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DIPEPTIDYL PEPTIDASE IV OF *STREPTOCOCCUS ANGINOSUS*: PURIFICATION AND CHARACTERIZATION

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Abstract: We found that N-unblocked nine *p*-nitroanilde derivatives of amino acids or peptides were hydrolyzed by the crude cell extracts of *Streptococcus anginosus* NCTC 10713. Then dipeptidyl peptidase IV was purified 323-fold by the procedures including ammonium sulfate concentration, anion exchange chromatography (twice), gel filtration (twice), hydrophobic interaction chromatography, and isoelectric focusing. The molecular weight was calculated as 84 kDa, and the isoelectric point was 4.9. The enzyme hydrolyzed mainly dipeptides containing proline residues at P1 position. It was strongly inhibited by serine enzyme inhibitors.

General protease inhibitors, metal chelators, thiol alkylating agent, reducing agent, and several metal ions had no effect on the enzyme activity. Optimum pH for the activity was found at 7.0. The enzyme was mostly inactivated by heating at 50 °C for 15 min.

*Key words: Streptococcus anginosu*s; peptidase; DPP IV; enzyme;

Abbreviations: dipeptidyl peptidase, DPP; *p*-nitroanilide, pNA

INTRODUCTION

Streptococcus anginosus, belonging to group of *Streptococcus milleri*, is an anaerobic oral indigenous species which is commonly identified in endodontic infections [1] and it may contribute to abscess formation in various body sites, including the oral cavity [2]. This organism also is noticed as a causative agent of infective endocarditis [3]. However, the pathogenic mechanism of this species is still unclear at the present time. Jacobs and Stobberingh tried to assess the relationship between clinical significance and production of hydrolytic enzymes such as ribonuclease, hyaluronidase, deoxyribonuclease, and chondroitin sulfatase produced by the 'anginosus' group of streptococci (*S. anginosus*, *S. constellatus*, and *S. intermedius*), but the comprehensible relation could not found concerning pathogenicity of *S. anginosus* and production of those enzymes [4]. In this study we examined formation, isolation, and characterization of peptidase (dipeptidyl peptidase IV), which is a proteinase-related enzyme of *S. anginosus* NCTC 10713.

MATERIALS AND METHODS

BACTERIAL STRAIN AND CULTIVATION METHODS

S. anginosus NCTC 10713 was inoculated into a medium of brain heart infusion (3.7%) supplemented with yeast extract (0.3%), 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 2 days.

PURIFICATION OF DPP

All steps of purification were carried out 4 °C if not otherwise specified.

Step 1: *Preparation of crude extract*. The cells were harvested by centrifugation at 10 000 x g for 10 min and rinsed twice with 0.15 M NaCl solution and suspended in 50 mM Tris-HCl buffer (pH 8.2). Then the cells were disrupted by sonication at 170 W for 20 min and the sonicate was centrifuged at

120 000 x g for 60 min.

Step 2: *Concentration with ammonium sulfate*. Ammonium sulfate was added to the crude extract prepared from 27 g cell at a concentration of 75% saturation of this reagent and stirred for 5 h. The precipitate was collected by centrifugation at 10 000 x g for 15 min and dissolved in 50 mM Tris-HCl buffer (pH 8.2), followed by dialysis against the same buffer over night. After dialysis, the insoluble material was removed by centrifugation.

Step 3: *First Q-Sepharose*. The clarified crude extract was applied to a column (2.5 x 16 cm) of Q-Sepharose fast flow, previously equilibrated with 50 mM Tris-HCl (pH 8.2).

The column was washed with 400 ml of the equilibration buffer, and the proteins were eluted with a linear concentration gradient of NaCl, which was generated by mixing 280 ml of Tris-HCl buffer (pH 8.2) containing 550 mM NaCl into an equal volume of Tris-HCl buffer (pH 8.2). The active fractions eluted by about 250 mM NaCl were combined and concentrated in vacuo, dialyzed against 50 mM Tris-HCl buffer (pH 7.7) containing 200 mM NaCl.

