Original

Purification of Thiol-Dependent Collagenolytic Enzyme
Isolated from Culture Supernatant of *Bacteroides*
gingivalis 381 and Its Inhibition by Some
Serum Components*

Bacteroides gingivalis の培養上清中に存在する SH-依存性の
コラーゲン溶解酵素の精製と血清成分による阻害*

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Received June 15, 1987; accepted August 10, 1987

概要: 成人型の辺縁性歯周炎患者の歯周ポケットより高頻度に分離される *Bacteroides gingivalis* の培養上清より, SH-依存性のコラーゲン溶解酵素を抽出し, 精製を試みた。本酵素タンパクは非常に凝集し易い性質のためイオン吸着クロマトグラフィーが困難であったが, 非イオン性の界面活性剤である 1-O-N-Octyl- β -D-glucopyranoside の添加により, 酵素活性に影響を与えることなく可溶化に成功した。酵素はアセトン分画, Sepharose CL-6B によるゲル濾過, 1-O-N-Octyl- β -D-glucopyranoside による可溶化, DEAE-Sephacel カラムクロマトグラフィー等により, タンパク当りの比活性で115倍に部分精製した。部分精製酵素は至適 pH 7.6~8.0で, 等電点は pI 4.4と5.5に存在し, SH-依存性は粗酵素と同様に非常に強いことがわかった。また, 種々の血清成分で強い活性阻害を受け, γ -グロブリンのみならずアルブミンによっても阻害を受けることが判明した。このことは歯周ポケット内で, 浸出液中の血清成分が *B. gingivalis* の病原性を減弱させるのに何らかの役割を果たしている可能性を示唆するものである。

Key words: Collagenolytic enzyme, *Bacteroides gingivalis*, Serum components

索引用語: コラーゲン溶解酵素, バクテロイデスジンジバリス, 血清成分

* A part of this study was presented at the 33rd General Session (October, 1984) of Japanese Society for Dental Health

* 本研究の一部は第33回日本口腔衛生学会総会 (昭和59年10月) において発表した。

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Introduction

There are several studies suggesting that enzymes are implicated in the etiology of gingivitis and marginal periodontitis. Some of these are concerned with mechanisms of enzymes which are increased in gingival and periodontal tissues^{1,2}, but a few emphasize the direct function of enzymes which are produced by oral bacteria.³ Among these enzymes, collagenase and protease are thought to be important because of their ability to directly destroy connective tissues. Our previous study⁴ demonstrated that culture media from *Bacteroides gingivalis* contained collagenolytic enzyme and that the enzyme activity was highly enhanced by the addition of reducing reagents having thiol groups. This enhancement appeared only in the *B. gingivalis* enzyme and not in the enzyme from other *Bacteroides* species. The same enhancement in protease from *B. gingivalis* was reported by several other groups.⁵⁻⁷ From this point of view, it was suggested that the collagenolytic enzyme produced by *B. gingivalis* was a different type of collagenase from those found in other microorganisms⁸ and tissues.⁹⁻¹¹

The collagenolytic enzyme from *B. gingivalis* showed activity in a reduced environment, because *B. gingivalis* is predominant in deep gingival pockets of patients with adult type of periodontal disease.¹²⁻¹⁴ This characteristic may be favorable in maintaining the direct function of the enzyme as a pathogen of periodontal disease. For further investigation, isolation and purification of this enzyme are required. However, the large molecules secreted into the bacterial culture medium of *B. gingivalis* possesses strong coaggregating nature which makes it difficult to isolate and purify enzymes.

The purpose of this paper is to describe the isolation and purification of a thiol-dependent collagenolytic enzyme produced by *B. gingivalis*. Inhibition of this enzyme by some serum components also is described.

Materials and Methods

Bacterial strains: *Bacteroides gingivalis* 381 was obtained from the State University of New York at Buffalo by the courtesy of Drs. R. J. Genco and J. Slots. It was maintained by weekly transfer on anaerobic basal medium¹⁵ with 5% sheep blood. Organisms in preculture were grown in anaerobic BHI broth supplemented with yeast extract, vitamin K₁, and hemin.¹⁵ All cultures were incubated at 37°C in an anaerobic box (Forma Scientific, Marietta, Ohio, USA) with an atmosphere of 5% carbon dioxide and 10% hydrogen in nitrogen.

