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Isolation and properties of a tripeptidyl peptidase from a periodontal pathogen *Prevotella nigrescens*

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Abstract

Prolyltripeptidyl amino peptidase activity was found in a crude extract of *Prevotella nigrescens* and this enzyme was purified by procedures including concentration with ammonium sulfate, ion exchange chromatography, gel filtration, and isoelectric focusing. This peptidase hydrolyzed Ala-Ala-Pro-*p*-nitroanilide as well as Ala-Phe-Pro-*p*-nitroanilide. Furthermore, several *p*-nitroanilide derivatives of dipeptides with a proline residue in the second position from the amino-terminal end (Xaa-Pro) were also cleaved detectably. The molecular mass of this tripeptidase was calculated as 56 kDa and its isoelectric point was 5.8. The enzyme was inactivated completely by heating at 60°C for 5 min and inhibited significantly by specific serine enzyme inhibitors.
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Keywords: *P. nigrescens*; Tripeptidase; Purification; Properties; Enzyme

1. Introduction

Several black-pigmented Gram-negative obligate anaerobes, such as *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Prevotella nigrescens*, are implicated in the pathogenesis of adult periodontal disease. The pathogenic factors of these species, including proteolytic enzymes [1–6], fimbriae [7,8], lipopolysaccharides [9–12], and cytokines [13–15], have been mainly investigated in *P. gingivalis*; the virulent substances of the other two species are not sufficiently known. *P. nigrescens* was recently proposed to be divided from *P. intermedia* because of some differences in DNA–DNA homology, production of enzymes including peptidases and lipases, and migration profile of malate dehydrogenase and glutamate dehydrogenase in electrophoresis between the two species [16], and the possibility of both *P. intermedia* and *P. nigrescens* in the etiology of periodontal disease has been discussed [17–19]. In addition, these bacteria have been isolated frequently from clinical materials of gingivitis and periodontitis, indicating that *P. nigrescens* is also a putative periodontal pathogen. At first, we tried to investigate proteolytic enzymes in *P. nigrescens*, which is responsible for tissue degradation as a pathogenic factor. However, the activity was too weak to isolate and characterize. Therefore, we undertook to study peptidase, a protease-related enzyme. In this report we describe the isolation and characterization of tripeptidyl peptidase (TPP) of *P. nigrescens*.

2. Materials and methods

2.1. Strain and cultivation methods

P. nigrescens ATCC 33563 was inoculated into a medium containing trypticase peptone (1.7%), yeast extract (0.3%), NaCl (0.5%), K_2HPO_4 (0.25%) and glucose (0.25%) supplemented with hemin (5 μg ml^{-1}) and menadione (0.5 μg ml^{-1}) as described by Sawyer et al. [20], and incubated at 37°C anaerobically in a glove box filled with a mixture of gases (N_2+H_2+CO_2, 85:10:5) for 3 days.

2.2. Preparation of crude extract and solubilization of the envelope

Cells (16 g in wet weight) collected from 2 l culture by centrifugation at 10 000×*g* were washed twice with 50 mM

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Tris–HCl buffer (pH 7.6) by centrifugation and suspended in the same buffer. The cells were then disrupted by sonication at 150 W for 15 min. The sonicate was centrifuged at 5000×*g* for 15 min and the supernatant was further centrifuged at 120 000×*g* for 60 min. The clarified supernatant was designated crude extract. The precipitate (envelope fraction) was solubilized with 3-[3-cholamidopropyl]-dimethylammonio]-propanesulfonate (CHAPS) as described elsewhere [21].

2.3. Purification of TPP

Ammonium sulfate was added to the crude extract to give 75% saturation and stirred for 3 h, followed by centrifugation at 10 000×*g*. The collected proteins were dissolved in 50 mM Tris–HCl buffer pH 8.2 and dialyzed overnight against 2 l of the same buffer with two changes. After dialysis, insoluble materials were removed by centrifugation at 20 000×*g* and the supernatant was applied to a column (1.5 by 30 cm) of Q-Sepharose fast flow which had been equilibrated with 50 mM Tris–HCl buffer (pH 8.2). After washing the column with the same buffer, the proteins were eluted with a linear concentration gradient of NaCl of 0–500 mM and 5-ml fractions were collected. The enzyme active fractions were pooled, concentrated in vacuo to half the volume, and dialyzed against 50 mM Tris–HCl buffer (pH 7.6) containing 200 mM NaCl. Then the dialyzed material was applied to a column (2.6 by 100 cm) of Sephacryl S-300, equilibrated with this buffer saline and eluted at a flow rate of 30 ml h^{-1}. Each 5 ml of eluates was collected. The active fractions were combined, concentrated in vacuo, and dialyzed against 50 mM Tris–HCl buffer (pH 7.6) containing 200 mM NaCl, and the gel filtration was repeated on Sephacryl S-300 in the same manner. The active fractions were dialyzed exhaustively against 1% glycine solution and applied to isoelectric focusing (120 ml capacity) using 1% (v/v) ampholine (pH 4.0–6.5). Electrophoresis was carried out at 700 V for the first 22 h and at 1000 V for the last 5 h. After electrophoresis, contents were cut into 1.5-ml fractions and the pH and activity of each fraction were measured.

