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Production and enzymatic properties of a prolyl tripeptidyl peptidase of *Streptococcus anginosus*

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Summary

Streptococcus anginosus is considered to be implicated in the etiology of oral infectious diseases as well as abscess formation in various body sites. We investigated the production and the enzymatic properties of PTP of *S. anginosus* NCTC 10713. This enzyme was found only in cell extract and active on tripeptide substrates containing proline residue at P1 position, particularly H–Ala–Ala–Pro–*p*–nitroanilide.

The enzyme was produced by all 8 species of tested streptococci, indicating occurrence of this enzyme is rather ubiquitous within streptococci.

This PTP was purified to homogeneity from the cell extract by the procedures including ammonium sulfate precipitation, chromatography, gel filtration and electrophoresis. The enzyme was inhibited by serine enzyme inhibitors and chelating reagents, indicating this PTP is a serine metalloenzyme with a molecular mass of 66 kDa. The enzyme was active against H–Ala–Ala–Pro–*p*–nitroanilide and H–Ala–Phe–Pro–*p*–nitroanilide in neutral pH solutions. The activity was completely lost by heating at 50°C for 10 min.

Introduction

Bacteria of the anginosus group of streptococci, formerly classified as *Streptococcus milleri*, are human oral indigenous facultative anaerobes. *S. anginosus* is known to possess the streptococcal group specific antigen F[1)]. This species is non–hemolytic but a potent causative agent of opportunistic infections in the oral cavity or in the female genital organs[2,3)], and also implicated in infective endocarditis[4–6)]. Jacobs et al. reported that *S. anginosus* was the most frequently isolated species within a collection of 518 *S. milleri* group isolates containing *S. anginosus*, *S. constellatus* and *S. intermedius*[7)]. Recently, an etiological relationship between this organism and esophageal and gastric

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Abbreviations : dipeptidyl peptidase, DPP ; prolyl tripeptidyl peptidase, PTP ; *p*–nitroanilide, pNA

cancer was noticed, based on the observations that *S. anginosus* DNA sequences were found in DNA samples from esophageal cancer and gastric cancer tissues, but no *S. anginosus* DNAs were detected from the none–cancerous portions of the corresponding body sites[8]. Hydrolytic enzymes such as deoxyribonuclease and chondroitin sulfatase have been regarded as the possible virulence factors of bacteria[9]. Peptidases of oral bacteria have been investigated mainly in strict anaerobic, Gram negative *Porphyromonas gingivalis* and its related species, which are considered to be the most important causative agents of periodontitis[10–15]. However, information about these enzymes of oral streptococci (viridans streptococci) is quite limited at the present time. We reported earlier production of many kinds of aminopeptidases in *S. anginosus*, and attempts were made to evaluate the isolation and properties of DPP IV[16]. In this report we describe the production, isolation and characterization of prolyl PTP and discuss its potential significance.

Materials and Methods

Bacterial strains and cultivation

Streptococcus anginosus NCTC 10713 was used mainly to assess PTP production and to provide the starting material for purification of PTP. Several other streptococcal species, including *S. gordonii* Challis, *S. oralis* (formerly *S. mitis*) ATCC 9811, *S. salivarius* JCM 5707, *S. pyogenes* JCM 5674, *S. sanguinis* JCM 5708, *S. mutans* Ingbritt and *S. sobrinus* 6715, also were employed for the test of PTP production. The medium for the routine cultivation was brain heart infusion fortified with yeast extract (0.3%), trypticase peptone, trypticase soy broth and nutrient broth also were used. All these media were purchased from Becton, Dickinson and Co. (Sparks, MD, USA). Anaerobic culture was performed in an anaerobic box filled with a mixture of gases containing $N_2 : H_2 : CO_2 = 85 : 10 : 5$ at 37°C, while aerobic culture was carried out under stationary conditions in atmosphere at 37 °C.

Preparation of crude extract

Cells harvested by centrifugation at 8,000 x g for 15 min were washed by centrifugation twice with 50 mM Tris–HCl buffer (pH 8.2) and suspended in the same buffer. Then the cells were disrupted by ultrasonic treatment at 150 W for 20 min, followed by centrifugation at 100,000 x g for 60 min. The centrifugal supernatant solution was designated crude extract.

Comparison of media in PTP production and growth

The cells were inoculated to several media (100 ml) fortified with 0.3% yeast extract and cultured anaerobicaly for 2 days. PTP activities in each whole culture were measured, whereas cells were collected by centrifugation and their weights were assayed.

