学位論文

Porphyromonas gingivalis のプロリルトリペプチジルペプチダーゼの産生,単離および性状

松尾和子

大学院歯学独立研究科　硬組織疾患病態解析学
（主指導教員：長谷川博雅）

松本歯科大学大学院歯学独立研究科博士（歯学）学位申請論文

Formation, isolation and characterization of a prolyl tripeptidyl peptidase of Porphyromonas gingivalis

Kazuko Matsuo

Department of Hard Tissue Research, Graduate School of Oral Medicine
(Chidf Academic Advisor：rofessor Hiromasa HASEGAWA)

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Matsumoto Dental University, for the degree Ph.D. in Dentistry

論文要旨

【目的】*Porphyromonas gingivalis* は、慢性歯周炎患者の歯周ポケットに高率に検出される偏性嫌気性グラム陰性菌で,本疾患の有力な原因菌とされており,プロテアーゼ活性が強い. *P. gingivalis* は糖および，遊離のアミノ酸を利用できないので，このプロテアーゼの作用によって生じたタンパク断片を，さらに小さなペプチドに加水分解し，窒素源やエネルギー源として利用すると考えられる.ペプチダーゼは,この過程に働く酵素であると思われるので,プロリル トリペプチジルペプチダーゼ（以下 PTP）について，その産生性，精製および性状について検討した.

【方法】*P. gingivalis* ATCC 33277 株の培養は，嫌気条件下で，ヘミンとメナジオンを加えたトリプチケース培地で行った. 3 日培養の菌体の超音波処理と 100,000g 遠心で，無細胞抽出液，エンベロープ，ベジクル，および培養上清を調製し，各画分の PTP 活性を H-Ala-Ala-Pro-pNA を基質として測定すると，ほとんどが無細胞抽出液に認められた. 3 日培養菌の無細胞抽出液を出発材料として，次の処方により PTP の精製を試みた. 始めに無細胞抽出液に硫安を 75%飽和に加え，5 時間攪拌後，生じた沈殿を 50 mM Tris-HCl buffer，pH 8.2 に溶解，透析し，同緩衝液に平衡化した Q-セファロースでクロマトグラフィーを行い，活性画分を，濃縮，透析後，セファクリル S-300 でゲル濾過を行った. この活性画分をフェニルセファロース CL-4B で疎水性クロマトグラフィーを行い，最終的に，等電点電気泳動を行ったところ，PTP 活性のピークは pH 5.7 の位置に認められた.

【結果】増殖と，PTP の産生を経日的に追跡すると，増殖は 2 日で，PTP 産生は 3 日で最大に達することが分かった. 精製標品は SDS-PAGE で単一のバンドを形成したので，PTP は純粋に精製され，その分子量は 45 kDa であることが判明した. 至適 pH は 7.0 から 9.0 までと広かったが，pH 6.5 以下，pH 9 以上では急速に活性が減少した. 熱安定性テストにおいて，50℃で加熱すると急速に失活し，5 分加熱で残存活性は 29%であり，60℃，5 分加熱では完全失活した. 基質特異性試験では H-Ala-Ala-Pro-pNA の他に H-Ala-Phe-Pro-pNA も分解し，その活性比は 100 : 83 であった. また，この両基質に対する Km 値はそれぞれ，0.16 mM，0.14 mM であり，Vmax/Km は 7.13 および 4.21 と算定された. この PTP はセリン酵素阻害剤により，活性が阻害されるが，システインプロテアーゼ阻害剤や金属キレーターによる阻害は認められなかった.

【考察】本研究で扱った *P. gingivalis* の PTP は，セリン酵素に分類されるプロリン特異的エキソペプチダーゼである. この酵素は菌の栄養素の摂取上有用と考えられ,その生存に寄与し,延いては病原因子の一つになる可能性がある.

Formation, isolation and characterization of a prolyl tripeptidyl peptidase of *Porphyromonas gingivalis*

KAZUKO MATSUO[1], AKIKO KISO[2], YUKINAGA SHIBATA[2], HIROMASA HASEGAWA[1,3] and SETSUO FUJIMURA[2]

[1]*Department of Hard Tissue Research, Graduate School of Oral Medicine, Matsumoto Dental University*

[2]*Department of Oral Microbiology, School of Dentistry, Matsumoto Dental University*

[3]*Department of Oral Pathology, School of Dentistry, Matsumoto Dental University*

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Abbreviations: dipeptidyl peptidase, DPP; tripeptidyl peptidase, TPP; prolyl tripeptidyl peptidase, PTP; *p*-nitroanilide, pNA; arginine gingipain, RGP; lysine gingipain, KGP; 50 mM Tris-HCl buffer (pH 8.2), Tris-HCl buffer

Summary

Formation, cellular locations, isolation and enzymatic properties of PTP of *Porphyromonas gingivalis*, an anaerobic periodontal pathogen were investigated.

