pattern of other substrates and molecular size could not be found.

The purified DPP was inactive against Xaa-Pro-pNA, but the activity against these substrates described in the text was detected in the cell extract. Moreover, the activities of the enzyme which cleave these substrates were higher than that of DPP. Therefore, it is likely that *P. gingivalis* elaborates several other aminopeptidases. In fact, Gazi et al. found an enzyme which cleaves Ala-Pro [25], and Suido et al. detected hydrolytic enzymes of Arg-Arg, Gly-Arg, Gly-Phe, Gly-Pro, Leu-Gly, and Lys-Pro [26] in cell extract of *P. gingivalis*. Functional investigation and isolation of these enzymes may be worthwhile in understanding the pathogenic properties of the periodontopathogen.

Table 3
Effect of various compounds and metal ions on DPP of *P. gingivalis*

Compound	Concentration	Residual activity (%)
Control	–	100
Diisopropylfluorophosphate	5.0 mM	69
3,4-Dichloroisocoumarin	0.4 mM	0
Bestatin	0.2 mM	97
Leupeptin	0.4 mM	109
Antipain	0.4 mM	92
E64[a]	0.4 mM	73
Aprotinin	1.0 mg ml^{-1}	85
ε-Aminocaproic acid	5.0 mM	98
Tosyl-L-lysine chloromethyl ketone	1.0 mM	18
EDTA	1.0 mM	82
EGTA[b]	1.0 mM	90
1,10-Phenanthroline	5.0 mM	77
p-Chloromerculibenzoate	1.0 mM	86
N-Ethylmaleimide	2.0 mM	94
2-Mercaptoethanol	5.0 mM	103
Cysteine	5.0 mM	100
Ca^{2+}	1.0 mM	117
Mg^{2+}	1.0 mM	117
Zn^{2+}	1.0 mM	93

[a] L-*trans*-epoxy-succinylleucylamido-(4-guanidino)butane.
[b] ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*,*N*′-tetraacetic acid.

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