2.4. Assay of enzyme activity

TPP activity was measured using Ala-Ala-Pro-*p*-nitroanilide (Ala-Ala-Pro-pNA) as substrate. Reaction mixtures containing 700 μl of 1 mM substrate in 50 mM Tris-maleate buffer (pH 7.5), 50 μl of enzyme source, and 150 μl of Tris-maleate buffer (pH 7.5) were incubated at 37°C for 30 min. After incubation, 100 μl of 7.5 M acetic acid was added to stop the reaction and the released *p*-nitroaniline was measured by absorption at 410 nm. One unit of enzyme activity was defined as the liberation of 1 μmol of *p*-nitroaniline min^{-1} under these conditions. Proteolytic activity was estimated using azocoll, azoalbumin, and azocasein as substrates by methods described earlier [2].

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

Purity of the sample and estimation of molecular mass were determined by SDS–PAGE (12.5% acrylamide), which was carried out according to the methods of Laemmli [22]. The marker proteins for the reference of molecular mass determination were phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa). Gels were stained with Coomassie brilliant blue R-250.

3. Results

3.1. Cellular localization of TPP

First the distribution of TPP in the crude extract, solubilized envelope fraction, and culture supernatant was examined. Relative amounts of activity in each fraction were 100, 12, and 3, respectively.

3.2. Purification of TPP

TPP was purified to homogeneity by the sequential procedure (Fig. 1). The specific activity (TPP activity (U) protein mg^{-1}) of the final purified sample increased to 546-fold over that of the crude extract. The recovery of

Fig. 1. SDS–PAGE of purified TPP. Lane A, marker proteins; lane B, purified TPP.

Table 1
Substrate specificity

Substrate	Activity (%)	V_{max} (U mg^{-1} min^{-1})	K_m (mM)	V_{max}/K_m
Ala-Ala-Pro-pNA	100	0.053	0.069	0.768
Ala-Phe-Pro-pNA	66	0.052	0.129	0.403
Ala-Ala-Phe-pNA	0			
Val-Leu-Lys-pNA	0			
Ala-Ala-Ala-pNA	0			
Lys-Pro-pNA	24	0.018	0.084	0.214
Arg-Pro-pNA	22	0.019	0.114	0.167
Ala-Pro-pNA	13			
Ser-Pro-pNA	13			
Phe-Pro-pNA	13			
Gly-Pro-pNA	3			
Ala-Phe-pNA	0			
Ala-Ala-pNA	0			
Ala-pNA	0			
Pro-pNA	0			
Bz-Arg-pNA	0			
Glt-Ala-Ala-pNA	0			
Suc-Ala-Ala-Ala-pNA	0			
Suc-Ala-Ala-Pro-pNA	0			
Tos-Gly-Pro-Lys-pNA	0			

the enzyme was 8.3%. The molecular mass was calculated as 56 kDa from the electrophoretogram.

3.3. Substrate specificity

Hydrolysis of chromogenic substrates by the purified enzyme is summarized in Table 1. The enzyme cleaved most actively Ala-Ala-Pro-pNA, and hydrolysis of Ala-Phe-Pro-pNA was also significant. Other *p*-nitroanilide derivatives of tripeptides and amino-terminally blocked tripeptidyl-pNA substrates were not split. Interestingly, the dipeptidyl-pNA with proline at the second position was found also to be hydrolyzed, if not strongly. Particularly compounds containing basic amino acids such as lysine and arginine at the first positions were more rapidly split than the others. No activity against azo dye-conjugated proteins was observed in the purified enzyme.

3.4. Effects of group-specific reagents on the enzyme activity

Specific serine enzyme inhibitors including diisopropylfluorophosphate, Pefabloc SC, and 3,4-dichloroisocoumarin exhibited significant inhibition of TPP activity; however, neither strongly inhibitory nor activating effects were found among the group-specific reagents tested.

The enzyme was not affected at all by metal chelators and protease inhibitors (Table 2).

