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Purification and Characterization of an Enzyme Produced by *Treponema denticola* Hydrolyzing Synthetic Collagenase Substrates

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Summary

A peptidase hydrolyzing synthetic substrates of collagenase was purified from cell extract of an oral spirochete, *Treponema denticola* by sequential procedures including anionic ionexchange chromatography, gel filtration, and hydrophobic interaction chromatography. The purified enzyme was most active at pH 8.0 and was inhibited by *p*-chloromercuribenzoate or EDTA. This enzyme was active on Pz-peptide and Z-Gly-L-Pro-L-Leu-Gly-L-Pro. It was observed that Gly-L-Pro was released from the latter substrate, but proteins, including collagens, were not found to be hydrolyzed by this enzyme.

Introduction

Genus *Treponema* spirochetes are commonly found in subgingival flora in man[1,3,5], and it has been shown that spirochetes increase in numbers during periodontal diseases[3,6]. *Treponema denticola* is a spirochete species frequently detected in periodontal lesions[9]. Thus, oral treponemes are thought to be associated with etiology of periodontal disease. However, little is known about pathogenic factors of this spirochete. Several reports have indicated that oral spirochetes including *T. denticola* elaborate enzymes responsible for tissue degradation[7,8,12,13,15,16]. We have also isolated *T. denticola* from adult periodontal lesions and investigated biological properties and diversity of protease production of these organisms[11]. The aim of this study was to characterize the enzyme hydrolyzing synthetic peptide substrates of microbial collagenase and to evaluate its enzymatic action on collagens.

Methods

Treponema denticola strain N-1-2 was isolated from the adult periodontal lesion in our laboratory[11]. The spirochete was grown in TYGVS medium[13] in anaerobic glove box filled with gases ($N_2+H_2+CO_2$; 85 : 10 : 5) at 37°C for 7 days.

Enzyme activity hydrolyzing Pz-peptide (4-phenylazobenzyloxy-carbonyl-L-prolyl-L-leucyl-glycyl-L-prolyl-D-arginine, Sigma Chem. Co.), a synthetic substrate for collagenase, was assayed based on the method of Wunsch and Heidrich[17]. Briefly, reaction mixture

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containing enzyme sample (0.1 ml), 2.5 mM Pz-peptide solution in 0.05 M Tris-hydrochloride buffer, (pH 7.2) (0.1 ml), and 0.05 M Tris-hydrochloride buffer, (pH 7.2) (0.3 ml) was incubated at 37°C for 30 min. To stop reaction, 0.5 ml of 0.4 M citric acid was added and shaken. Then 2.0 ml of ethyl acetate was introduced into this mixture and mixed exhaustively followed by low speed centrifugation. The ethyl acetate phase containing Pz-L-Pro-L-Leu generated by the hydrolytic action of the enzyme was taken carefully and absorbance at 320 nm was assayed to estimate Pz-L-Pro-L-Leu. One unit of enzyme was defined as the amount that increased 0.001 absorbance unit per min under these conditions. Hydrolytic activity against other peptide substrates was assessed by ninhydrin reaction[10]. Protein degradation by the purified enzyme was determined by SDS-PAGE method[4] which was also employed to examine the purity of the enzyme samples and determination of molecular weight. Marker proteins used for molecular weight determination were phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa).

Crude extract of *T. denticola* N-1-2 was prepared by the following procedure; the spirochetes were harvested by centrifugation at 12,000 G for 15 min. Cells (12 g) washed twice with 0.05 M Tris-hydrochloride buffer (pH 8.4) were suspended in the same buffer. Then cells were disrupted by sonication at 9 KHz for 15 min. The sonicate was centrifuged at 100,000 G for 60 min and the supernatant (crude extract) was collected and used for a starting material of purification.

Reversed-phase high performance liquid chromatography (HPLC), equipped with Shim-Pack CLC-ODS column (Shimadzu Co.), equilibrated with 0.1% trifluoroacetic acid-35% acetonitrile was used for detection of peptides. The peptides in the eluate were monitored by absorbance at 210 nm.

Results

Pz-peptidase activity was found in crude extract. No significant amount of activity was detected in culture supernatant or in 100,000 G pellet of sonicate.