Almost all activities of this enzyme were detected in the crude extract of the cell, but the other bacterial fractions such as culture fluids, envelopes and vesicles were not found to contain PTP.

PTP was purified from the crude extract prepared by sonication and centrifugation through five steps including concentration, ion exchange chromatography, gel filtration, hydrophobic interaction chromatography and isoelectric focusing to homogeneity.

The enzyme was a serine enzyme since it was inhibited strongly by Pefabloc SC, diisopropyl fluorophosphate and 3,4-dichloroisocoumarin. It hydrolyzed H-Ala-Ala-Pro-pNA and H-Ala-Phe-Pro-pNA. The molecular mass was determined as 45 kDa and isoelectric point was 5.7.

Optimum pH was moderately broad, and maximum activity was observed in the range of pH 7.0 to 9.0.

The residual activity after heating at 50℃ for 5 min was 29%, but heating at 60℃ resulted in complete loss of the activity.

Introduction

Gram-negative, black-pigmented obligatory anaerobes including *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Prevotella nigrescens* have been considered putative etiological agents of human periodontitis, of which *P. gingivalis* is the most potent pathogen of this disease, based on ecological properties in the oral cavity and etiological characteristics including production of proteolytic enzymes[1-6] and endotoxin[7-10]. In addition, DNAs of the periodontopathogenic bacteria were detected from oral samples of patients with Buerger disease (obstructive thromboangitis) and these patients had moderate to severe periodontitis[11]. These findings suggest that the periodontopathogens are implicated as causative agents of systemic disease as well as of oral disease.

As pathogenic factors of *P. gingivalis* (formerly *Bacteroides gingivalis*), proteinases (RGP and KGP), once designated "trypsin-like enzymes"[12] have been most intensively studied[13-17]. *P. gingivalis*, is an asaccharolytic organism, utilizes peptides but not free amino acids as its energy source[18-21]. Proteins surrounding the organisms are degraded by these proteinases to fragments which must be further hydrolyzed into smaller peptides by peptidases to provide adequate nutrients and energy sources. In this sense, peptidases of *P. gingivalis* are significant enzymes.

Previously we reported the isolation and characterization of DPP degraded H-Lys-Ala-pNA with weak activity against H-Ala-Ala-pNA and H-Val-Ala-pNA of *P. gingivalis*[22]. Suido et al. pointed out that *P. gingivalis* actively elaborated many kinds of

DPPs, but only poor TPPs were found[23]. However, PTP activity was confirmed in the soluble fraction of the cell of *P. gingivalis* ATCC 33277, thus we will describe formation, isolation and properties of PTP of this species in this report.

Materials and Methods

Bacterial strains and cultivation methods

Porphyromonas gingivalis ATCC 33277 was mainly used through the studies. *P. gingivalis* 381, W50 and W83 were also employed for additional studies. These strains were maintained on blood agar plates and grown in a liquid medium consisting of trypticase peptone (17 g/l), yeast extract (3 g/l), NaCl (5 g/l), K_2HPO_4 (2.5 g/l), hemin (5 mg/l) and menadione (0.5 mg/l)[24]. Cultivation was carried out anaerobically in a glove box filled with a mixture of gasses containing N_2:H_2:CO_2=85:10:5 at 37℃ for 3 days. Bacterial growth was monitored by optical density at 600 nm (OD_{600}).

Assays

Protein concentration was determined by absorbance at 280 nm, using bovine serum albumin as a standard protein (A_{280} of 1mg/ml bovine serum albumin solution = 0.624).

The substrates for DPP and PTP, *p*-nitroanilide derivatives of

amino acids and peptides, were obtained from Bachem, AG, Bubendorf, Switzerland, and Peptide Institute, Inc. Osaka, Japan. The peptidase activities were determined photometrically[25,26]. One unit of the enzyme activity was defined as the liberation of 1 µmol of *p*-nitroaniline per min as described earlier[14,16]. Reaction mixtures containing 50µl of enzyme source, 700µl of 1 mM substrates in 50 mM Tris-maleate buffer (pH 7.5) and 150µl of 50 mM Tris-maleate buffer (pH 7.5) were incubated at 37℃ for 30 min. To stop the reaction, 100µl of 7.5 M acetic acid was added, and released *p*-nitroaniline was assayed by absorbance at 410 nm. H-Ala-Ala-Pro-pNA was used routinely to assay PTP activity, if not otherwise stated.

