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Supplementary studies on an extracellular proteinase of *Prevotella intermedia*: formation and some enzymatic properties

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Summary

Formation and some enzymatic properties of a proteinase of *Prevotella intermedia* ATCC 25611 were examined using Azocoll, a solid chromogenic non–specific proteinase substrate.

Anaerobic globe box supported better growth of cell and production of proteinase than AnaeroPak. Nearly all proteinase activity was found in the extracellular culture fluid, but not in the cell extract and the envelope. Production of proteinase started immediately after inoculation of the bacteria to the medium and continued until the middle stage of the stationary phase of the growth. Proteinase production was strongly inhibited by addition of glucose or fructose to the medium, moderately by sucrose and weakly by galactose.

This proteinase was inactivated entirely by heating at 60℃ for 20 min. Ethyleneglycol bis (*β*–aminoethyl ether)–*N,N,N,N'*–tetraacetate (EGTA), Zn^{2+} and Cu^{2+} completely inhibited the proteolysis. No effect on the activity was observed by Antipain, SDS, *N*–tosyl–L–phenylalanyl chloromethyl ketone (TPCK), Ca^{2+} and Mg^{2+}.

Introduction

Even though a major putative pathogenic agent of periodontitis is *Porphyromonas gingivalis*[1–6], *Prevotella intermedia* has also been implicated in periodontal diseases, as well as *Prevotella nigres-*

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cens[7–10]. Theses species are gram–negative obligate anaerobic rods and they form jet black colonies on the blood agar plates. Among various physiologically active substances of *P. intermedia* and *P. nigrescens*, hydrolytic enzymes, particularly proteinases[11–14] and hemoglobin binding protein[15], are considered to be important pathogenic factors.

In the previous report, we described partial characterization of a metallo–serine proteinase of *P. intermedia*[16]. In this report, we describe successive studies on this enzyme concerning the formation and further characterization to supplement the previous report[16].

Materials and Methods

The subcultured fresh bacterial strain *Prevotella intermedia* ATCC 25611 was cultured in anaerobox filled with a mixture of gases containing $N_2 : H_2 : CO_2 = 85 : 10 : 5$ or in jar in which AnaeroPack (Mitsubishi Gas Chem. Co., Inc. Tokyo, Japan), oxygen–absorbing and carbon dioxide–generating agent were put. The strain was grown at 37℃ for 3 days 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). In the case of addition of sugars to the medium, each sugar was sterilized by passing through a Ministart filter with a pore size of 0.45 μm (Sartorius Stedium Biotech GmbH, Göttingen, Germany).

The cells grown for 3 days were harvested by centrifugation at 10,000 xg for 15 min were suspended in Tris–maleate buffer pH 7.2, followed by disruption using an ultrasonic generator 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 further at 100,000 xg for 60 min. The supernatant and precipitate were designated cell extract and the envelope, respectively. From 4.9 g cells (2,000 ml culture), yields of the cell extract and the envelope were 48.9 ml and 760 mg, respectively.

Proteinase activity was determined using a solid non–specific proteinase substrate, Azocoll[17] (Calbiochem Co. Calif. USA). Reaction mixtures contained 5 mg Azocoll, 100 μl enzyme source and 900μl of 50 mM Tris–maleate buffer pH 7.2 were incubated at 37℃ for appropriate time. Thereafter, the mixtures were centrifuged at 15,000 xg for 5 min and the released azodye from the substrate in the centrifugal supernatants was estimated by absorbance at 520 nm (A_{520}). One unit of proteolytic activity was defined as the increase of the A_{520} by 1.0 per min. Similarly, hydrolysis of remazol brilliant blue hide powder was tested photometrically at 520 nm.

Semi–purified samples from the culture fluid prepared by the methods as described earlier[16] with a minor modification were used to investigate the properties of proteinase. Briefly, the concentrated culture fluid by ammonium sulfate precipitation was subjected to ion–exchange chromatography on Q–Sepharose and Sephacryl S–300 gel filtration. Finally, it was electrophoresed on an isoelectric focusing column in which proteinase was concentrated at a fraction of pH 7.2.