Crude extract was applied to a column (2.6 by 20 cm) of Q-Sepharose equilibrated with 0.05 M Tris-hydrochloride buffer (pH8.4). The column was washed thoroughly with this buffer and subsequently eluted with linearly increasing concentration of NaCl in this buffer. The enzyme was found to elute at about 0.8M NaCl from the column. The active fractions were combined, concentrated, and dialyzed against 0.05M Tris-hydrochloride buffer (pH7.2) containing 0.2M NaCl. The concentrated material was applied to a column (2.6 by 80cm) of Sephacryl S-300 and eluted with the same buffer saline. The specific activity (enzyme activity units per 1mg protein) rose to 52fold of that of crude extract, however, this sample showed several stained protein bands on SDS-PAGE. The NaCl was added to this sample up to 1.0M and subjected to hydrophobic interaction chromatography on Phenyl Sepharose CL-4B column (1.6 by 8cm). When the column was washed with 0.05M Tris-hydrochloride buffer (pH7.2) containing 1.0M NaCl, about 70% of the total protein of the applied sample eluted accompanying only negligible amount of Pz-peptidase. The column was then eluted with 0.05M Tris-hydrochloride buffer (pH7.2). The enzyme eluted as a sharp peak. The active fractions were combined and applied to a column (1 by 5cm) of hydroxyapatite equilibrated with 0.05M Na-phosphate buffer (pH7.0). When the column was eluted with stepwise increasing concentration of Na-phosphate buffer (pH7.0), Pz-peptidase eluted with 0.12M Na-phosphate buffer (pH7.0). The active fractions from the column were combined and dialyzed against 0.05M Tris-hydrochloride buffer (pH7.2). This sample was a purified Pz-peptidase. The enzyme was purified 93-fold compared to crude extract by the purification procedure.

As shown in Fig. 1, the purified Pz-peptidase appeared to be homogeneous on SDS-PAGE, and molecular weight was calculated to be 65kDa.

The optimum pH for the reaction of the enzyme was found at pH8.0. The activity could not be detected below pH6.0, however, about 40% the maximum activity at pH8.0 was seen even though at pH9.5.

Effects of various group specific reagents and metal ions are summarized in Table 1. The enzyme was inhibited completely by *p*-chloromercuribenzoate, indicating sulfhydryl group(s) of this enzyme molecule may be important for its hydrolytic activity, although the enzyme was not activated by reducing agents such as mercaptoethanol or dithiothreitol. Inhibition by EDTA is also obvious.

Pz-peptide was incubated with some proteins and peptides to determine its substrate specificity. All the proteins including human collagens (Type I, Type III, and Type IV), gelatin, bovine albumin, bovine fibrinogen, human Ig A, and human Ig G were not hydrolyzed. Z-Gly-L-Pro-L-Leu-Gly-L-Pro was found to be hydrolyzed, but it could not hydrolyze Z-Gly-L-Pro-L-Leu-Gly and Z-Gly-L-Pro-L-Leu. Hydrolytic products of Z-Gly-L-Pro-L-Leu-Gly-L-Pro which had been digested for 1h and 4h by Pz-peptidase were analyzed by HPLC. As illustrated in Fig. 2, only Gly-L-Pro and Z-Gly-L-Pro-L-Leu were generated from Z-Gly-L-Pro-L-Leu-L-Pro. These results suggest that Pz-peptidase splits L-Leu-Gly bond of Z-Gly-L-Pro-L-Leu-Gly-L-Pro.

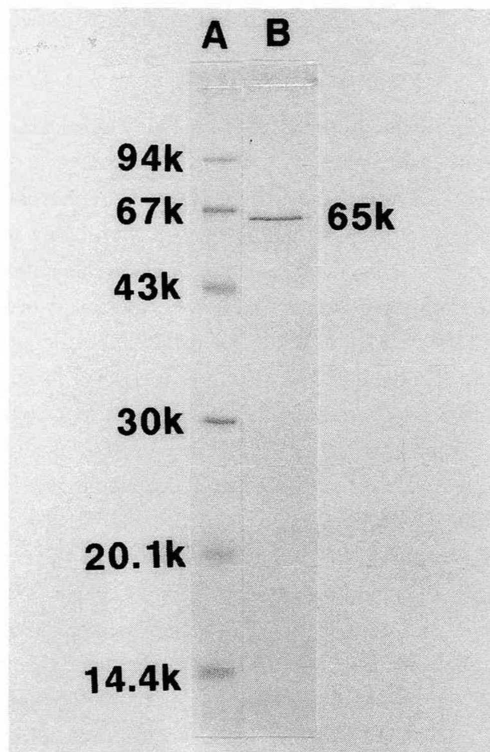

Fig. 1 : SDS-PAGE of the purified Pz-peptidase.
Lane A : Marker proteins
Lane B : Purified Pz-peptidase

Table 1 : Effects of group specific reagents and metal ions on Pz-peptidase activity

Reagents	Conc. (mM)	Activity(%)
Control	……	100
Phenylmethylsulfonyl fluoride	0.2	87
p-Chloromercuribenzoate	0.2	0
Tosyl-L-lysine chloromethyl ketone	0.2	83
Tosyl-L-phenylalanine chloromethyl ketone	0.2	79
EDTA	2.0	8
Mercaptoethanol	2.0	82
Dithiothreitol	2.0	108
$CaCl_2$	2.0	101
$MgCl_2$	2.0	94