Step 4: *First Sephacryl S-300*. The concentrated Q-Sepharose fraction was subjected to a column (2.6 x 98 cm) of Sephacryl S-300, equilibrated with 50 mM

Tris-HCl buffer (pH 7.7) containing 200 mM NaCl and eluted with this buffer saline at a flow rate of 20 ml/h. Fractions containing DPP activities were collected and dialyzed against 50 mM Tris-HCl (pH 8.2) containing 0.7 M ammonium sulfate.

Step 5: *Phenyl Sepharose CL 4B*. The fraction of gel filtration was applied to a column of Phenyl Sepharose CL 4B (0.9 x 11 cm), pre-equilibrated with 50 mM Tris-HCl buffer (pH 8.2) containing 0.7 M ammonium sulfate to carry out hydrophobic interaction chromatography on this column. The column was eluted with a descending gradient of ammonium sulfate from 0.7 M to 0 M (100 ml each side). The active fractions eluted by about 290 mM ammonium sulfate were collected and dialyzed against 50 mM Tris-HCl (pH 8.2).

Step 6: *Second Q-Sepharose*. Chromatography on Q-Sepharose fast flow was repeated similarly to the first chromatography using a smaller column (0.9 x 12 cm). NaCl gradient was 0 to 500 mM (100 ml each side).

Step 7: *Second Sephacryl S-300*. Gel filtration of the concentrated and dialyzed active fraction from the second Q-Sepharose chromatography was repeated on Sephacryl S-300 column as the same methods as the first gel filtration. The fraction containing the enzyme was exhaustively dialyzed against water to remove electrolytes in the sample.

Step 8: *Isoelectric focusing*. The dialyzed material was applied to an isoelectric focusing column (120 ml capacity). Electrophoresis was conducted using 1 % (v/v) ampholine (pH 4.0-6.0) under constant voltage (400 V) for 48 h, and the column was cooled with tap water (about 10 °C). After electrophoresis, activity, protein amount, and pH of each fraction was measured.

Assay of Enzyme Activity

Routine assay of DPP activity was performed using Gly-Pro-pNA as a substrate. Reaction mixtures containing 700 μl of 1 mM substrate in 50 mM Tris-maleate buffer (pH 7.5), 50 μl of enzyme, and 150 μl of Tris-maleate buffer (pH7.5) were incubated at 37 °C for 30 min. The reaction was stopped by the addition of 10 μl of 7.5 M acetic acid. The released *p*-nitroaniline was measured by absorbance at 410 nm. One unit of enzyme activity was defined as the liberation of 1 μmol of *p*-nitroaniline/min under these conditions [5]. Various kinds of Xaa-Pro-pNA, except for Gly-Pro-pNA were kindly provided by Dr. K. M. Fukasawa (Matsumoto Dental University).

Hydrolysis of azodye-conjugated proteins was examined by the methods described earlier [6].

Determination of Optimum pH for the Activity

Correlation of the pH and the enzyme activity was investigated from pH 4.0 to 10.0 using the following buffers: acetate buffer (pH 4.0-6.0), Na-phosphate buffer (pH 6.0-7.5),

Tris-HCl buffer (pH 7.5-9.0), and Na-bicarbonate buffer (pH 9.0-10.0).

Protein Determination

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

Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis (SDS-PAGE)

Purity of the purified sample and determination of molecular weight were carried out by SDS-PAGE (12.5%) [7]. The molecular mass markers used 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).

Results

Detection of Peptidase and Protease Activities

First, peptidase and proteolytic activities in the crude extract of *S. anginosus* NCTC 10713 were surveyed. We confirmed that the degraded substrates were limited to N-blocked substrates, as summarized in Table 1. Among these substrates Gly-Pro-pNA, Lys-Ala-pNA, and Val-Ala-pNA were also hydrolyzed by the purified enzyme.

The dye conjugated proteins including azocoll, azoalbumin, azocasein, hyde powder RBB, elastin-Congo red were only weakly degraded. A dipeptidyl peptidase (DPP) with Gly-Pro-pNA hydrolyzing activity was not detected in the culture supernatant, but only in the crude extract.

Table 1. Degradation of various *p*-nitroanilide derivative substrates by the crude extract of *S. anginosus*.