Assays

Bacterial growth was monitored by optical density at 600 nm and protein concentration was determined by absorbance at 280 nm using bovine albumin as a standard.

Synthetic substrates, which are dipeptides and tripeptide conjugated with *p*–nitroaniline through amide bonds, were used as substrates for DPP and PTP, respectively. The substrates for DPP and PTP were obtained from Bachem, AG, Bubendorf, Switzerland. The other substrates were products of Peptide Institute, Inc. Osaka, Japan. The peptidase activities were determined photometri-

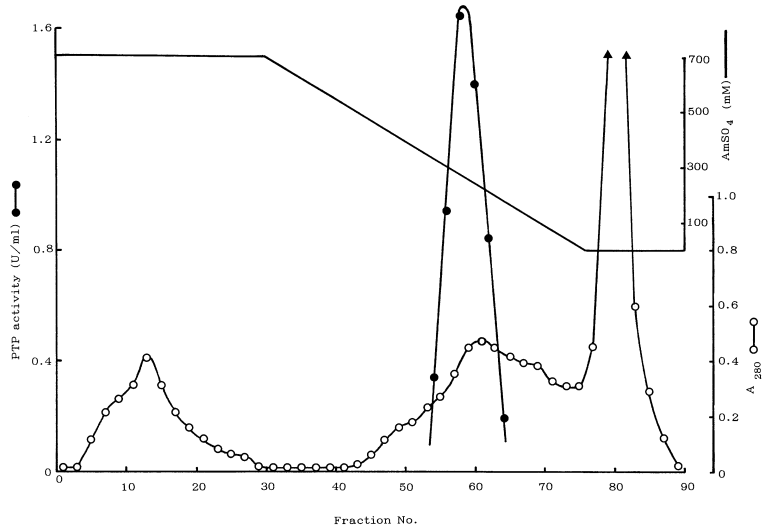
cally[17, 18]. One unit of the enzyme activity was defined as the liberation of 1 μmol of *p*−nitroaniline per min as briefly described earlier[14, 16]. Reaction mixtures contained 50 μl of enzyme source, 700 μl of 1 mM substrates in 50 mM Tris−maleate buffer (pH 7.0) and 150 μl of 50 mM Tris−maleate buffer (pH 7.0) were incubated at 37°C for 30 min. To stop the reaction, 100 μl 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.

Isolation of PTP

All procedures were conducted at 4°C, if not otherwise specified, and used buffer was 50 mM Tris−HCl buffer (pH 8.2), abbreviated to only Tris−HCl buffer.

Ammonium sulfate was added to the crude extract (150 ml) prepared from 20 g (wet weight) cells at a concentration of 75% saturation of this reagent and stirred for 5 h, followed by centrifugation at 10,000 x g for 15 min. The precipitate was dissolved in Tris−HCl buffer and dialyzed against the same buffer. The dialyzed sample was applied to a Q−Sepharose column (2.4 × 15 cm), which had been equilibrated with Tris−HCl buffer. Afterwards, the column was washed with the same buffer until the absorbance of effluent from the column at 280 nm reached below 0.05. Then the column was eluted by a linear gradient of NaCl concentration from 0 mM to 700 mM which was generated by mixing 220 ml of Tris−HCl buffer containing 700 mM NaCl into an equal volume of Tris−HCl buffer. PTP was eluted at around 400 mM NaCl. The active fractions were pooled and dialyzed against Tris−HCl buffer, followed by addition of ammonium sulfate to 700 mM. This material was subjected to hydrophobic interaction chromatography on Phenyl Sepharose CL−4B column (0.9 × 15 cm), equilibrated with Tris−HCl buffer containing 700 mM ammonium sulfate. After the column was washed thoroughly with this buffer, the column was eluted by a descending linear gradient of ammonium sulfate from 700 mM to 0 mM (50 ml each side) (Fig. 1). PTP active fractions eluted at about 270 mM ammonium sulfate were combined, concentrated in vacuo and dialyzed against Tris−HCl buffer containing 200 mM NaCl and subjected to gel filtration on a Sephacryl S−300 column (2.6 × 95 cm) equilibrated with the dialysis buffer. The elution volume of PTP was confirmed to be

Fig. 1 : Hydrophobic interaction chromatography of PTP on Phenyl Sepharose CL−4B.