RGP and KGP were determined using Bz-Arg-pNA and Tos-Gly-Pro-Lys-pNA, respectively[13,25].

Non-specific proteolytic activity was measured using azocoll as a substrate. The reaction mixtures containing 4 mg of the substrate, 100 µl of enzyme source and 900 µl of 50 mM Tris-maleate buffer (pH 7.5) were incubated at 37℃ for 30 min. After incubation, reaction mixtures were cooled immediately in an ice-water bath to stop the reactions, followed by centrifugation at 15,000 xg at 4℃ for 3 min. The absorbance of the supernatants at 520 nm (A_{520}) was measured, and the activity was defined as the increase of the A_{520} by 1.0 per min. Similarly, hydrolysis of remazol brilliant blue hide powder (RBB-hide powder) was evaluated by increase at A_{595}.

Preparation of bacterial fractions

All procedures described below were performed at 4℃.

The cells harvested from 2,000 ml culture by centrifugation at 10,000 xg for 15 min [11.7 g (wet weight)] were suspended in Tris-HCl buffer and subjected to sonication at 150 W for 15 min. After centrifugation of the sonicate at 6,000 xg for 15 min to remove cell debris and unbroken cells, the supernatant was centrifuged at 100,000 xg for 60 min. The supernatant and precipitate (2.0 g) were designated the crude extract and the envelope, respectively. Meanwhile ammonium sulfate was added to the supernatant of the whole culture at 40 % saturation of this drug to prepare the vesicle fraction, and stirred for 5 h followed by centrifugation at 10,000 xg for 20 min. The precipitate (220 mg) was suspended in Tris-HCl buffer and dialyzed against the same buffer and referred to as the vesicle[27].

The concentration of ammonium sulfate of the supernatant was brought to 75% saturation and stirred for 8 h. The mixture was centrifuged at 10,000 xg for 20 min and dissolved in Tris-HCl buffer and dialyzed against the same buffer, and designated concentrated culture supernatant.

Extraction of TPP from cells

Cells were suspended in Tris-HCl buffer at a concentration of 50 mg/ml. Several reagents were added separately to the aliquots of this suspension and the mixtures were shaken gently at room

temperature for 1 h followed by centrifugation at 30,000 xg for 30 min. The ratios of the activities in each supernatant to that of sonicate were compared.

Purification

To prepare the starting material of purification of PTP, ammonium sulfate was added to crude extract of strain ATCC 33277 at 75% saturation and stirred for 8 h, followed by correction of precipitate by centrifugation at 10,000 xg for 15 min and dialysis against Tris-HCl buffer as described above for the preparation of concentrated culture supernatant. The concentrated material was applied to a column of Q-Sepharose previously equilibrated with Tris-HCl buffer. After the column was rinsed thoroughly, proteins adsorbed to the column were developed with linear gradient of NaCl in Tris-HCl buffer from 0 to 500 mM (Fig. 1). PTP active fractions eluted with around 300 mM NaCl were combined, concentrated in vacuo and dialyzed against Tris-HCl buffer containing 150 mM NaCl. The active fractions were concentrated in vacuo, dialyzed against Tris-HCl buffer containing 150 mM NaCl, subjected to gel filtration on Sephacryl S-300 equilibrated with the same buffer saline. The active fractions from gel filtration procedure were collected and dialyzed against Tris-HCl buffer containing 700 mM ammonium sulfate and applied to hydrophobic interaction chromatography on a Phenyl Sepharose CL-4B column equilibrated with Tris-HCl buffer containing 700 mM ammonium

sulfate. When the column was eluted with a descending gradient of ammonium sulfate from 700 mM to 0 mM, PTP activity was detected in the fractions eluted with about 50 mM ammonium sulfate. The active fractions were dialyzed against 150 mM glycine solution and further purified by isoelectric focusing at 300 V for 24 h using ampholine generating a pH gradient from 3 to 10 in the column. In this procedure, PTP was found to be focused at pH 5.7 zone (Fig. 2). This fraction was dialyzed against Tris-HCl buffer and referred to as the purified PTP.

Sodium dodecyl sulfate polyacryl amide gel electrophopresis (SDS-PAGE)

SDS-PAGE was employed to monitor enzyme purification and determination of molecular mass[28]. Concentration of acrylamide was 12.5% and the gels were stained with Coomassie brilliant blue R-250. The marker proteins for the reference of molecular mass estimation were phosphorylase *b* (97 kDa), bovine albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa), which were purchased from GE Healthcare, Buckinghamshire, UK.