Degraded	Not degraded
Arg, Pro, Leu, Gly-Pro*, Lys-Ala*, Val-Ala*, Ala-Ala-Ala, Ala-Ala-Pro, Ala-Phe-Pro	Val, Ala, Gly-Phe, Ala-Phe, Ala-Ala Val-leu-lys, Bz-Arg, Suc-Ala, Suc-Ala-Ala, Suc-Gly-Pro, Suc-Ala-Ala-Ala, Suc-Ala-Ala-Pro-Phe, Glt-Ala-Ala-Pro-Leu, Metsuc-Ala-Ala-Pro-Val, Tos-Gly-Pro-Lys

*:degraded also by the purified DPP IV

Purification of DPP

DPP was isolated from the crude extract to homogeneity as shown in Fig. 1. The enzyme was purified 323-fold with a 3 % yield over the crude extract (Table 2). The molecular weight was calculated as 84 kDa from the electrophoretogram.

Substrate Specificity

As summarized in Table 3, DPP hydrolyzed not a few *p*-nitroanilide derivatives of dipeptides containing proline residue in the P1 position. However, the corresponding tripeptides such as Ala-Ala-Pro-pNA and Ala-Phe-Pro-pNA were not hydrolyzed. The enzyme also split Val-Ala-pNA and Lys-Ala-pNA, with relative activity of 7% and 5% to its activity against Gly-Pro-pNA, respectively. The activity against Pro-pNA, Leu-pNA, Arg-pNA, and Ala-Ala-Ala-pNA, which were degraded by the crude extract, was found to be negative. No proteolytic activity was shown by this enzyme.

Effects of Reagents on the Enzyme

The effects of various proteinase inhibitors, group specific reagents, detergents, and metal ions are presented in Table 4. The enzyme was not affected by the

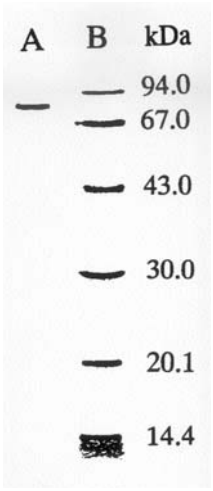

Fig.1. SDS-PAGE of purified DPP IV. Lane A, purified DPP IV; Lane B, marker proteins.

Table 3. Substrate specificity of DPP IV.

Substrate	Vmax (U/mg/min)	Km(mM)	Vmax/Km
Gly-Pro-pNA	1.786	0.556	3.212
Ala-Pro-pNA	1.042	0.750	1.389
Lys-Pro-pNA	1.471	0.370	3.976
His-Pro-pNA	1.408	0.217	6.488
Glu-Pro-pNA	0.704	0.714	0.986
Ser-Pro-pNA	1.176	0.217	5.419
Met-Pro-pNA	1.020	0.179	5.698
Phe-Pro-pNA	0.556	0.330	1.684

Table 4. Effects of various reagents and metal ions on DPP IV.

Compound	Concentration	Residual activity (%)
Control	–	100
Leupeptin	0.2 mM	108
Antipain	0.2 mM	83
L-trans-Epoxy-succinylleucyl-amido-(4-guanidino)butane	0.2 mM	100
Bestatin	0.2 mM	110
Diisopropylfluorophosphate	1.0 mM	0
3,4-Dichloroisocoumaline	1.0 mM	3
SDS	1.0 %	0
Triton X-100	1.0 %	68
EDTA	1.0 mM	108
1,10-Phenanthroline	1.0 mM	98
N-ethylmaleimide	1.0 mM	103
Mercaptoethanol	1.0 mM	95
Ca^{2+}	1.0 mM	96
Mg^{2+}	1.0 mM	107
Cu^{2+}	1.0 mM	85
Mn^{2+}	1.0 mM	78
Zn^{2+}	1.0 mM	78

Table 2. Purification of DPP IV of *S. anginosus*.