Assays of collagenolytic activity: Collagenolytic activity was measured as previously described by Toda *et al.*⁴ ¹⁴C-Acetylated rat-skin collagen was prepared¹⁶⁻¹⁸ and used as substrate for the enzyme assay. The reaction mixture consisted of 0.2ml of 0.2% ¹⁴C-acetylated collagen solution (about 10,000 dpm) in 0.05M Tris-HCl buffer pH 7.8, containing 0.2mM NaCl, 5mM CaCl₂ and 1mM dithiothreitol (DTT) and aliquots of enzyme preparation. The final volume was made up to 0.4ml with the same buffer. After incubation at 35°C for 1h, the reaction was stopped by adding 20μl of 0.4M ethylene diamine tetraacetate (EDTA) sodium salt, pH 7.8, in dioxane. The mixture was kept at the same temperature for another 60 min and then placed at room temperature. The reaction product in the resulting mixture was extracted with 0.4ml of dioxane by vigorous shaking followed by centrifugation at 3,000×g for 20min. Aliquots (0.5ml) of the supernatant was transferred into counting vials which contained ACS II Liquid Counting Scintillant and measured for radioactivity. As a blank for each determination to exclude neutral proteases activity, a parallel reaction mixture included 20mM EDTA, and the collagenolytic activity was corrected by subtracting the apparent activity of each blank determination. One unit of collagenolytic enzyme was defined as the amount which digested 1μg of

collagen per min at 35°C. Specific activity was expressed as units per mg protein of enzyme solution.

Protein in the sample was estimated by Lowry's method as modified by Hartree.¹⁹⁾ Recrystallized bovine albumin was used as the standard for protein assay.

Preparation of anti-*B. gingivalis* serum globulin: Rabbits were immunized with whole cells of *B. gingivalis* 381. Cells were formalin-killed and suspended in saline at a density of 20mg wet weights per ml. Rabbits were injected subcutaneously above the vertebral column with approximately 0.5ml of cell suspension incorporated into an equal volume of Freund complete adjuvant. One booster immunization of the same immunogen was carried out 1 month later. Serum was collected approximately one month after the booster immunization. The γ -globulin fraction was obtained from the serum by high performance liquid chromatography using a Glas Pac TSK DEAE-5PW.

Purification and isolation procedures of collagenolytic enzyme: Preculture of *B. gingivalis* 381 was transferred into 1,500ml of the same anaerobic BHI as used for preculture and cultivated anaerobically at 37°C to a stationary phase. The culture was then centrifuged for 15min at 10,000g and the supernatant was harvested for the following purification. Unless stated otherwise, the centrifugation and all subsequent purification were carried out at 4°C.

To the culture supernatant (1,350ml), one and a half volume of acetone, which had been chilled to -20°C, was added gradually by gentle stirring. The temperature of the mixture was kept at -2°C~-10°C during this procedure. The mixture was then centrifuged at 10,000g for 30min at -10°C. The resulting precipitate was dissolved in 40ml of 10mM Tris-HCl buffer, pH 7.4 containing 1mM DTT and dialyzed overnight against the same buffer. The dialyzed sample was applied to a Sepharose CL-6B gel column (5×100cm) which had been equilibrated with the same buffer. The flow rate was adjusted to 45ml per hour and 15ml fractions were collected.

The collagenolytic enzyme-active fractions from the gel filtration were collected and centrifuged at 100,000g for 120min. The subsequent sediment was dissolved with 0.8% 1-O-N-octyl- β -D-glucopyranoside (Oct-Glu) in the above buffer. The mixture was again centrifuged at the same conditions and the supernate was applied to a column (2.6×50) packed with DEAE-Sepharose which had been equilibrated with 10mM Tris-HCl buffer, pH 7.4, containing 1mM DTT and 0.8% Oct-Glu. After the column was washed with 300ml of the same buffer, elution was carried out, in a stepwise manner, by successive addition of 0.1, 0.2, and 0.4M NaCl in the same buffer. The flow rate was adjusted to 30ml per hour and the 6ml fractions were collected. The absorbance at 280nm of the elution was monitored by an ultraviolet absorption meter.

