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Characterization of a proteinase using a partially purified sample of *Prevotella intermedia*

AKIKO KISO[1], KAZUKO MATSUO[2], SATOSU ONOZAWA[2], YUKINAGA SHIBATA[1], HIROMASA HASEGAWA[2,3] and SETSUO FUJIMURA[1]

[1]*Department of Oral Microbiology, School of Dentistry, Matsumoto Dental University*
[2]*Department of Hard Tissue Research, Graduate School of Oral Medicine, Matsumoto Dental University*
[4]*Department of Oral Pathology, School of Dentistry, Matsumoto Dental University*

Prevotella intermedia is an anaerobic oral indigenous species and forms marked jet black colonies on blood agar plates. This species has been regarded as one of the etiological agents of periodontitis[1–4].

Collagenase, arginine gingipain (RGP) and lysine gingipain (KGP) of *Porphyromonas gingivalis*,the most significant periodontal pathogen, are potent pathogenic factors[5–7]. Even though *P. intermedia* and *P. nigrescens* were shown to have higher levels of proteolytic activities than other species of *Prevotella* such as *P. loescheii*, *P. oralis*, *P. melaninogenica*, *P. buccae* and *P. denticola*[8], an insufficient number of reports on proteinases have been published. Jensen et al. demonstrated formation of enzyme hydrolyzing IgG of *P. intermedia* and *P. nigrescens*[9]. Degradation of hemoglobin by *P. intermedia* was noticed as a part of a provision mechanism of iron source for this microorganism[10]. We found a proteinase in the culture fluids of *P. intermedia* which appeared to possess different enzymatic properties from the proteolytic enzymes as mentioned above.

P. intermedia ATCC 25611 was grown at 37°C for 3 days anaerobically in a glove box filled with a mixture of gases containing N_2 : H_2 : CO_2 = 85 : 10 : 5. The medium employed consisted of 17 g of Trypticase peptone, 3 g of yeast extract, 2.5 g of K_2HPO_4, 5 g of NaCl, 5 mg of hemin and 0.5 mg menadione per liter. After the full growth of cells, culture fluids were obtained as supernatant solutions of the cultures by centrifugation at 15,000 xg for 15 min.

Proteolytic activity was measured routinely using azocoll, a non–specific proteinase substrate. That reaction mixtures contained 4 mg of the substrate, 100 μl of enzyme source and 900 μl of 50 mM Tris–maleate buffer, pH 7.2, were incubated at 37°C for 30 min. After incubation, reaction mixtures were cooled immediately in an ice–water bath to stop the reaction, followed by centrifugation at 15,000 xg at 4°C for 3 min. The absorbance of the supernatants at 520 nm (A_{520}) was measured, and the activity was defined as the increase of the A_{520} by 1.0 per min. Similarly, hydrolysis of remazol brilliant blue hide powder (RBB–hide powder) was evaluated by increase at A_{595}.

RGP and KGP activities were detected using synthetic substrates, Bz–Arg–*p*–nitroanilide and Tosyl–Gly–Pro–Lys–*p*–nitroanilide, respectively, as descried earlier[11,12]. Fibrinolysis and caseinolysis were evaluated using 1 % bovine fibrin plates prepared with thrombin and agar plates embed-

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ded 1 % casein, respectively. The lysis of both proteins was confirmed by emergence of clear zones around sample–spotted points by overflowing of 10 % of perchloric acid.

Partial purification of proteinase was carried out at 4°C by the procedures described below. Ammonium sulfate was added to the culture fluid at 75 % saturation of this reagent and stirred for 7 h, followed by centrifugation at 10,000 xg for 15 min to collect resultant precipitate. The centrifugal precipitate was dissolved in 50 mM Tris–HCl buffer (pH 8.2) and dialyzed against the same buffer over night. The dialyzed sample was applied to a Q–Sepharose column, which had been equilibrated with Tris–HCl buffer. Afterward, the column was washed with the same buffer until the absorbance of effluent from the column at 280 nm reached below 0.05. Then the column was eluted by a linear gradient of NaCl concentration from 0 to 700 mM. A main peak of azocoll degrading activities was found in the column washings with Tris–HCl buffer and small amount (less than 10 % of the activity in the washings) was detected in the eluted fractions with about 300 mM NaCl. The former active fractions passed through the column were concentrated using an evaporator and dialyzed against Tris–HCl buffer containing 200 mM NaCl. The concentrated material was applied to a column (2.6 by 100 cm) of Sephacryl S–300. By this gel filtration, brown material derived from the culture medium was removed from the active fractions, clear and colorless sample was obtained. The molecular size of proteinase was estimated by the separate operation of gel filtration on the same Sephacryl column using aldolase (158 kDa), albumin (68 kDa), ovalbumin (45 kDa) and cytochrome c (12.5 kDa) as reference proteins. Detection of these proteins was performed photometrically at 280 nm (cytochrome c was done at 410 nm). Molecular weight of the proteinase was calculated as 28 kDa from the plots of molecular weights of the reference proteins versus Kav $(Ve-V_0)/(Vt-V_0)$, here, Ve = elution volume of the proteins, V_0 = void volume of the column, Vt = total bed volume (Fig. 1). The active fraction obtained by gel filtration was referred to as semipurified proteinase.