Table 2
Effect of various group-specific reagents on TPP of *P. nigrescens*

Compound	Concentration	Relative activity (%)
Control	–	100
Diisopropylfluorophosphate	1.0 mM	43
	5.0 mM	31
	10.0 mM	31
Pefabloc[a] SC	1.0 mM	67
3,4-Dichloroisocoumarin	1.0 mM	64
Tosyl-L-lysine chloromethyl ketone	1.0 mM	53
N-Ethylmaleimide	1.0 mM	97
1,10-Phenanthroline	5.0 mM	94
EDTA	1.0 mM	102
E64[b]	0.2 mM	105
Antipain	0.4 mM	98
Leupeptin	0.2 mM	106
Aprotinin	1.0 mg ml^{-1}	90
Bestatin	0.2 mM	104
Mercaptoethanol	1.0 mM	95

[a] 4-(2-Aminoethyl)-benzenesulfonyl fluoride.
[b] L-*trans*-Epoxy-succinylleucylamido-(4-guanidino)butane.

No effect was observed by the addition of Ca^{2+} and Mg^{2+} at 1–5 mM.

3.5. Other enzymatic properties

The optimal pH for the activity was found to be pH 7.0–7.5. No significant activity was observed below pH 6.0 or over 8.5. Heating the enzyme at 60°C for 5 min resulted in complete loss of the activity; however, no significant loss took place during storage at 4°C for 24 h or at −40°C for several weeks.

4. Discussion

We purified TPP hydrolyzing preferably Ala-Ala-Pro-pNA and Ala-Phe-Pro-pNA, but it exhibited no activity against N-terminally blocked tripeptide substrate, suc-Ala-Ala-Pro-pNA, indicating this enzyme is not an endopeptidase. Since the enzyme was inhibited, if not strongly, by diisopropylfluorophosphate, Pefabloc SC, and 3,4-dichlorocoumarin, it is classified as a serine enzyme.

Several reports have described proteolytic enzymes or peptidases of *P. intermedia*, a species quite close to *P. nigrescens*. The envelope-associated elastase was a serine enzyme with a molecular mass of 31 kDa and hydrolyzed elastin powder and synthetic substrates for elastase including glutaryl-Ala-Ala-Pro-Leu-pNA and metsuc-Ala-Ala-Pro-Val-pNA, but it did not hydrolyze casein, albumin, and collagen (type I) [23]. Using SDS–PAGE methods, Jansen et al. observed that IgG, IgA, and IgM were degraded by a 38-kDa neutral protease located on the surface of the cell envelope of *P. nigrescens* and *P. intermedia* [24]. Similar properties can be found between both elastase and immunoglobulin protease; however, the latter enzyme is not inhibited by specific serine enzyme inhibitors. Suido et al. reported that four strains of *P. intermedia*, which included one strain classified as *P. nigrescens* at the present time, exerted various activities of peptidase such as dipeptidyl peptidase (DPP) I, II, III, and IV which hydrolyzed Gly-Phe, Lys-Ala, Arg-Arg, and Gly-Pro, respectively. In addition, these strains were also found to produce a TPP hydrolyzing Gly-Pro-Leu [25]. Recently a prolyl DPP IV, which split Lys-Pro-pNA, Arg-Pro-pNA, Met-Pro-pNA, and Ala-Pro-pNA, but did not Gly-Pro-pNA, was isolated. The molecular mass of this serine enzyme was 74 kDa and its optimal pH for activity was 7.0–7.5. [26]. The fragments generated from proteins by these proteases are expected to be degraded more to di- or tripeptides, which can be utilized as nutrients by *Prevotella* species and other oral indigenous flora.

TPP and DPP of *P. gingivalis*, a *P. intermedia*-related species, have also been purified and characterized. Banbula et al. purified prolyl TPP from a cell extract of whole cells by Triton X-100 [27]. Its molecular mass was 82 kDa but another form, of 76 kDa derived from the 82-kDa enzyme through proteolytic modification, existed. This TPP was a serine enzyme, and it cleaved Ala-Phe-Pro-pNA and various synthetic peptides containing a proline residue in the third position from the amino-terminal end. They discussed how this enzyme may contribute to the pathogenesis of periodontal disease. DPP-7 of *P. gingivalis* was active against synthetic substrates with an aliphatic or aromatic residue in the second position from the amino-terminal end, such as Ala-Ala, Ala-Phe, Gly-Phe, Lys-Ile, and Phe-Leu [28]. A DPP similar to this enzyme, but with strict substrate specificity, was isolated [29]. It was active against only the substrates with alanine at the second position from the amino-terminal end. Among the tested synthetic substrates, only Lys-Ala, Ala-Ala, and Val-Ala were hydrolyzed by this 64-kDa serine enzyme. Arginine aminopeptidase and DPP IV activities [30] and arginine carboxypeptidase [31] of *P. gingivalis* were also found. These enzymes may also play a significant role co-operating with well known endogenous proteases (arginine gingipain, lysine gingipain) in the provision of nutrients, particularly energy, because this species is lacking in saccharolytic function. Furthermore, comparative studies of the histopathological effects of the wild-type strain and DPP IV-deficient mutant of *P. gingivalis* on abscess formation in the mouse revealed that this enzyme participates in virulence [32]. Thus, peptidases may play substantial roles in both nutritional and pathological respects.