Chemicals and reagents: All reagents used were of analytical grades and generally purchased from Sigma Chemical Co., St. Louis, Mo., U. S. A. ¹⁴C-Acetic anhydride and ACS II Liquid Counting Scintillant were from Amersham/Searle, Arlington Heights Ill., U. S. A., and all serum components were from Miles' Laboratories, Inc., Elkhart, Indiana, U. S. A. 1-O-N-Octyl- β -D-glucopyranoside was purchased from Nakarai Chemicals, Kyoto, Japan.

Results and Discussion

Table 1 shows the summary of isolation and partial purification of collagenolytic enzyme from culture media of *B. gingivalis* 381. In the gel filtration on Sepharose CL-6B, the collagenolytic activity was eluted at the void volume fraction, from which the molecule size of the collagenolytic enzyme was estimated to be more than 1×10^6 . However, it was found by the following procedures that this large molecule was due to the aggregation of the proteins secreted into the culture medium by *B. gingivalis*. When the active fraction from gel filtration was applied directly onto a ion-exchange column, the enzyme

Table 1 Purification of SH-dependent collagenolytic enzyme from *Bacteroides gingivalis* 381

Steps	Total volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg. prot.)	Yield (%)	Purification (-fold)
Crude (Culture supernatant)	1,350	12,825	5.22×10^4	4.0	100	1
Acetone fractionation	44	484	1.15×10^4	47.5	22.0	11.6
Gelfiltration on Sepharose CL-6B	189	88.5	1.01×10^4	113	19.3	27.8
Solubilization and Supercentrifug.	9.5	46.0	6.01×10^3	131	11.5	32.0
Chromatography on DEAE-Sephacel	9.0	7.29	3.41×10^3	467	6.53	115

protein failed to pass through the absorbent and stayed at the top of the column. That is, the disaggregation of the enzyme protein was required prior to the ion-exchange chromatography.

A Similar phenomenon was reported on the particle fraction from *Bacteroides melaninogenicus* by Hausmann and Kaufman.²⁰ Their attempts to disaggregate and to release collagenase activity were unsuccessful. We have also tried to disaggregate collagenase active fraction with detergents for further purification. For ion-exchange chromatography, only non-ionic detergents such as Triton X, N-D-glucosyl-N-methylalkylamide compounds, and alkyl- β -D-glucosides were useful. Among the detergents we tried, 0.8% Oct-Glu was the most effective to disaggregate the enzyme without losing collagenolytic activity.

By taking advantage of the fact that the collagenolytic enzyme easily aggregates in the usual buffer, supercentrifugation of the gel filtrate was performed to concentrate the enzyme. The resulting sediment yielded more than 80% of the activity when it was solubilized in the buffer containing 0.8% Oct-Glu. After the solubilization, collagenolytic activity was recovered in the supernatant when the mixture was supercentrifuged again. The buffer for chromatography on DEAE-Sephacel also contained this detergent in order to avoid re-aggregation of the enzyme. As shown in the chromatographic pattern on DEAE-Sephacel of the extract (Fig. 1), the active fraction of collagenolytic enzyme was successfully eluted in the buffer containing 0.2M NaCl. Through these purification procedures, collagenolytic enzyme was purified 115-fold with a specific activity of 7.30×10^4 dpm per mg protein.

Fig. 1 Column chromatography of the solubilized enzyme sample with 1-O-N-octyl- β -D-glucopyranoside on DEAE-Sephacel. Elution was carried out in a stepwise manner by successive addition of 0.1, 0.2, and 0.4M NaCl in 10mM Tris-HCl buffer, containing 1mM DTT and 0.8% 1-O-N-octyl- β -glucopyranoside. The flow rate was adjusted to 30ml/h and 6ml fractions were collected.

In order to determine the homogeneity of the purified sample, electrophoresis on polyacrylamide gel (PAGE) was performed according to the method of Davis.²¹⁾ The purified enzyme easily re-aggregated and could not be electrophoresed without sodium dodecyl sulfate (SDS) when the Oct-Glu was removed from the enzyme solution. An Attempt to replace SDS with Oct-Glu in the solution for PAGE was unsuccessful and resulted in tailings of the sample proteins. Therefore, electrophoresis with SDS was achieved. Although not shown in the Figure, the partially purified enzyme still had several bands on SDS-PAGE. However, there is no way to know if these bands were of collagenolytic enzyme or contaminated proteins, because treatment of collagenolytic enzyme with SDS totally inactivated the activity irreversibly.