Step	Protein (mg)	Total activity (U)	Sp. act. (U/mg)	Purification (fold)	Yield (%)
Crude extract	4662	112	0.024	1	100
Ammonium sulfate	2789	106	0.038	2	95
1st Q-Sepharose	208	65.8	0.316	13	59
1st Sephacryl S-300	57.2	40.3	0.705	29	36
Phenyl Sepharose	15.3	16.2	1.059	44	15
2nd Q-Sepharose	7.6	13.1	1.724	72	12
2nd Sephacryl S-300	4.2	9.4	2.238	93	8
Isoelectric focusing	0.4	3.1	7.750	323	3

Sp. Act.;specific activity

standard proteinase inhibitors and bestatin. The inhibition by diisopropylfluorophosphate and 3,4-dichloroisocoumaline was quite obvious. An ionic detergent SDS completely inhibited the enzyme, but the inhibition by the nonionic detergent Triton X-100 was moderate. Other reagents containing chelators, thiol-alkylating reagent, reducing reagent, and metal ions did not influenced substantially. Possible inhibition by Gly-Pro was negative (data not shown).

Optimum pH of the Enzyme

Optimal pH for the purified enzyme was observed at 7.0. The activity could not be determined in the alkaline pH ranges (above pH 9.0) since the substrate was degraded automatically in the high pH buffer solutions.

Thermo-stabilities of the Enzyme

The purified enzyme was stable at 25 °C for 10 h or at -40 °C for at least a month. However, 94 % of the activity was lost when it was heated at 50 °C for 15 min.

Discussion

S. anginosus, one of the anaerobic viridans streptococci, is detected in the nazopharynx, pharynx, vaginal mucous membrane, and the gastrointestinal tract as well as in the dental plaque, and this species is frequently associated with purulent infections [8, 9]. However, the pathogenic factor of this species is quite unknown.

First, we examined the occurrence of proteolytic enzyme in the culture fluid, the cell extracts, and the envelope of *S. anginosus* NCTC 10713 using proteins and many synthetic chromogenic substrates of proteases, which, however, resulted in failure. Subsequently, we noticed production of different kinds of peptidase by this strain, such as aminopeptidase, dipeptidyl peptidase, and tripeptidyl peptidase (Table 1), in which the amount of relative activity of Gly-Pro peptidase was prominent. Furthermore, it was indicated that Gly-Pro peptidase was important as the pathogenic factor of the periodontal disease [10, 11]. Therefore isolation and characterization of this enzyme was undertaken. However, the presence of unknown proteinases remains still possible, because peptidases can not directly degrade intact proteins, but they hydrolyse the digested protein fragments generated by proteinases, that is, existence of peptidases implies existence of proteinases.

Isolation and characterization of peptidases including DPP IV of oral indigenous bacteria have been performed mainly in the periodontopathogens and the possible pathological implications of these enzymes for periodontitis have also been discussed [12-18]. Although little information of peptidases of viridans streptococci is available, Suido et al. reported that *S. mitis*, *S. sanguis* and *S. salivarius* exhibited active production of DPP IV [19]. The formation of arginine aminopeptidase and leucine aminopeptidase was confirmed in *Streptococcus sanguis* [20 21]. In *S. mitis*, aminopeptidase hydrolyzed peptide bonds at carboxyl side of arginine and lysine was partially purified [22]. Recently, arginine aminopeptidase of *S. gordonii*, which is a close related species to *S. sanguis*, was purified and well characterized [23]. These enzymes also were detected in our present study.

The purified DPP hydrolyzed preferably the substrates with proline residue at P1 position (Xaa-Pro-pNA). However, proline residue can be replaced with alanine (Val-Ala-pNA and Lys-Ala-pNA), even though the rates of hydrolysis for these substrates are much lower than Xaa-Pro-pNA. In addition, the enzyme was quite sensitive to inhibition by DFP and 3,4-dichloroisocmaline. These findings indicate the enzyme should be classified as DPP IV. Interestingly, the identity of the N-terminal amino acid residue of the substrate is not so significant for the enzyme actvity (Table 3). These findings are rightly consistent with the properties of DPP IV revealed by Yoshimoto et al. [24].

Some strains of *S. anginosus* including NCTC 10713, which was used in this work, exhibit β-hemolytic activity [25]. Our preliminary experiments using partially purified sample from *S. anginosus* demonstrated that the hemolysin was recovered from the culture supernatant and destroyed by trypsin treatment, and it lysed erythrocytes of many animal species (unpublished observations). We are going to achieve complete purification of this hemolysin, followed by its characterization and precise identification in the near future, since the hemolysins are widely accepted as virulence factor.

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