Results and Discussion

Quantitative comparison of growth and proteinase production between using anaerobox and AnaeroPak

The ratios of yields of cells (wet weight) cultured using AnaeroPak and in anaerobox were 2.0 and 1.0, respectively. The corresponding values of proteinase activities in the culture fluids were 1.5

and 1.0, respectively. Since culture in anaerobox was better in the growth and proteinase production, it was employed for the cultivation throughout the experiments.

Cellular locations of proteinase

Almost all proteinase activity was detected in the culture fluides. The activity only less than 1% in the culture fluid was found in the cell extract and the envelope. These findings suggest that an active transport function is provided to secrete extracellularly for proteinase in this organism.

Time course of proteinase production and cell growth

Accumulation of proteinase in the culture fluid started just after inoculation and it continued linearly until Day 2 of the cultivation and ceased at Day 3. However, the growth stopped at Day 1, and the cell weight decreased after stationary phase from Day 3, possibly due to the cell lysis (Fig. 1).

Effects of sugars on the proteinase formation and cell growth

Considering positive saccharolytic activity of *P. intermedia*, effects of addition of glucose, galactose fructose and sucrose to media on the proteinase production and the growth were evaluated (Table 1). It is obvious that proteinase formation was inhibited strongly by the addition of glucose and fructose, and depressed moderately by sucrose. However, substantially no effect on the growth was observed by these sugars. Since lowering of the final pHs of the culture media and degree of in-

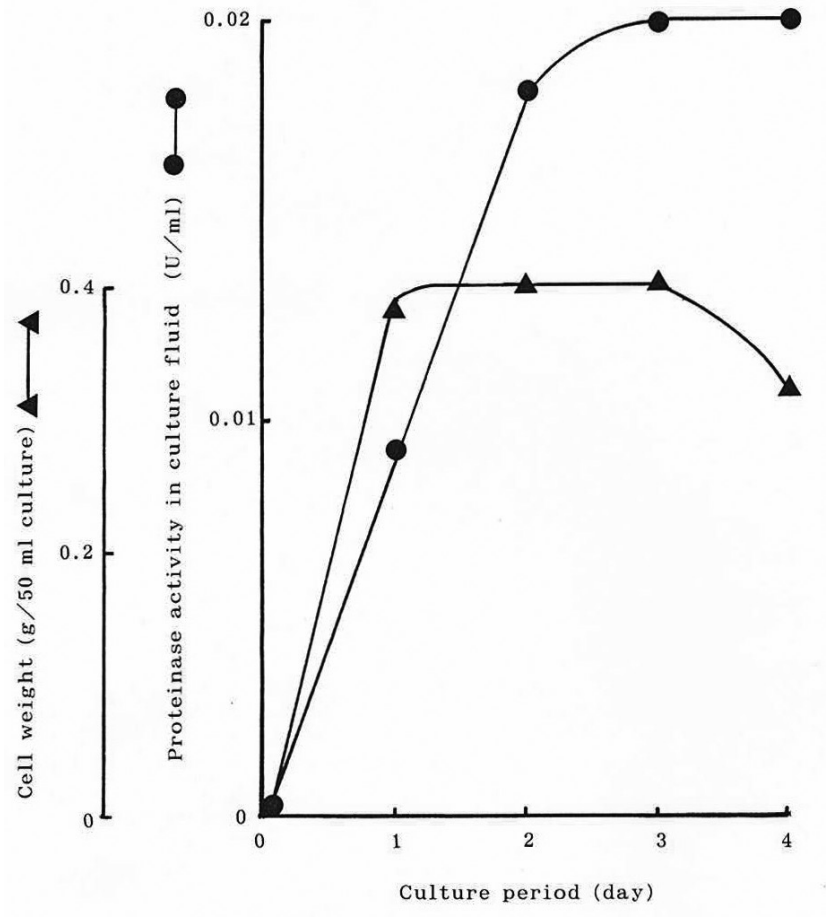

Fig. 1：Time course of proteinase production and growth of *P. intermedia* ATCC 25611.