Symbols : ○———○ ; A_{280}, ●———● ; PTP activity against H−Ala−Ala−Pro−pNA, ——— ; concentration of ammonium sulfate ($AmSO_4$).

the same as that of bovine albumin in a separate run on the same column. The active fractions were dialyzed against 1% glycine solution exhaustively and the dialyzed sample was applied to an isoelectric focusing column (120 ml capacity). A mixture of Ampholine pH 3.5–5.0 and 4.0–6.5 (GE Healthcare AB, Uppsala, Sweden) was used to generate pH gradient during electrophoresis. The electrophoresis was carried out according to the methods of Vesterberg et al.[19] under a constant 600 V for 24 h, cooling the column with tap water (about 8°C).

The electrophoresis profiles obtained upon isoelectric focusing exhibited two major and one minor protein peaks. PTP activity was found at one of the major peaks with a pI of 4.9.

SDS–PAGE

SDS–PAGE was employed to monitor enzyme purification and determination of molecular mass[20]. Concentration of acrylamide was 12.5% and the gels were stained with Coomassie brilliant blue R–250. The marker proteins (GE Healthcare, Buckinghamshire, UK) for the reference of molecular mass estimation were phosphorylase *b* (97.0 kDa), bovine albumin (66.0 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (30.0 kDa), soybean trypsin inhibitor (20.1 kDa) and α–lactalbumin (14.4 kDa).

Estimation of the optimum pH for PTP activity

The following buffers were introduced into the reaction mixtures to a final concentration at 100 mM and influence of differences in pH values of the incubation mixtures on the enzyme activity was evaluated ; 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

Cellular locations of PTP

PTP activity was detected only in the crude extract, but not in the culture supernatant or in the cell surface materials (100,000 x g precipitate of the sonicate). It was possible to find the activity in suspension of intact cells and whole cultures.

Comparison of cell growth and PTP production in the several culture media

The ratios PTP production to cell yield [PTP (units) / wet weight cell (g)] in the following media ; brain heart infusion, trypticase peptone, trypticase soy broth and nutrient broth added yeast extract were 4.3, 0.3, 1.6 and 0.9, respectively. Since the ratio of PTP production to cell yield was obtained by brain heart infusion, this medium was employed to prepare PTP samples in the future.

Time course of PTP production, growth (OD_{600}) and changes of pH of the medium are summarized in Table 1. PTP production and growth reached maximum levels at Day 2. Significant lowering of pH of the medium may be due to accumulation of acid compounds generated from the ingredient glucose in brain heart infusion.

Assessment of PTP production by different species of streptococci and effect of anaerobic culture

The peptidase hydrolyzed H–Ala–Ala–Pro–pNA was found in the whole cultures in all the tested strains of streptococci as well as peptidase for H–Ala–Phe–Pro–pNA (Table 2), indicating PTP is

Table 1 : Time course of PTP production, growth (OD_{600}) and pH change of the medium.

	Day 0	Day 1	Day 2	Day 3
PTP(U)	0	3.9	5.5	5.6
OD_{600}	0	1.79	1.98	2.11
pH	7.2	5.7	5.7	5.8

medium ; brain heart infusion plus yeast extract

Subculture of *S. anginosus* NCTC 10713 (100 μl) was inoculated to medium (100 ml) and cultured. Aliquots of culture were taken every day. PTP activity, OD_{600} and pH were determined using whole culture immediately after sampling.

Table 2 : Production of PTP by streptococci.

	PTP production (%)	
Strain	H–Ala–Ala–Pro–pNA	H–Ala–Phe–Pro–pNA
S. anginosus NCTC 10713	100	100
S. gordonii Challis	89	81
S. oralis ATCC 9811[1)]	83	88
S. salivarius JCM 5707	64	81
S. pyogenes JCM 5674	44	54
S. sanguinis JCM 5708	44	50
S. mutans Ingbritt	42	69
S. sobrinus 6715	42	58

[1)] formerly *S. mitis* ATCC 9811

Table 3 : Purification of PTP of *S. anginosus* NCTC 10713.

Step	protein (mg)	total activity (U)	sp. act[1)]. (U/mg)	purification (fold)	yield (%)
Crude extract	9,593	198.2	0.021	1	100
Ammonium sulfate	6,012	163.4	0.027	1.3	82.4
Q–Sepharose	408.9	98.5	0.241	11.5	49.7
Phenyl Sepharose CL–4B	52.5	43.4	0.827	39.4	21.9
Sephacryl S–300	7.4	16.9	2.284	108.8	8.5
Isoelectric focusing	0.7	4.8	6.857	326.5	2.4

1) : specific activity

produced by a wide range of streptococcal species. The relative enzyme activities of the tested strains for H–Ala–Ala–Pro–pNA and H–Ala–Phe–Pro–pNA were found to be around 3 : 1.