Effects of group specific reagents on PTP

After incubation of mixtures of various group specific reagents and purified samples at 37℃ for 15 min, the residual enzyme activities were compared with the control without reagent

treatment.

Optimum pH for the enzyme activity

For evaluation of the influence differences in pH values of the incubation mixtures of the enzyme activity, the following buffers were introduced into the reaction mixtures to a final concentration at 100 mM: acetate buffer (pH 4 to 6), Tris-maleate buffer (pH 6.5 to 7.5), Tris-HCl buffer (pH 8 to 9) and carbonate-bicarbonate buffer (pH 9.5 to 10).

Results

Time course of growth and PTP production

As illustrated in Fig. 3, daily progress of cell growth and PTP production were examined. Both growth and production continued vigorously till Day 2 of the cultivation. After that PTP production reached a plateau. However, growth ceased and tended toward decrease by lysis of cells.

Extraction of PTP from cells

From the evaluation of cellular locations of PTP, the enzyme was found to remain in the cytoplasm. Efforts were made to search for effective means of extraction of the enzyme using several reagents. The most adequate extraction was obtained by two detergents, 3-[3-cholamidopropyl]-dimethylammoniol]-propanesulfonate (CHAPS)

and Triton X-100. However, mechanical disruption by sonication resulted in the highest yield of PTP (Table 1).

Cellular locations of PTP

Nearly all PTP activity was detected in the crude extract prepared by sonication and centrifugation. While the base of PTP activity in the crude extract was 100, levels in the envelope, vesicle and culture fluid were only 4.1, 0.3 and 0.3, respectively.

Comparison of PTP production by 4 strains of P. gingivalis

PTP activities in the crude extracts prepared by sonication and centrifugation from 2.5 g of *P. gingivalis* strains of ATCC 33277, 381, W50 and W83 were compared. The extracts of all the strains were shown to elaborate this enzyme. The total units of PTP in each extract were 27.3 U, 10.8 U, 23.0 U and 15.7 U, respectively.

Degradation of p-nitroanilide derivatives of amino acids and peptides

Among the tested synthetic and dye-conjugated protein substrates for peptidase and proteinase, respectively, the crude extract degraded H-Ala-Ala-Pro-pNA, H-Ala-Phe-Pro-pNA, H-Gly-Pro-pNA, H-Arg-pNA, H-Lys-pNA, H-Lys-Ala-pNA, Bz-Arg-pNA, Bz-Lys-pNA, Tos-Gly-Pro-Lys-pNA, Azocoll and RBB-hide powder. However, H-Leu-pNA, H-Ala-Ala-Ala-pNA, Met-Ala-Ala-Pro-Val-pNA or Glt-Ala-Ala-Pro-Leu-pNA were not

hydrolyzed.

Purification and purity of PTP

PTP was purified from the crude extract of cells through six steps. The enzyme was purified 410 fold with a recovery of 1.3 %. The purification is summarized in Table 2. SDS-PAGE analysis of the purified sample by isoelectric focusing revealed a single stained band. The molecular mass was calculated as 45 kDa (Fig. 4).

Inhibition by various reagents of PTP

Effects of group specific reagents on PTP are summarized in Table 3. Significant inhibition of PTP was observed by serine enzyme inhibitors such as Pefabloc SC[2)], diisopropyl fluorophosphate and 3,4-dichloroisocoumarin. No effect was noticed by other chemicals.

Substrate specificity and kinetic constants

Among tested *p*-nitroanilide derivatives of amino acids and peptides, the purified enzyme only actively hydrolyzed Ala-Ala-Pro-pNA and Ala-Phe-Pro-pNA. Kinetic constants for two substrates are presented in Table 4. H-Ala-Ala-Ala-pNA, H-Ala-Ala-Phe-pNA, H-Gly-Phe-pNA, H-Lys-Ala-pNA, H-Gly-Pro-pNA, Bz-Arg-pNA, Tos-Gly-Pro-Lys-pNA, Suc-Ala-Ala-Pro-pNA and Suc-Ala-Ala-Ala-pNA were not degraded.

Neither azocoll nor remazol brilliant blue hide powder was hydrolyzed.

Thermostability of the enzyme

When the enzyme was heated at 50℃ for 5 min, the residual activity was 29% for the unheated sample. Treatment at 60℃ for 5 min resulted in complete inactivation.

Optimum pH

Optimum pH for the enzyme activity was seen from pH 7.0 to 9.0. However, the activity decreased rapidly in the ranges of lower than pH 6.5 and higher than 9.0. The similar results were observed using H-Ala-Phe-Pro-pNA as substrate in substitution for H-Ala-Ala-pNA.