Table 1 : Effects of sugars on production of proteinase and cell growth

Sugar	Concentration (%)	Proteinase (%)	Growth (%)	Final pH
None	—	100.0	100.0	7.1
Glucose	0.05	58.8	96.4	6.8
	0.20	16.8	81.6	6.3
Galactose	0.05	98.7	84.5	6.8
	0.20	76.8	78.3	6.7
Fructose	0.05	37.1	77.5	6.7
	0.20	14.1	83.2	6.2
Sucrose	0.20	44.2	95.0	5.5

Initial pHs of each medium were adjusted to 7.2 prior to cultivation.
The sugars employed were confirmed not to inhibit the proteolytic activity in separate experiments.

hibition of proteinase formation were necessarily not parallel, it seems that no direct relationship between the two was assumed.

In addition, glucose–6–phoshate was examined at a concentration of 0.20 %, it also depressed the proteinase formation (60.7 % inhibition) with no effect on the cell growth.

Additional examinations of effects of reagents on proteinase

Effects of some reagents which were not dealt with in the previous report[16] were tested. Complete inhibition was confirmed by a chelater, EGTA, as well as by EDTA[16]. However, contrarily, Shibata et al.[11] found no inhibition by EDTA and EGTA of elastolytic proteinase of *P. intermedia*, and in hemoglobin degradation enzyme of the same species[14], only around 20 % inhibition was noticed. Zn^{2+} (1 mM) and Cu^{2+} (1 mM) also completely inhibited, but no effect on the proteinase was observed by Antipain (2 mM), TPCK (2 mM), SDS (0.5 %), Ca^{2+} (1 mM) and Mg^{2+} (1 mM). When the enzyme source was mixed with human serum albumin prior to addition of azocoll and incubated, no significant reduction of the activity against azocoll was shown indicating that no competition between the two proteins for this proteinase seems to be considered.

Thermostability

Heating of the purified sample an 60℃ for 20 min resulted in the complete loss of the proteolytic activity.

Hydrolytic activity

As far as we examined, this proteinase was active against only Azocoll and remazol–conjugated hide powder. Particular notice was paid that substrates employed for the characterization of elastolytic enzyme of *P. intermedia* (Glt–Ala–Ala–Pro–Leu–pNA) and Metsuc–Ala–Ala–Pro–Val–pNA[11] and Congo red–conjugated elastin were not degraded by this enzyme. These findings suggest that the proteinase discussed in the present report is another enzyme of the elastolytic enzyme. Substrates for arginine–specific and lysine–specific gingipaines of *P. gingivalis*, a closed species of *P. intermedia* were also not hydrolyzed by this proteinase. Furtheremore, no activity against over 20 commercially available synthetic chromogenic substrates for proteinase was detected.

The proteinase described in the present report may function in the nutritional role of this spe-

cies and/or in the progress of periodontitis.

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抄録：*Prevotella intermedia* の細胞外タンパク分解酵素の補充的研究：産生と酵素性状

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Prevotella intermedia ATCC 25611のタンパク分解酵素の産生および性状についてアゾコルを酵素基質として調べた．嫌気ボックスとアネロパックで培養したところ，前者の方が増殖も酵素の産生は優れていた．酵素活性のほとんどは菌体外の培養上清に認められ，無細胞抽出液とエンベロープには検出されなかった．酵素産生は培養開始直後から開始され，定常期中ごろまで続いた．また，この酵素の産生は培地にグルコースやフルクトースを加えると強く抑制され，シュクロースでは中等度の，ガラクトースでは弱い抑制が観察された．温度感受性テストにより，60℃，20分加熱で失活し，EGTA，Zn^{2+}，Cu^{2+} で活性は阻害されるが，アンチパイン，SDS，TPCK，Ca^{2+}，Mg^{2+} による影響はなかった．