References

[1] Bedi, G.S. and Williams, T. (1994) Purification and characterization of a collagen-degrading protease from *Porphyromonas gingivalis*. J. Biol. Chem. 269, 599–606.
[2] Fujimura, S., Shibata, Y. and Nakamura, T. (1992) Comparative studies of three proteases of *Porphyromonas gingivalis*. Oral Microbiol. Immunol. 7, 212–217.
[3] Hinode, D., Hayashi, H. and Nakamura, R. (1991) Purification and characterization of three types of proteases from culture supernatants of *Porphyromonas gingivalis*. Infect. Immun. 59, 3060–3068.
[4] Kadowaki, T., Yoneda, M., Okamoto, K., Maeda, K. and Yamamoto, K. (1994) Purification and characterization of a novel arginine-specific cysteine protease (argingipain) involved in the pathogenesis of periodontal disease from the culture supernatant of *Porphyromonas gingivalis*. J. Biol. Chem. 269, 21371–21378.
[5] Lewis, J.P., Dawson, J.A., Hannis, J.C., Muddiman, D. and Macrina, F.L. (1999) Hemoglobinase activity of the lysine gingipain protease (Kgp) of *Porphyromonas gingivalis* W83. J. Bacteriol. 181, 4905–4913.
[6] Pike, R., McGraw, W., Potempa, J. and Travis, J. (1994) Lysine- and arginine-specific proteases from *Porphyromonas gingivalis*. Isolation characterization, and evidence for the existence of complexes with hemagglutinins. J. Biol. Chem. 269, 406–411.
[7] Naganuma, K., Amano, S., Takeda, H., Kitano, S. and Hanazawa, S. (2000) Role of transcriptional factor activation protein-1 in endogenous expression of the interleukin-1 β gene involved in *Porphyromonas gingivalis* fimbriae-stimulated bone resorption in the mouse calvarial system. Oral Microbiol. Immunol. 15, 54–57.
[8] Fan, Q., Sims, T., Sojar, H., Genco, R. and Page, R.C. (2001) Fimbriae of *Porphyromonas gingivalis* induce opsonic antibodies that sig-

