

Proteolytic Activity of Dental Plaque Material

Part V. Purification of Proteinases from Dental Plaque Material

by Gel Filtration through Sephadex Columns

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With the aid of gel filtration through Sephadex columns G-100 and G-200, two different enzyme fractions capable of hydrolyzing gelatin and hemoglobin were obtained from dental plaques. These two fractions were obtained from plaques taken from either one person or pooled from several persons. The enzymes had different molecular weights and different pH maxima. One of the enzymes was obtained in relatively pure form; its activity was enhanced by calcium.

Periodontal disease is a morbid condition including an inflammatory process in the tooth supporting structures¹ and one of the most important causes of this disease is the dental plaque.²⁻⁵ A dental plaque is defined as an accumulation of microorganisms embedded in a tough gelatinous substance on the surface of the tooth.⁶⁻⁸ The plaque is also always in direct contact with the soft tissues. It contains minute amounts of food debris, epithelial and inflammatory cells⁹ and is called a dental calculus when mineralized.^{10,11}

In four earlier articles¹²⁻¹⁵ the proteolytic activity from dental plaques had been investigated as well as the influence of activators, inhibitors, and pH on this activity. In the studies presented earlier no separation of the enzymes was made, but the activity in the supernatant and the pellet after low-speed centrifugation of the plaque was studied separately.

The present communication describes the separation of proteolytic enzymes in water extracts of plaque material by filtration through Sephadex columns.¹⁶⁻¹⁸ The proteolytic activity within the different fractions was measured using gelatin and hemoglobin as substrates and some of the properties of the enzymes were studied.

MATERIAL AND METHODS

Dental plaque material was collected from 70 persons with normal and clinical diseased gingiva as described in a previous report.¹² In 20 experiments plaques, each from only

one person, were analyzed individually. The wet weight was determined and 0.1 M tris-(hydroxymethyl)aminomethane-HCl buffer (TRIS), pH 8.1, was added to the plaque suspension. The crude suspension was ground in a Virchow's glass mortar in order to separate bacterial aggregations¹³ and was then centrifuged at 2500 *g* for 15 min (Original Wifug, type XI, 4 × 100). In one experiment the suspension was centrifuged at 15 000 *g* (M S E centrifuge). The supernatant was concentrated in a Visking tube against dextrose at room temperature.

Gel filtration. Sephadex G-100 and G-200 were obtained from Pharmacia (Uppsala, Sweden). One filtration (Fig. 1) was performed at 4°C but all others were carried out at

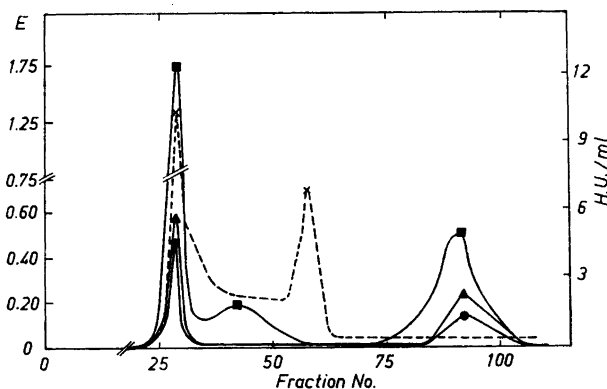


Fig. 1. Gel filtration of pooled dental plaque material, on a 1.5 × 197 cm Sephadex G-100 column at 4°C. Eluent was 0.1 M tris-HCl buffer in 0.5 M NaCl, pH 8.1, and 1 % butanol. The elution flow rate was 10 ml/h. The clarified plaque material was centrifuged at 15 000 *g* for 15 min, and 3.0 ml of the supernatant was applied to the column. Optical density: ■ 220 *mμ*, ▲ 260 *mμ*, ● 280 *mμ*. The enzyme reaction was run for 18 h. Gelatinase activity expressed in Hultin units (H.U.): ×

room temperature since the enzyme activity did not decrease under these conditions. The size and other data for the Sephadex columns are given in the figure legends. The extracts were applied to the top of the gel bed between the bed surface and the overstanding buffer by injection with a special syringe.

Elution was made at + 20°C with 0.1 M tris-HCl buffer in 0.5 M NaCl, pH 8.1 or 9.0. Butanol (1 %) was used as bactericidal agent.

The optical density of the fractions was measured at 220, 260, and 280 *mμ* in a Beckman spectrophotometer model DU.

Enzyme assay. For the proteolytic assay two substrates were used: *gelatin* (U.S.P. granular) from Fischer, Sc. Co., N.J., U.S.A. and *hemoglobin* from Difco Laboratories, Detroit, Michigan, U.S.A.