The experiments described below were achieved by using this semipurified sample as an enzyme source.

As illustrated in Fig. 2, hydrolysis of azocoll was faster than that of RBB–hide powder by about two fold.

The proteinase was found to be positive in fibrinolysis and caseinolysis, as well as the dye–conjugated protein substrates, however, it exerted neither RGP nor KGP activity.

Effects of the group specific reagents on the proteinase are summarized in Table 1. Obvious inhibition was observed by Pefabloc SC, diisopropyl fluorophosphate and 3,4–dichloroisocoumarin, indicating this proteinase was a serine enzyme. The proteinase is considered to be a metalloenzyme, since the enzyme was inhibited strongly by chelating agents including EDTA and 1,10–phenanthroline.

Examinations of effect of pH on the proteolytic activity revealed that the optimum pH for the reaction was observed 7.0 to 7.5 (Fig. 3). Employed buffers as follows were used at a final concentration of 100 mM ; acetate buffer (pH 4.0 to 6.0), Tris–maleate buffer (pH 6.5 to 7.5), Tris–HCl buffer (pH 8.0 to 9.0) and carbonate–bicarbonate buffer (pH 9.5 to 10.0).

From the thermostability tests, it took 25 min to reduce 50 % of the proteolytic activity at 50°C, however, at 60°C, only 5 min heating was required for 50 % reduction.

A proteinase of *P. intermedia* active to immunoglobulin G located mainly on the surface of the cell was a cysteine enzyme but not a serine enzyme[9]. A novel proteinase degrading elastin was purified from cell surface materials of the same species[13]. It was also a serine enzyme but without hydrolytic activity against azodye–conjugated proteins used in the present report. An extracellular pro-

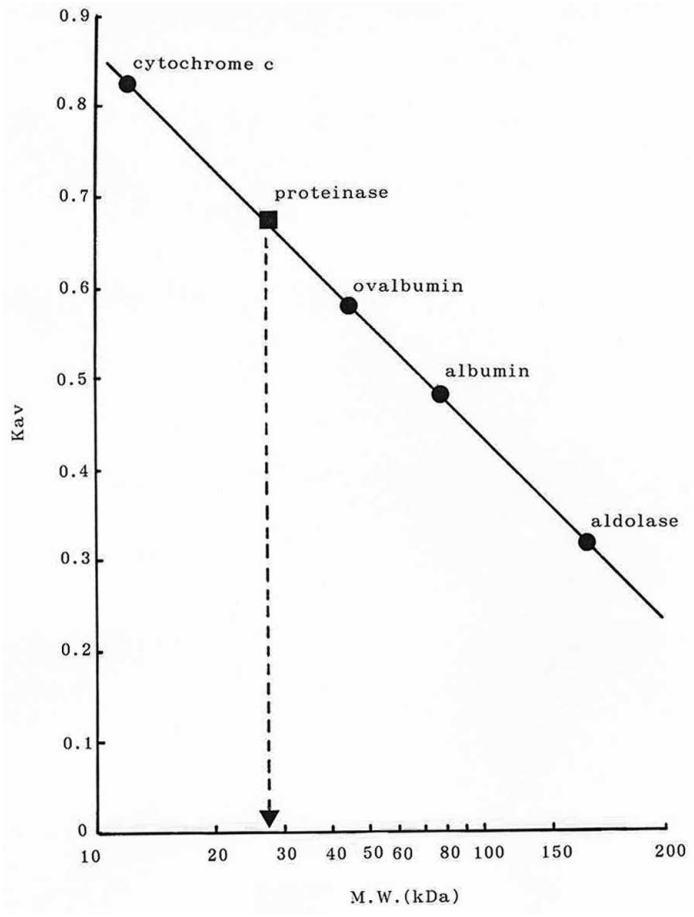

Fig. 1 : Determination of molecular weight of proteinase by gel filtration on Sephacryl S–300.

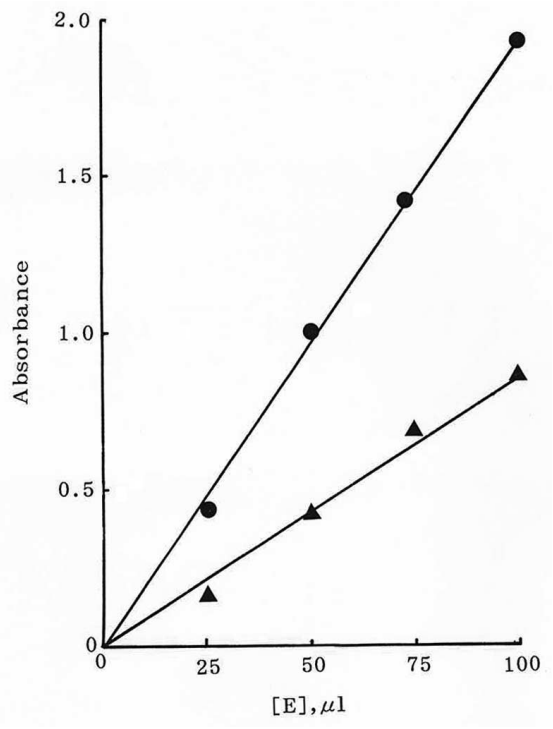

Fig. 2 : Hydrolysis of azocoll and RBB–hide powder.
Symbols : ●——● ; azocoll, ▲——▲ ; RBB–hide powder

Table 1 : Effects of group specific reagents on proteinase.

