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Isolation and Properties of an Extracellular Gelatinase from a Strain of *Prevotella intermedia* Which Coelaborates Elastase

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Summary

Gelatinase in the culture supernatant of *Prevotella intermedia* was purified to homogeneity by the combined procedure of ethanol precipitation and three steps of chromatography. The enzyme was a cystein protease with a molecular mass of 45 kDa. The activity was inhibited by divalent metal chelators and its inhibition was recovered by the addition of Ca^{2+}. The optimum pH for activity was 7.0—7.5 and the enzyme was inactivated by heating at 60℃ for 10 min. It hydrolyzed actively azocoll and hide powder besides gelatin. Hydrolysis of type IV collagen, if not strong, was also observed.

Introduction

To date, not a few bacterial species were specified for putative perioidontopathogens. Among these, *Porphyromonas gingivalis, Prevotella intermedia,* and *Actinobacillus actinomycetemcomitans* are regarded as the main members responsible for developing periodontal disease[1,14]. Proteolytic enzymes and hemaggultinins of *P. gingivalis* as virulence factors have been extensively investigated, but only poor attention has been paid to those of *P. intermedia,* closely related spesies to *P. gingivalis*. We noticed the tissue destructive enzymes and reported frequency of occurrence, purification, and characterization of elastase of *P. intermedia* isolated from pus sample of adult periodontal lesion[13]. We undertook to isolate and characterize the general proteolytic enzyme of this microorganism for better understanding of the proteolytic systems of this species.

Materials and Methods

Bacterial strain and cultivation conditions

Prevotella intermedia EL2-1, isolated in our laboratory from pus sample of adult periodontal lesion[13] was used in the present study. This strain elaborates cell-boundary elastase but is negative in trypsin-like protease. The organism was cultured in general anaerobic medium (GAM ; Nissui Seiyaku Co., Tokyo) supplemented with hemin (5 μg/ml) and menadione (0.5 μg/ml) at 37℃ for 7

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days anaerobically.

Qualitative analysis of gelatinase

The samples were poured into the wells (5 mm in diameter) of the agar plates containing 0.5% gelatin and incubated at 37℃ for about 18 h. After that the plates were flooded with the saturated ammonium sulfate solution. Existence of gelatinase activity was determined by the formation of clear zones around the wells.

Quantitative assay of gelatinase

Hydrolysis of azocoll estimated photometrically at 520 nm was employed to quantify the activity of gelatinase[5]. One unit of the activity was defined as the increase of the A_{520} by 0.001 per min.

Cadmium-ninhydrin reaction method

To compare the hydorolytic activity of gelatinase against various proteins and peptides, amino groups of the generated free amino acids derived from the substrates by the action of gelatinase were determined by ninhydrin reaction in the presence of cadmium[4].

HPLC gel filtration

To analyse the cleaved fragments of gelatin after digestion by the purified gelatinase, HPLC gel filtration on a GS-520H column was employed. Peaks of the hydrolytic products were monitored by A_{215}.

Results and Discussion

1. *Purification of gelatinase*

All purification procedure was conducted at 4℃, if not otherwise stated. Whole culture was spun at 12,000 g and the culture supernatant was collected. Chilled (−20℃) ethanol was introduced into the culture supernatant (2,000 ml) slowly under gentle stirring to a final concentration of 70%. After standing for 1 h, the suspension was centrifuged at 12,000 g for 30 min at −5℃. The resulting precipitate was dissolved in 50 mM Tris-HCl buffer (pH 7.2) and dialyzed against the same buffer. Then the dialyzed material was applied to a column of Q-Sepharose equilibrated with 50 mM Tris-HCl buffer (pH 7.2) and eluted with a linear concentration gradient of NaCl concentration. The active fractions from the column were collected, combined, and dialyzed briefly against 50 mM Tris-HCl buffer (pH 7.2). NaCl was added to this dialyzed sample to a concentration of 0.5 M and applied to a column of Phenyl Sepharose CL-4B equilibrated to 50 mM Tris-HCl buffer (pH 7.2) containing 0.5 M NaCl. The column was eluted with a linear concentration gradient (descendent) of NaCl from 0.5 M to 0 M. The active fractions were desalted by dialysis against 50 mM Tris-HCl buffer (pH 7.2) and subjected to a column of hydroxyapatite. After the column was washed with 50 mM Tris-HCl buffer (pH 7.2), the column was eluted with a linear concentration of gradient of PO_4. The peak of the gelatinolytic activity emerged around 20 mM of PO_4. The active fractions were combined and dialyzed against 50 mM Tris-HCl buffer (pH 7.2). This was the purified sample of gelatinase. The purified sample contained 400 μg protein with 50, 250 U activity and it was homogeneous on SDS-PAGE (Fig. 1).

2. *Properties of gelatinase*

The molecular mass was estimated to be 45 kDa on SDS-PAGE. The purified gelatinase showed the 11, 700-fold activity of the crude culture supernatant in comparison of the specific activities between the two samples. The recovery of the activity was 12%.

The optimum pH of the activity was 7.0 to 7.5. The enzyme was completely inactivated by

heating at 60℃ for 10 min.