Gelatinase activity was determined in Ostwald viscosimeters at 37°C according to a method introduced by Hultin¹⁹ and used in studies by Hultin and Lundblad.²⁰⁻²² The enzymatic activities were calculated according to Hultin's formula (Söder)¹³ and the values so obtained were expressed in Hultin units (H.U.).

Hemoglobin splitting activity was measured by the method of Anson,²³ using urea-denatured bovine hemoglobin. The reaction mixture consisted of 1.0 ml extract, 2.0 ml buffer (*vide infra*) and 1.0 ml of a 4.0 % hemoglobin stock solution. This stock solution was made by mixing 4.0 g hemoglobin dissolved in distilled water with 36.0 g urea dissolved under heating in 50 ml distilled water. To this solution were added 8.0 ml 1 M NaOH and 0.1 ml 10 % merthiolate (Lilly) and the volume adjusted to 100 ml with distilled water. The solution was stirred for one hour.

The enzymatic digestion was stopped by adding 5.0 ml of 0.3 M trichloroacetic acid (TCA). The amount of aromatic amino acids released was determined spectrophotometrically at 280 *mμ* in the clear filtrate and expressed as the corresponding amount of tyrosine.

The following buffers were used at the pH's indicated for the gelatinase assay: 0.2 M glycine-HCl pH 3.0–4.0; 0.2 M succinic acid-NaOH, pH 4.0–6.0; 0.1 M sodium arsenate-HCl, pH 6.0–7.2; 0.2 M tris-HCl, pH 7.2–9.1; 0.2 M glycine-NaOH, pH 9.0–10.0.

For hemoglobinase assay: 0.4 M glycine-HCl, pH 3.2; 0.2 M succinic acid-NaOH, pH 4.0–6.5; 0.2 M tris-HCl, pH 7.2–9.0; 0.4 M glycine-NaOH, pH 10.0.

EXPERIMENTS AND RESULTS

In the first experiment a Sephadex G-100 column was used to fractionate pooled dental plaque material collected from 3 patients (Fig. 1). Two different absorption maxima were found in the collected filtrate and gelatinase activity was also found in two different intervals. The first proteolytic enzyme (protease I) was found in fractions 25–35 coincident with the first large protein peak, and the other enzyme (protease II) was contained in fractions 55–63. The latter was found between the two protein peaks. These results are representative for a series of over twenty independent experiments.

Earlier experiments had shown that Ca^{2+} had a stimulatory effect on the proteolytic activity, and therefore the influence of different concentrations of Ca^{2+} on pooled fractions was studied in concentrations ranging from 0.025 M up to 2.0 M CaCl_2 (Fig. 2). Protease I (fractions 28–33) was unaffected by Ca^{2+} while protease II (fractions 57–59) was very strongly activated. The proteolytic activity in the area between the peaks (fraction 46) was unaffected by Ca^{2+} until fraction 55, the beginning of protease II, which was slightly activated by Ca^{2+} .

Fig. 3 illustrates the effect of 0.5 M Ca^{2+} on the gelatinase activity on fractions separated by gel filtration. Protease I was not appreciably influenced

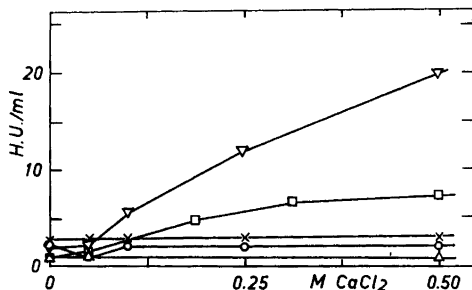


Fig. 2. The effect of Ca^{2+} on the gelatinase activity in fractions from the experiment shown in Fig. 1. The gelatinase activity is expressed in Hultin units (H.U.). Fractions Nos. 28–32 \times , 33 \circ , 46 \triangle , 55 \square , 57–59 ∇ .

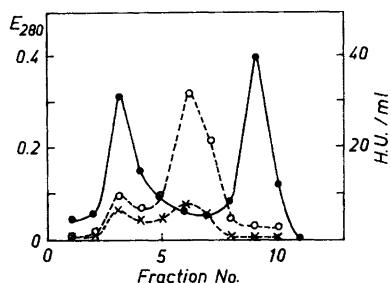


Fig. 3. Gel filtration of dental plaque extracts on a 0.60×85 cm column at 20°C . The starting volume was 1.5 ml. The eluent was 0.1 M tris-HCl buffer in 0.5 M NaCl, pH 8.1, and 1% butanol with a flow rate of 5 ml/h.