Discussion

We isolated PTP from the crude extract of *P. gingivalis* by chromatography, gel filtration and isoelectric focusing. Among the purification steps, gel filtration on Sephacryl S-300 was rather effective, and the specific activity rose about 19 fold by this means. Since the enzyme was inhibited strongly by Pefabloc SC, diisopropyl fluorophosphate and 3,4-dichloroisocoumarin, PTP is a serine enzyme, as are other peptidases of this species[29-32].

As shown in PTP of *P. gingivalis*, bacterial peptidases are generally,

not sensitive to inhibition by metal chelators. Exceptionally, PTP of *S. anginosus*, a metalloenzyme, was inhibited by EDTA, EGTA and 1,10-phenanthroline[26].

PTP investigated in the present report is an exopeptidase since peptides with a blocked amino group are not hydrolyzed, and it requires proline residue in the P1 position.

PTP of *Prevotella nigrescens* which is a quite close species of *P. intermedia*[33] was isolated and characterized[34]. It also was a serine enzyme and degraded H-Ala-Ala-Pro-pNA and H-Ala-Phe-Pro-pNA like PTP of *P. gingivalis.* However, differences are apparent in the molecular weights and pH-activity relationship[34].

PTP of *P. gingivalis* was already purified from the cell extract using Triton X-100 and precisely characterized[32]. This enzyme had two molecular masses, 81.8 kDa and 75.8 kDa. The authors corroborated that the smaller molecule was possibly derived from the larger one by proteolytic truncation of 6 kDa amino-terminal peptide. However, we found only one molecule, and its molecular mass was estimated as 45 kDa by SDS-PAGE. Even though the reason for this discrepancy (in the molecular masses) is still obscure, possible explanations include the different methods of preparation of the starting material for purification: chemical extraction by detergent and mechanical extraction by sonication. Furthermore, different strains of the source of the starting material for purification may also be a likely reason for this discrepancy, since three strains of *P. gingivalis* (HG66, W50 and

ATCC 33277) were used in the cellular location tests of PTP, but it can not be specified which strain was used for the purification of PTP from the description of this report. In this report, optimum pH for the activity determined using H-Ala-Phe-Pro-pNA was found in the range of 6.0 to 8.0. This value nearly coincided with our results.

We confirmed also that PTP was extractable by detergent treatment including Triton X-100 (Table 1) but recovery rate appeared to be significantly lower than with treatment by sonication.

PTP degrades the fragments of proteins occurred by the endogeneous proteinases and generates the smaller peptides in collaboration with other peptidases. These peptides may provide energy source for *P. gingivalis* which is asaccharolytic and incapable of transporting free amino acids[35].

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Figure legends

Fig. 1: Q-Sepharose chromatography.

Symbols: ●—●; PTP activity, ---; A_{280}, —; NaCl.

Fig. 2: Isoelectric focusing.

Symbols: ●—●; PTP activity, ▲—▲; pH, ---; A_{280}.

Fig. 3: Time course of production of PTP and growth.

Symbols: ●—●; PTP activity, ○—○; growth.

Fig. 4: SDS-PAGE of purified PTP.

Lane A, marker proteins; Lane B, purified PTP.