nificantly enhance phagocytosis and killing by human polymorphonuclear leukocytes. Oral Microbiol. Immunol. 16, 144–152.
[9] Bramanti, T.E., Wong, G.G., Weintraub, S.T. and Holt, S.C. (1989) Chemical characterization and biologic properties of lipopolysaccharide from *Bacteroides gingivalis* stain W50, W83 and ATCC 33277. Oral Microbiol. Immunol. 4, 183–192.
[10] Hamada, S., Okahashi, N., Fujiwara, T., Nishihara, T. and Koga, T. (1988) Selective induction of prostagrandin E production in C3H/HeJ mouse macrophages by lipopolysaccharides from *Bacteroides gingivalis*. Oral Microbiol. Immunol. 3, 196–198.
[11] Shenker, B.J. and Slots, J. (1989) Immunomoduratory effects of *Bacteroides* products in in vitro human lymphocyte functions. Oral Microbiol. Immunol. 4, 24–29.
[12] Yamazaki, K., Ikarashi, F., Aoyagi, T., Takahashi, K., Nakajima, T., Hara, K. and Seymoure, G.J. (1992) Direct and indirect effects of *Porphyromonas gingivalis* lipopolysaccharide on inkerleukin-6 production by human gingival fibroblasts. Oral Microbiol. Immunol. 7, 218–224.
[13] Bom-van Noorloos, A.A., van der Meer, J.W., van de Gevel, J.S., Schepens, E., van Steenbergen, T.J. and Burger, E.H. (1990) *Bacteroides gingivalis* stimulates bone resorption via interleukin-1 production by mononuclear cells. The relative role for *B. gingivalis* endotoxin. J. Clin. Periodontol. 17, 409–413.
[14] Gemmell, E. and Seymour, G.J. (1993) Interleukin 1, interleukin 6 and transforming growth factor-beta production by human gingival mononuclear cells following stimulation with *Porphyromonas gingivalis* and *Fusobacterium nucleatum*. J. Periodont. Res. 28, 122–129.
[15] Walsh, L.J., Stritzel, F., Yamazaki, K., Birds, P.S., Gemmell, E. and Seymour, G.J. (1989) Interleukin-1 and interleukin-1 inhibitor production by human adherent cells stimulated with periodontopathic bacteria. Arch. Oral Biol. 34, 679–683.
[16] Shah, N.H. and Gharbia, S.E. (1994) Biochemical and chemical studies on strains designated *Prevotella intermedia* and proposal of a new pigmented species, *Prevotella nigrescens* sp. nov. Int. J. Syst. Bacteriol. 42, 542–546.
[17] Haffajee, A.D. and Socransky, S.S. (1994) Microbial etiological agents of destructive periodontal diseases. Periodontology 2000 5, 78–111.
[18] Paquet, C. and Mouton, C. (1997) RAPD fingerprinting for the distinction of *Prevotella intermedia* sensu stricto from *Prevotella nigrescens*. Anaerobe 3, 271–278.
[19] Goncalves, R.B., Robitaille, M. and Mouton, C. (2000) Identical clonal types of *Porphyromonas gingivalis* or *Prevotella nigrescens* recovered from infected root canals and subgingival plaque. Oral Microbiol. Immunol. 14, 197–200.
[20] Sawyer, S.J., MacDonald, J.B. and Gibbons, R.J. (1962) Biochemical characteristics of *Bacteroides melaninogenicus*. A study of thirty-one strains. Arch. Oral Biol. 7, 685–691.
[21] Fujimura, S., Shibata, Y., Hirai, K. and Nakamura, T. (1996) Binding of hemoglobin to the envelope of *Porphyromonas gingivalis* and isolation of the hemoglobin-binding protein. Infect. Immun. 64, 2339–2342.
[22] Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.
[23] Shibata, Y., Fujimura, S. and Nakamura, T. (1993) Purification and partial characterization of an elastolytic serine protease of *Prevotella intermedia*. Appl. Environ. Microbiol. 59, 2107–2111.
[24] Jansen, H.-J., Grenier, D. and van der Hoeven, J.S. (1995) Characterization of immunoglobulin G-degrading protease of *Prevotella intermedia* and *Prevotella nigrescens*. Oral Microbiol. Immunol. 10, 138–145.
[25] Suido, H., Nakamura, M., Mashimo, P.A., Zambon, J.J. and Genco, R.J. (1986) Arylaminopeptidase activities of oral bacteria. J. Dent. Res. 65, 1335–1340.
[26] Shibata, Y., Miwa, Y., Hirai, K. and Fujimura, S. (2003) Purification and partial characterization of dipeptidyl peptidase from *Prevotella intermedia*. Oral Microbiol. Immunol. (in press).
[27] Banbula, A., Mak, P., Bugno, M., Silberring, J., Dubin, A., Nelson, D., Travis, J. and Potempa, J. (1999) Prolyl tripeptidyl peptidase from *Porphyromonas gingivalis*. A novel enzyme with possible pathological implications for the development of periodontitis. J. Biol. Chem. 274, 9246–9252.
[28] Banbula, A., Yen, J., Olesky, A., Mak, P., Bugno, M., Travis, J. and Potempa, J. (2001) *Porphyromonas gingivalis* DPP-7 represents a novel type of dipeptidylpeptidase. J. Biol. Chem. 276, 6299–6305.
[29] Fujimura, S., Hirai, K. and Shibata, Y. (2002) Dipeptidyl peptidase with strict substrate specificity of an anaerobic periodontopathogen *Porphyromonas gingivalis*. FEMS Microbiol. Lett. 209, 127–131.
[30] Grenier, D., Gauthier, P., Plamondon, P., Nakayama, K. and Mayrand, D. (2001) Studies on the aminopeptidase activities of *Porphyromonas gingivalis*. Oral Microbiol. Immunol. 16, 212–217.
[31] Masuda, T., Yoshioka, M., Hinode, D. and Nakamura, R. (2002) Purification and characterization of arginine carboxypeptidase produced by *Porphyromonas gingivalis*. Infect. Immun. 70, 1807–1815.
[32] Yagishita, H., Kumagai, Y., Konishi, K., Takahashi, Y., Aoba, T. and Yoshikawa, M. (2001) Histopathological studies of dipeptidyl aminopeptidase IV (DPPIV) of *Porphyromonas gingivalis* in a mouse abscess model: Use of a DPPIV-deficient mutant. Infect. Immun. 69, 7159–7161.