Optical density: \bullet 280 μ . Gelatinase activity without Ca^{2+} expressed in H.U.: \times . Gelatinase activity with 0.5 M CaCl_2 added, expressed in H.U.: \circ . The enzyme reactions were run for 18 h.

by Ca^{2+} at this concentration, whereas protease II activity was increased four-fold.

The effect of various concentrations of Ca^{2+} up to 2 M on protease II activity is shown in Fig. 4 B. The enzyme was obtained from the fractionation shown in Fig. 4 A. In this experiment maximum enzyme activity was reached

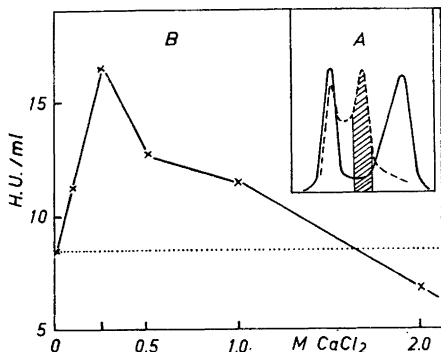


Fig. 4. A: Gel filtration experiment of dental plaques extracts on a 0.60×160 cm Sephadex G-200 column at 20°C . The starting volume was 2.0 ml. The eluent was 0.1 M tris-HCl buffer in 0.5 M NaCl, pH 8.1, and 1 % butanol with a flow rate of 4 to 3 ml/h.

Optical density: — 280 μ . Gelatinase activity in H.U.: ---. The enzyme reaction was run for 18 h. Pooled fractions from the shaded area (protease II) were taken for activation experiment in Fig. 4 B.

B: The influence of increasing amount of CaCl_2 on protease II activity. The enzyme reaction was run for 18 h and the gelatinase activity is given in H.U.: x.

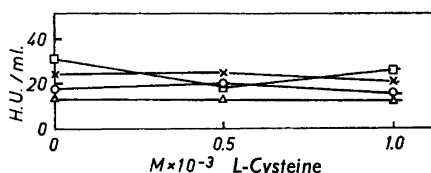


Fig. 5. The effect of various concentration of cysteine-HCl on fractions from the experiment shown in Fig. 1. The gelatinase activity is expressed in H.U. Fraction No. 34 x, 47 o, 53 Δ, 56 □, pH 7.2.

around 0.25 M Ca^{2+} ; at higher concentrations the activity decreased and at concentrations over 1.5 M the activity was lower than the value without Ca^{2+} .

Cysteine-HCl in concentrations of $0.5\text{--}1.0 \times 10^{-3}$ M had no activating effect of protease I or protease II at pH 7.2 (Fig. 5).

Dental plaque material obtained from one patient was filtered through a Sephadex column and the gelatinase activity and protein distribution in the collected fractions are shown in Fig. 6. The localization of protease I and II was similar to that shown in Fig. 1 for pooled plaque material with the same relative position to the protein curve. This experiment was repeated with twenty different patients and with the same results.

Protease II was equally active against gelatin and hemoglobin, while protease I was active against gelatin but had much lower hemoglobinase activity (Fig. 7).

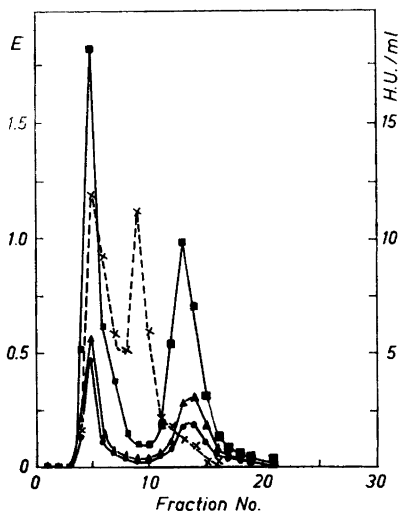


Fig. 6. Gel filtration of plaque extract from one patient after centrifugation at 2500 *g*. The wet weight of the plaque was 230.7 mg. A 0.60 × 160 cm Sephadex G-100 column was used with a total bed volume of 42 ml. Elution flow rate at 20°C was 4.5 ml/h. Eluent 0.1 M tris-HCl buffer in 0.5 M NaCl, pH 9.0, and 1 % butanol. The amount of protein in the sample was 4.41 mg (according to a nomogram by Warburg and Christian²⁷) and the starting volume was 1.5 ml. Optical density: ■ 220 mμ, ▲ 260 mμ, ● 280 mμ. The enzyme reaction was run for 18 h. Gelatinase activity is expressed in H.U.: ×.