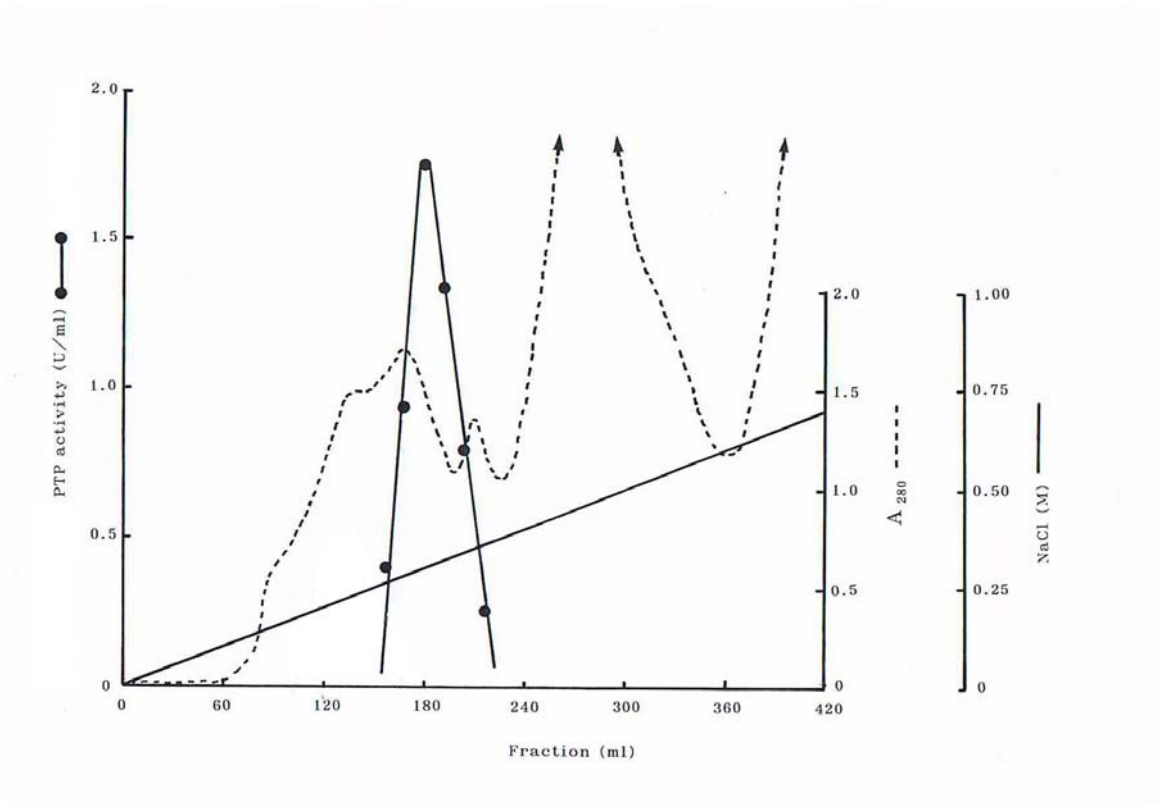
PTP activity (U/ml)
2.0
1.5
1.0
0.5
0
0
60
120
180
240
300
360
420
Fraction (ml)
A 280
NaCl (M)
1.00
0.75
0.50
0.25

Fig.1

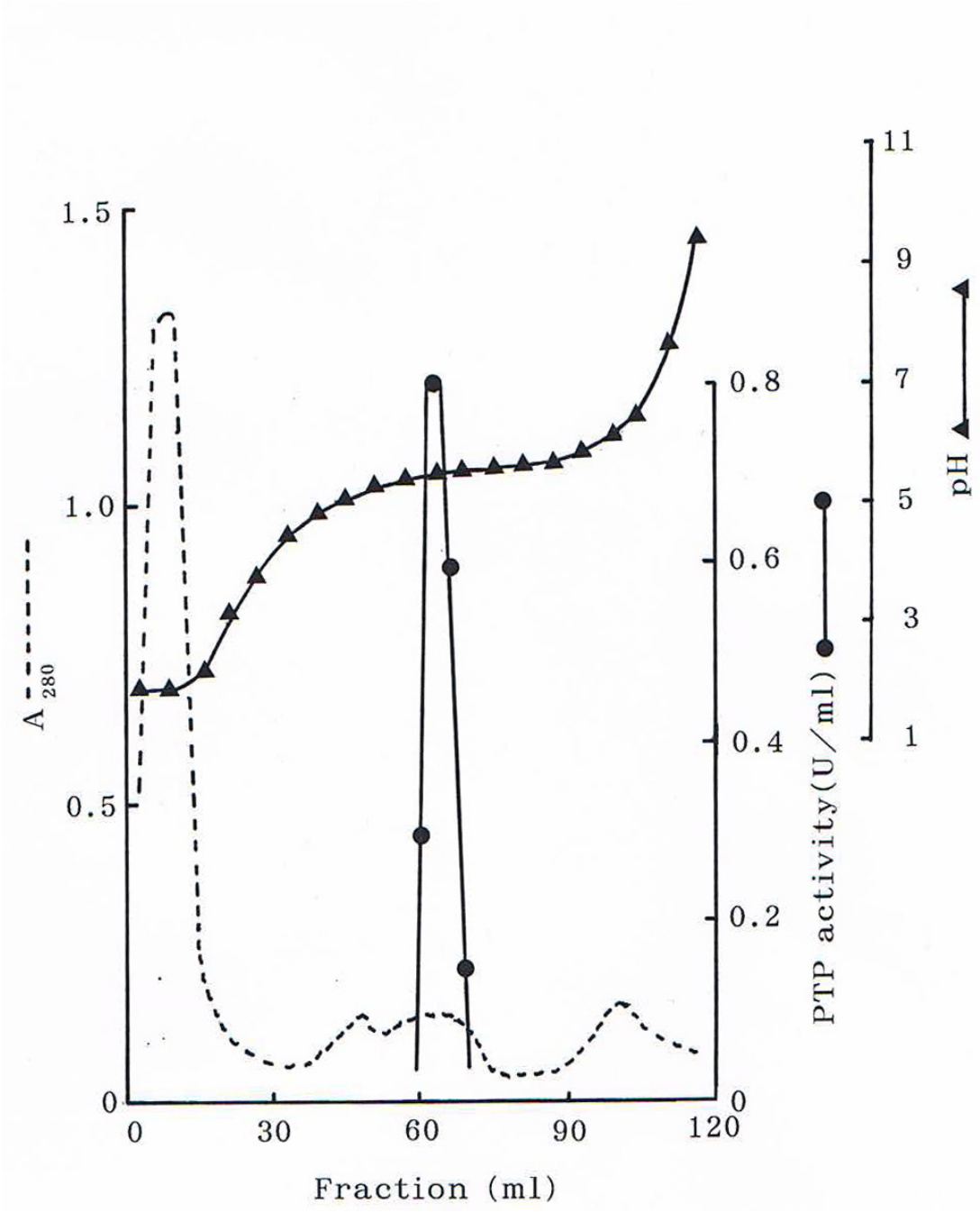
1.5
1.0
0.5
0
A_{280}
0.8
0.6
0.4
0.2
0
PTP activity(U/ml)
11
9
7
5
3
1
pH
0
30
60
90
120
Fraction (ml)