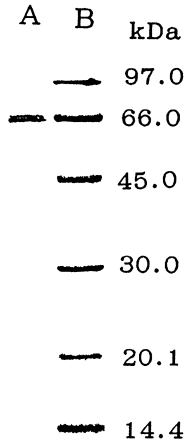
Efforts were made to examine the effects of anaerobic and aerobic culture on cell growth and PTP production in *S. anginosus* NCTC 10713. The same level of growth was obtained in anaerobic and aerobic culture. But approximately 4 times the amount of PTP was produced in the anaerobic culture than under aerobic condition (data are not shown).

Purification, purity and molecular mass of PTP

Purification of PTP is summarized in Table 3. The enzyme was purified 326.5 fold with a recovery of 2.4%.

SDS–PAGE of this fraction showed a single stained band as the same mobility with bovine albumin (Fig. 2). The molecular mass of PTP was determined as 66 kDa, judging from these observations.

Fig. 2 : SDS–PAGE of the purified PTP.



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

Table 4 : Effects of group specific reagents and metal ions on PTP.

Reagent	Concentration	Activity (%)
Control	–	100
Leupeptin	0.2 mM	77
Antipain	0.2 mM	41
	1.0 mM	16
E64[1)]	0.2 mM	97
Bestatin	0.2 mM	102
Pefabloc SC[2)]	1.0 mM	86
	10.0 mM	6
Diisopropyl fluorophosphate	1.0 mM	94
	10.0 mM	10
3,4–Dichloroisocoumarin	1.0 mM	86
	10.0 mM	0
EDTA	1.0 mM	41
	10.0 mM	20
EGTA	1.0 mM	49
	10.0 mM	13
1,10–Phenanthroline	1.0 mM	3
Ca^{2+}	5.0 mM	106
Mg^{2+}	5.0 mM	98
Zn^{2+}	5.0 mM	9
Mn^{2+}	5.0 mM	56
SDS	1.0 %	9
N–ethylmaleimide	5.0 mM	100
Mercaptoethanol	5.0 mM	98
Dithiothreitol	5.0 mM	103

1) : L–*trans*–epoxy–succcinylleucylamido–(4–guanidino)butane
2) : 4–(2–aminoethyl)–benzenesulfonyl–fluoride

Effects of group specific reagents and metal ions on PTP

Effects of various reagents on PTP are summarized in Table 4. Marked inhibition was found by treatment with specific serine enzyme inhibitors such as Pefabloc SC, diisopropyl fluorophosphate and 3,4–dichloroisocoumarin. Inhibition by metal ion chelators including EDTA, EGTA and 1, 10–phenanthroline was also apparent. Antipain, a papain inhibitor, abrogated significantly the PTP activity.

Table 5: Kinetic constants.

Substrate	Km (mM)	Vmax (U/mg/min)	Vmax/Km	Relative activity (%)
H–Ala–Ala–Pro–pNA	0.20	0.56	2.85	100
H–Ala–Phe–Pro–pNA	0.50	0.33	0.66	28
H–Ala–Ala–Ala–pNA	N.D.[1)]	N.D.	N.D.	0
H–Ala–Ala–Phe–pNA	N.D.	N.D.	N.D.	0
H–Gly–Phe–pNA	N.D.	N.D.	N.D.	0
H–Lys–Ala–pNA	N.D.	N.D.	N.D.	0
H–Gly–Pro–pNA	N.D.	N.D.	N.D.	0
Bz–Arg–pNA	N.D.	N.D.	N.D.	0
Suc–Ala–Ala–Pro–pNA	N.D.	N.D.	N.D.	0
Suc–Ala–Ala–Ala–pNA	N.D.	N.D.	N.D.	0
Tos–Gly–Pro–Lys–pNA	N.D.	N.D.	N.D.	0

1) : not determined

Substrate specificity

The purified PTP was actively hydrolyzed H–Ala–Ala–Pro–pNA. Kinetic constants of PTP for H–Ala–Ala–Pro–pNA and H–Ala–Phe–Pro–pNA are shown in Table 5. The substrates without proline residue at the P1 position, DPP substrates and the N–terminus blocked substrates were not found to be hydrolyzed.

Other properties

The activity was maintained at 0°C at least for 2 days ; however, it was completely inactivated by heating at 50°C for 10 min.

Optimum pH for the activity was found between 6.5 to 7.5 (maximum activity was observed in a pH 7.0 buffer). No activity was shown in acidic buffers below pH 5.0 or alkaline buffer solutions over pH 9.0.