Effects of various enzyme inhibitors or group specific agents on gelatinase are summarized in Table 1. The enzyme was completely inhibited by a sulfhydryl group inhibitor, *p*-chloromercuribenzoate. Disulfide bond reducing agent (cystein) activated significantly. EDTA also inhibited completely and EGTA and phenanthroline inhibited strongly. Phosphorylating reagents of serine residue (diisopropylfluorophosphate and phenylmethylsulfonyl fluoride) and alkilating reagents (tosyl-L-lysine chloromethyl ketone and tosy-L-phenylalanine chloromethyl ketone) did not influence the activity. These findings suggest that gelatinase is a metal enzyme and cysteine protease. The inhibition by EDTA was completely recovered by the addition of Ca^{2+} and half recovery was possible by Mg^{2+}.

Substrate specificity of gelatinase was assessed using eight natural proteins, two pigment-conjugated protein and a synthetic peptide for collagenase assay (Z-Gly-Pro-Leu-Gly-Pro) (Table 2). The enzyme was found to be active against only gelatin and general protease substrates (azocoll and azure-conjugated hide powder). Considerable amount of the activity was seen against type IV

Table 1: Effects of various reagents and metal ions on the enzyme activity

Reagents	Conc (mM)	Relative activity(%)
Control	-	100
Diisopropylfluorophospahe	0.2	102
Phenylmethylsulfonyl fluoride	0.2	88
p-Chloromercuribenzoate	0.2	0
Tosyl-L-Lysine chloromethyl ketone	0.2	103
Tosyl-L-phenylalanine chloromethyl ketone	0.2	88
EDTA	2.5	0
EGTA	2.5	8
1, 10-Phenanthroline	2.5	9
2-Mercaptoethanol	2.0	128
Dithiothreitol	2.0	110
Cysteine	2.0	156

Table 2: Substrate specificity of gelatinase

Substrates	Relative activity(%)
Gelatin	100
Milk casein	11
Bovine albumin	0
Bovine hemoglobin	0
Bovine fibrinogen	0
Human collagen (type I)	0
Human collagen (type III)	5
Human collagen (type IV)	13
Azocoll	138
Hide powder azure	120
Z-Gly-Pro-Leu-Gly-Pro	0

(The activity was measured by cadmium-ninhydrin method.)

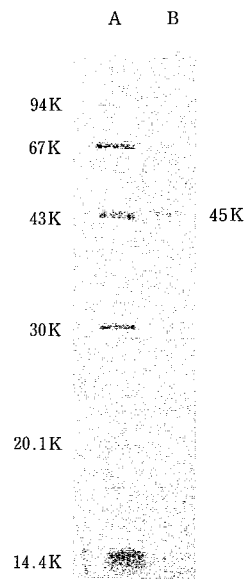

Fig. 1: SDS-PAGE of the purified gelatinase. Lane A: Standard proteins, phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydlase (30 kDa), soybean trypsin inhibitor(20.1 kDa), and α-lactalbumin(14.4 kDa). Lane B: Purified sample of gelatinase. Gel was stained with Coomassie.

collagen. From these substrate species profiles, this enzyme should be referred to as gelatinase.

When the degradation products of gelatin after incubation with gelatinase were analyzed on HPLC gel filtraion, two hydrolytic products with molecular mass less than 100 kDa emerged (data are not shown). Since the molecular mass of the undigested gelatin was found to be about 440 kDa on the same HPLC gel filtration, these results indicate that hydrolysis of gelatin might progress significantly. However, hydrolytic products could not be perceived on SDS-PAGE as Coomassie-stained bands.

We previously reported isolation and characterization of elastase of *P. intermedia* EL2-1 (the same strain used in the present study)[13] which is an envelope-associated and a serine enzyme. Therefore, elastase is obviously different enzyme from gelatinase. Thus, the protease systems of *P. intermedia* may be diverse as *Porphyromonas gingivalis,* a putative etiologic agent of periodontal disease. Proteases of this species have been vigorously investigated and characterization of collagenase[2,9,15], trypsin-like enzyme[3,6,8,16], and lysine-specific protease[7,10,18] were well documented. In oral microbiota, the metalloprotease with a molecular mass of 38 kDa which can hydrolyze gelatin was isolated from *Propionibacterium acnes*[12]. This enzyme was a serine protease and hydrolyzed natural proteins, displaying the distinct properties from the *P. intermedia* gelatinase.

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抄録：エラスターゼ産生性 *Prevotella intermedia* の菌体外ゼラチナーゼの分離と性状

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エラスターゼ産生性の *Prevotella intermedia* の培養上清中のゼラチナーゼをエタノール沈殿法と3種のクロマトグラフィーの組み合わせで精製し，単一標品を得た．本酵素は分子量45kDa のシステインプロテアーゼであった．2価金属キレーターで活性は阻害されるが，Ca^{2+}の添加により阻害は回復する．反応の至適 pH は7.0～7.5で，60℃，10分の加熱で失活する．ゼラチンの他にアゾコール，ハイドパウダーを加水分解し，タイプIVコラーゲンにも活性を示した．