Fig. 2

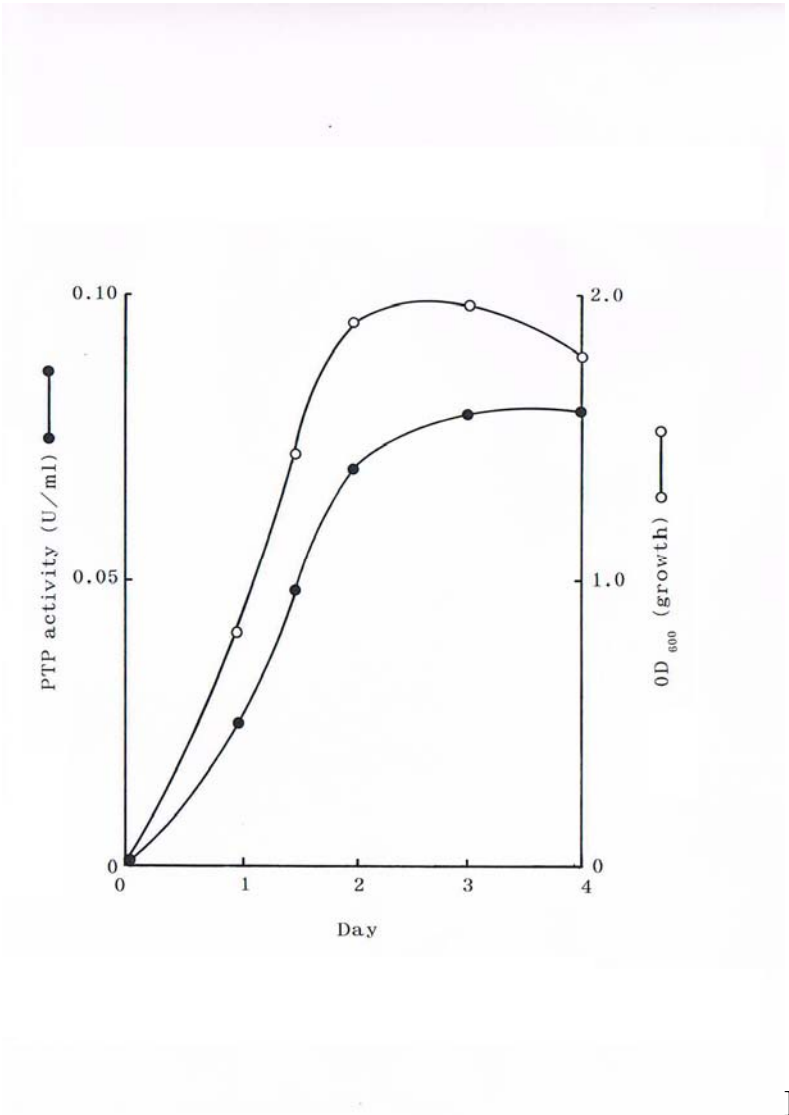

Fig. 3

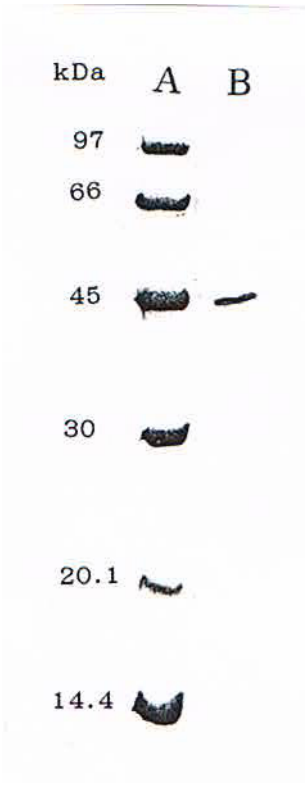

Fig. 4

Table 1: Extraction of PTP from cells

Treatment	PTP recovery (%)
Tris-HCl buffer	3.1
NaCl (150 mM)	10.8
EDTA (10 mM)	5.5
Mercaptoethanol (15 mM)	5.0
CHAPS (0.5 %)	42.9
Triton X-100 (0.5 %)	37.7
Sonication	100.0

Table 2：Purification of PTP

Step	protein (mg)	total activity (U)	sp. act*. (U/mg)	purification (fold)	recovery (%)
Crude extract	184.8	19,888	0.009	1.0	100.0
Ammonium sulfate	120.1	8,928	0.013	1.4	65.0
Q-Sepharose	33.8	609	0.056	6.2	18.3
Sephacryl S-300	13.1	12.5	1.050	117.0	7.1
Phenyl Sepharose	8.8	3.9	2.256	251.0	4.7
Isoelectric focusing	2.4	0.65	3.090	410.0	1.3

*:specific activity (U/mg protein)

Table 3: Effects of group specific reagents on PTP.

Reagent	Concentration	Activity (%)
Control	—	100
Leupeptin	2 mM	100
Antipain	10 mM	100
Bestatin	2 mM	106
E64[1)]	2 mM	114
Pefabloc SC[2)]	2 mM	5
Diisopropyl fluorophosphate	10 mM	13
3,4-Dichloroisocoumarin	2 mM	6
EDTA	10 mM	97
1,10-Phenanthroline	2 mM	93
Mercaptoethanol	2 mM	98
SDS	0.5%	83