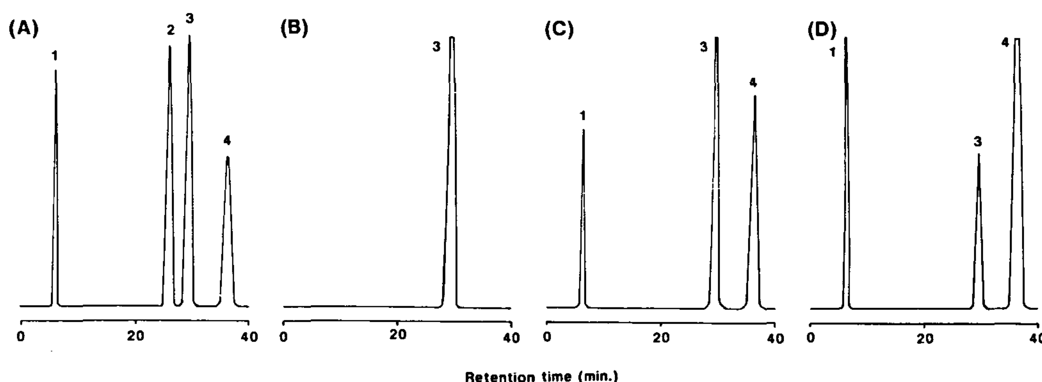

Fig. 2 : Reversed-phase chromatography (HPLC) of the hydrolytic products by Pz-peptidase of Z-Gly-L-Pro-L-Leu-Gly-L-Pro.
(A) Standard peptides,
(B) Z-Gly-L-Pro-L-Leu-Gly-L-Pro with Pz-peptidase (0 h)
(C) Z-Gly-L-Pro-L-Leu Gly-L-Pro with Pz-peptidase (1 h)
(D) Z-Gly-L-Pro-L-Leu-Gly-L-Pro with Pz-peptidase (4 h)
1 : Gly-L-Pro
2 : Z-Gly-L-Pro-L-Leu-Gly
3 : Z-Gly-L-Pro-L-Leu-Gly-L-Pro
4 : Z-Gly-L-Pro-L-Leu

Discussion

In the present investigation, we describe the purification and the characterization of Pz-peptidase of a strain of *T. denticola,* isolated from an adult periodontal lesion. The molecular weight was 65kDa and was characterized as a thiolmetallo-enzyme, judged from inhibition tests by various group specific reagents. The purified Pz-peptidase may cleave L-Leu-Gly bond as other microbial collagenase[7,8,14]. This was confirmed by the analyses of hydrolyzed products of the other peptide, Z-Gly-L-Pro-L-Leu-Gly-L-Pro. The enzyme splits the peptide bonds of amino group side of Gly-L-Pro.

Up to date, some enzymes of treponemes which are thought to be associated with destruction of gingival connective tissue have been purified and characterized ; Ohta et al.[13] purified an enzyme

hydrolyzing a trypsin synthetic substrates from *T. denticola*. This enzyme was a serine protease and inhibited by leupeptin. The enzyme, however, could not hydrolyze proteins such as casein, albumin, hemoglobin, and gelatin.

Chymotrypsinlike enzyme was isolated from cell extrat of *T. denticola*. This enzyme was active not only on chymotrypsin synthetic substrates but on natural proteins including transferrin, fibrinogen, Ig A, Ig G, gelatin, and albumin[16]. Followingly, location of this enzyme within the cell was studied by means of immunohistochemical technique, which revealed the enzyme attached to the outside the cell envelope[2]. Furthermore, this enzyme was shown to degrade basement membrane Type IV collagen, indicating chymotrypsinlike protease of *T. denticola* may play an important role in the invasion and destruction of basement membrane[2].

Makinen et al. reported that *Treponema vincentii* and unidentified species of *Treponema* produced collagenase which were found to cleave collagens as well as synthetic substrates of collagens[7,8]. However, Pz-peptidase in the present study did not show the hydrolytic activity toward collagens, but this enzyme may contribute to destruction of periodontal tissue through degradation of collagen with the cooperation of collagenase in gingival fluid secreted from tissue or produced by other bacterial species.

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抄録：*Treponema denticola*の産生するコラゲナーゼ基質分解酵素の精製と性状

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口腔スピロヘータ（*Treponema denticola*）の細胞抽出物によりコラゲナーゼの合成基質を加水分解する酵素を，イオン交換クロマトグラフィー，ゲル濾過，疎水結合クロマトグラフィーによって精製した．精製酵素はpH8.0で最大活性を示し，パラクロロマーキュリベンゾエイト，EDTAによって阻害された．この酵素はPZ-ペプチド，Z-Gly-L-Leu-Gly-L-Proに作用し，後者基質よりGly-L-Proを遊離することがわかった．しかし，コラーゲンを含む蛋白質は本酵素によって分解されなかった．