Discussion

PTP of *S. anginosus* discussed in this report was detected in cellular soluble fraction and was a proline specific enzyme. It was inhibited by serine enzyme inhibitors and chelators.

Few observations concerning screening test of proteolytic activities in viridans streptococci are available. Cowman and Baron demonstrated proteolytic activity in one strain of *Streptococcus sanguis* (presently, *Streptococcus sanguinis*) using casein and azodye–conjugated casein as substrates[21)]. However, in the evaluation of proteinase and peptidase activities of many kinds of oral bacteria using synthetic substrates for trypsin, chymotrypsin and elastase, proteolytic activities were not detected in *Streptococcus mitis*[22)]. Our investigation course to search for proteolytic enzymes as a potent pathogenic factor in *S. anginosus* was also unsuccessful (data are not shown). Therefore, whether the peptidase dealt in this study functions biologically in vivo is still uncertain, because supplies of the peptide substrates for the enzyme are not expected, if really proteases are not produced by this organism. However, the provision of peptide substrates presumably is possible by proteolytic enzymes given by commensal bacteria. In fact, when investigated using two kinds of proteinases (arginine specific gingipain and lysine specific gingipain) of *P. gingivalis*, an oral indigenous species are known to exert strong proteolytic activities[23–26)].

PTP described in this report, which was isolated from the cell extract of *S. anginosus* NCTC

10713, was also found in other tested species of streptococci, indicating this enzyme commonly is produced by bacteria of this genus. Very little information about PTP of streptococci is available, but the presence of DPP hydrolyzes Xaa–Pro peptide was confirmed in *S. mitis*, *S. salivarius*, *S. sanguinis*[27], *S. gordonii*[28] and *S. anginosus*[16].

PTP production by *S. anginosus* was confirmed by all the tested streptococci including group A and viridans group (Table 2), indicating this enzyme is formed widely among streptococci.

PTP of *P. gingivalis* was isolated from the detergent extract of cells. It hydrolyzed H–Ala–Phe–Pro–pNA (no description about the activity against H–Ala–Ala–Pro–pNA) and was inhibited by serine enzyme inhibitors but not by metal chelators. Activity appeared to have a broad pH optimum from pH 6.0 to 8.0[10]. Both PTP of *P. gingivalis* and *S. anginosus* are serine enzymes. Even though PTP is undoubtedly a distinct enzyme from DPP, interestingly, PTP and DPP IV of *S. anginosus*[16] are classified as serine enzymes as well as DPP–7[11] and Xaa–Ala specific DPP of *P. gingivalis*[14]. Since this PTP was inhibited by chelators, the enzyme is a serine metalloenzyme.

Several reports have suggested that peptidases function as virulent factors ; DPP IV positive wild type of *P. gingivalis* caused abscess formation and it led to frequent death by extent in mice. However, DPP IV deficient mutant exhibited faster recovery from infection[13, 29]. In addition, eukaryotic DPP IV has been considered to be involved in pathogenicity based on its T cell activation and interaction with tissue proteins such as collagen and fibronectin[30]. These findings indicate that peptidases are significant proteins as pathogenic factors.

Since *S. anginosus* was observed earlier to hydrolyze also H–Ala–Ala–Ala–pNA[16], it is worthwhile to undertake an examination of the properties of this enzyme for better understanding of the peptidase system of this organism.

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抄録：*Streptococcus anginosus* のプロリルトリペプチジルペプチダーゼの産生と酵素性状

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[3]（松本歯大・口腔細菌）

Streptococcus anginosus NCTC 10713株のプロリルトリペプチジルペプチダーゼの産生と酵素性状を調べ，次の結果を得た．

この酵素は菌体の無細胞抽出液中にのみ認められ，プロリン残基をP1位にもつトリペプチド，特にH–Ala–Ala–Pro–*p*–nitroanilideに活性を示した．菌の増殖は好気性培養でも嫌気性培養でも差はなかったが酵素産生は嫌気性培養の方が良好であり，8種のレンサ球菌属にその産生が認められたので，本菌属では広く産生されていると思われる．無細胞抽出液から硫酸アンモニウム沈殿，イオン交換クロマトグラフィー，疎水性クロマトグラフィー，ゲル濾過，等電点電気泳動で酵素を分離精製したところ，等電点はpH 4.9，分子量は66 kDaであった．活性はセリン酵素阻害剤とキレート剤による阻害が顕著で，セリンメタロ酵素であると考えられる．反応至適pHは中性域にあり，活性は50℃，10分の加熱で完全に失われた．