1):L-*trans*-epoxy-succinyl leucylamido-(4-guanidino)butane

2):4-(2-aminoethyl)-benzenesulfonyl-fluoride hydrochloride

Table 4: Kinetic constants

Substrate	Activity (%)	Km (mM)	Vmax (U/mg/min)	Vmax/Km
H-Ala-Ala-Pro-pNA	100	0.16	1.14	7.13
H-Ala-Phe-Pro-pNA	83	0.14	0.59	4.21

抄録：*Porphyromonas gingivalis*のプロリルトリペプチジルペプチダーゼの産生，単離および性状

松尾和子[1]，木曽有紀子[2]，柴田幸永[2]，長谷川博雅[1]，藤村節夫[2]

[1](松本歯大・硬組織疾患病態解析学)

[2](松本歯大．口腔細菌)

偏性嫌気性歯周病原菌 *Porphyromonas gingivalis*のプロリルトリペプチジルペプチダーゼ（PTP）の産生，単離と酵素性状について検討した．

PTP 活性のほとんどは，細胞の粗抽出液に検出され，培養上清，エンベロープやベジクルには極僅かしか分布していなかった．菌体の超音波処理と 100,000 xg 遠心で得た粗抽出液を出発材料にして，PTP を硫酸アンモニウムによる塩析濃縮，イオン交換クロマトグラフィー，ゲル濾過，疎水性クロマトグラフィー，等電点電気泳動にて精製したところ，PTP は Pefabloc Sc, diisopropyl fluorophosphate, 3,4-dichloroisocoumarin で強く阻害されたので，セリン酵素に分類される．

分子量は 45 kDa で等電点は 5.7 であった．精製標品は H-Ala-Ala-Pro-pNA と H-Ala-Phe-Pro-pNA に活性を示し，それぞれの Vmax (U/mg/min) / Km (mM)は 7.13 および 4.21 であった．反応の至適 pH は 7.0～9.0 に見られた． 熱安定性試験では，50℃，5 分，加熱後の残存活性は 29%であったが，60℃，5 分では，完全失活することが分かった．

本研究で扱った *P. gingivalis* の PTP は，セリン酵素に分類されるプロリン特異的エキソペプチダーゼである．この酵素は，菌の栄養素の摂取上有用と考えられ，その生存に寄与し，延いては，病原性の一因子となる可能性がある．