Reagent	Concentration	Activity (%)
Control	–	100
TLCK	1 mM	0
Leupeptin	2 mM	40
E64[1)]	2 mM	97
Pefabloc SC[2)]	2 mM	3
Diisopropyl fluorophosphate	5 mM	0
3,4–Dichloroisocoumarin	2 mM	1
EDTA	1 mM	7
1,10–Phenanthroline	2 mM	14
N–Ethylmaleimide	2 mM	104
PHMB[3)]	2 mM	107
Mercaptoethanol	2 mM	98

1) : L–*trans*–epoxy–succinyl leucylamido–(4–guanidino)butane
2) : 4–(2–aminoethyl)–benzenesulfonyl–fluoride hydrochloride
3) : *p*–hydroxymercuribenzoic acid

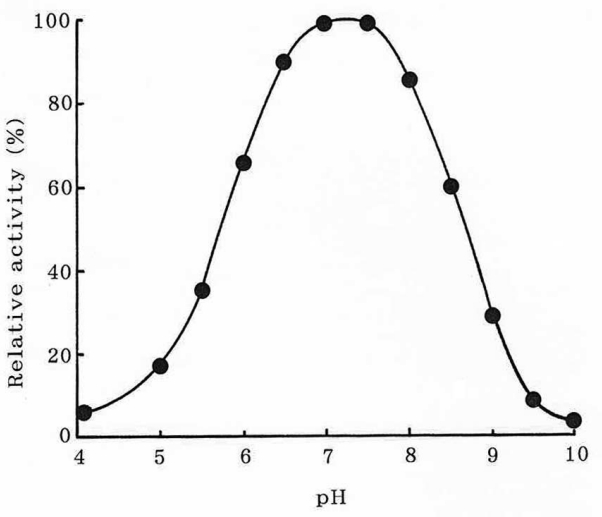

Fig. 3 : pH–activity relationship.

teinase of *P. intermedia* responsible for hydrolysis of hemoglobin was investigated in relation to the provision of heme for this species[10)]. However, these three proteinases are considered to be different enzymes from the proteinase described in this report, based on sensitivity profiles to various inhibitory reagents.

Occurrence of the proteinases may contribute to the arrangement for supplying nutritional substances collaborating with dipeptidyl peptidase[14)] of this species, since peptidases can not exhibit their functions under the shortage of peptides generated by proteolytic enzymes.

It remains to be evaluated the cellular locations of the proteinase, exhaustive purification and attempts to search synthetic substrates suitable for this proteinase.

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抄録：*Prevotella intermedia* のタンパク分解酵素の部分精製と性状

木曽有紀子[1]，松尾和子[2]，小野沢　諭[2]，柴田幸永[1]，長谷川博雅[2,3]，藤村節夫[1]
[1]（松本歯大・口腔細菌）
[2]（松本歯大・硬組織疾患制御再建学）
[3]（松本歯大・口腔病理）

歯周病原菌の一つである偏性嫌気性グラム陰性桿菌 *Prevotella intermedia* ATCC 25611株の培養上清中のタンパク分解酵素を硫酸アンモニウム塩析，イオン交換クロマトグラフィー，セファクリルゲル濾過で部分精製し，その酵素学的性状を調べた．タンパク分解活性はアゾ色素結合コラーゲン（アゾコル）を用いた．レマゾールブリアントブルー結合ハイドパウダーの分解も見られたが，その活性はアゾコルに対して約50%であった．また，フィブリン溶解とカゼイン分解活性も認められた．

分子量はゲル濾過法で28 kDa と算定され，本酵素はセリン酵素阻害剤と金属キレーターで強く阻害されたが，システイン酵素の阻害剤および還元剤による影響はなかったので，メタロ・セリン酵素に分類される．反応の至適 pH は7.0～7.5にあり，酵素の50%失活に要する時間は，50℃加熱で25分を要したが，60℃では 5 分であった．

P. intermedia のペプチダーゼについての報告はかなりなされているが，無論ペプチダーゼはタンパク質に直接作用するのではなく，ペプチダーゼが働くにはタンパク質からのペプチドの蓄積が必要である．しかし，それを供給する本菌のタンパク分解酵素についての知見はまだ不十分であり，さらに詳しい性状把握ががなされるべきである．我々も今後，このレポートで扱ったタンパク分解酵素の，完全精製と合成基質の探索をし，より詳細な研究を推進する必要があると考える．