Isoelectric focusing of the partially purified enzyme was carried out with a 110ml column (LKB-Produkter, Bromma, Sweden) by the method of Vesterberg and Svensson,²²⁾ using 1% ampholite with a pH range from 3 to 10 in a sucrose gradient. As shown in Fig. 2, most of the enzyme protein was focused at pI 4.4, but some activity was found also at the fraction of pI 5.5. Some other protein peaks which had no collagenolytic activity were also found. This result suggests that the partially purified collagenolytic enzyme still had contaminated proteins and might have some isoenzymes.

In our previous paper,⁴⁾ some effects of crude collagenolytic enzyme in the culture medium of *B.*

Fig. 2 Isoelectric focusing of the partially purified collagenolytic enzyme. A portion of partially purified material (approximately 3.0mg protein) was subjected to a 110 ml column with 1% ampholite of a pH range from 3 to 10 in a sucrose gradient. The run was performed at a voltage of 350 V for 40h.

Fig. 3 pH dependency of the partially purified collagenolytic enzyme. Two kinds of buffers were used for this experiment, 0.1M Tris-maleate buffer (pH 6.0-8.0, $\blacktriangle$ --- $\blacktriangle$) and 0.1M Tris-HCl buffer (pH 7.2-8.8, $\circ$ — $\circ$).

gingivalis on reducing agents having thiol-groups and thiol-blocking reagents were described. Although it is not shown in the Table, partially purified enzyme showed the same effect on these reagents as that described previously⁴⁾ for the crude enzyme. Fig. 3 shows the pH-dependency on the activity of the partially purified collagenolytic enzyme. The dependency was studied using two kinds of buffers, 0.1M Tris-maleate (pH 6.0-8.0) and 0.1M Tris-HCl (pH 7.2-8.8) in the reaction mixture. The enzyme had optimum activity at a pH range from 7.6-8.0 in Tris-HCl buffer.

Human α_2 -macroglobulin is a common inhibitor of all kinds of proteinases including specific collagenase of mammalian origin.²³⁾ The inhibition of specific collagenase is caused by the interaction of

collagenase and α_2 -macroglobulin.²⁴⁾ Furthermore, some human serum components interact with some oral anaerobes including *B. gingivails* and aggregate bacterial cells including *B. gingivalis*.^{14,25)} Therefore, the inhibitory effect of some serum components on the collagenolytic enzyme from *B. gingivalis* was determined. Table 2 summarizes the inhibition of the partially purified collagenolytic enzyme by some serum components as the concentration in the reaction mixture which produced 50% inhibition (IC_{50}). The activity of the partially purified enzyme was strongly inhibited by not only globulin fractions but also by albumin. The inhibition by rabbit albumin was stronger than that by globulin fractions. The inhibition of the enzyme by γ -globulin was non-specific. As shown in the Table, serum γ -globulin prepared from immunized rabbit with *B. gingivalis* cells inhibited the collagenolytic enzyme at the same order as that from the unimmunized one.

The phenomenon that some serum components strongly inhibited the collagenolytic activity is of particular interest from the viewpoint of oral defence mechanisms. The deep gingival pockets where *B. gingivalis* resides are usually abundant in the exudate with some serum components. These serum components might be effective in decreasing the pathogenicity of *B. gingivalis* by inhibiting the collagenolytic activity of *B. gingivalis*.

Acknowledgement

This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan, 1986.

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Table 2 Effect of some serum components on collagenolytic enzyme isolated from *Bacteroides gingivalis*

Serum component	IC_{50} * (mg/ml)
Human γ -globulin	0.056
Human α_2 -macroglobulin	0.041
Rabbit γ -globulin	0.103
Rabbit anti- <i>B. gingivalis</i> serum γ -globulin	0.092
Rabbit albumin	0.015

* Concentration of serum component in the reaction mixture which produced 50% inhibition. The value was obtained by plotting the data of at least four different concentrations on a logarithmic probit paper.

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