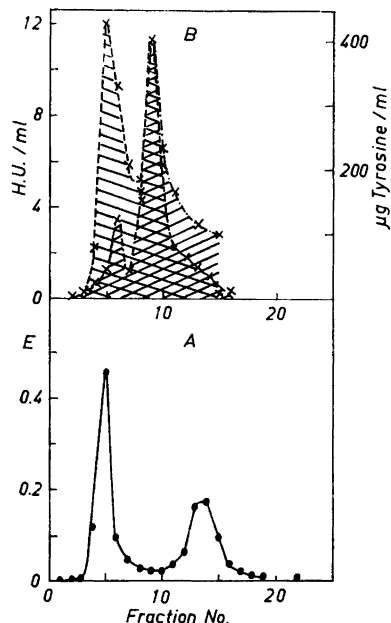


Fig. 7. A: Gel filtration of a dental plaque extract obtained from one patient. A 0.60 × 160 cm Sephadex G-100 column at 20°C. The starting volume 1.5 ml. The eluent was 0.1 M tris-HCl buffer in 0.5 M NaCl, pH 9.0 and 1 % butanol with a flow rate of 4.5 ml/h. Optical density: ● 280 mμ. B: Gelatinase activity in H.U.: \ \ \ \ . Hemoglobinase activity in μg tyrosine: / / / . The enzyme reaction was run for 18 h.

The data listed in Table 1 show that after one gel filtration protease II was isolated with a specific activity of approximately 500–850 H.U./mg protein, whereas the specific activity of protease I was about 30 H.U./mg protein.

The effect of heat on the gelatinase activity of protease I and II was determined by heating the pooled fractions containing these enzymes in a water bath at temperatures from 40° to 70°C for up to 30 min and then measuring the enzyme activity at 37°C (Figs. 8 and 9).

For both protease I and II the activity increased about 130–140 % after 15 min at 40°C, but after 30 min the activity of protease I and II had decreased to about 80 % and 100 %, respectively, of the initial value. At 50°C the enzyme activity showed an initial decrease, but after 15 min there was a marked

Table 1. The gelatinase activity of the different fractions from Sephadex G-100 filtration from three typical experiments. The specific activity is expressed as H.U./mg protein. In fractions Nos. 26–95 a 1.5×197 cm Sephadex G-100 column was used. The elution flow rate was 10 ml/h. In fractions 5–15 and 5–10 a 0.60×160 cm Sephadex G-100 column was used. The elution flow rate was 5.5 ml/h and 6.0 ml/h, respectively.

Fraction No.	Protein mg/ml	Activity H.U./ml	Spec. activity H.U./mg protein
26	0.05	0.4	8.0
27	0.07	0.7	10.0
28	0.19	4.0	21.1
29	0.31	10.3	32.8
30	0.19	9.3	49.0
31	0.035	6.0	172
35	0.01	3.6	360
40	0.01	3.4	340
50	0.01	2.9	290
55	0.01	3.5	350
56	0.01	4.5	450
57	0.01	5.1	510
58	0.01	7.0	700
59	0.01	5.8	580
60	0.01	5.0	500
65	0.01	0.5	50
70	0.01	0.3	50
90	0.03	0.6	20
95	0.06	0.6	10
5	0.39	5.3	13.6
6	0.60	16.0	26.7
7	0.13	12.6	97.0
9	0.03	12.8	426
10	0.02	16.8	840
11	0.02	6.0	300
12	0.05	2.6	52.0
15	0.10	1.2	12.0
5	0.14	5.5	39.3
6	0.18	8.5	47.2
7	0.03	4.8	160
8	0.025	7.0	280
9	0.02	8.7	435
10	0.02	10.6	530

rise in the protease I activity and a smaller rise in protease II to over 100 % and about 70 %, respectively, of the initial value. The same effect for both enzymes occurred at 60°C after 15 min heating; the enzyme activity increased to 60 % and 80 % of the initial activity of protease I and II, respectively, but decreased after longer heating periods. At 70°C almost no activity remained after 2 min.

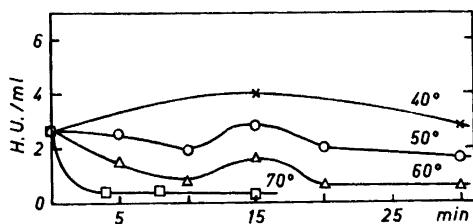


Fig. 8. The influence of heating at different temperatures over varying periods of time on the gelatinase activity of protease I in pooled fractions.

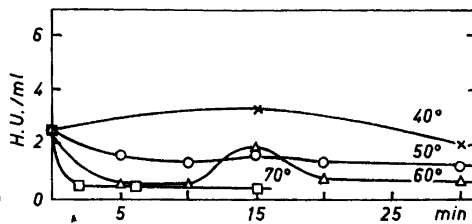


Fig. 9. The influence of heating at different temperatures over varying periods of time on the gelatinase activity of protease II in pooled fractions.

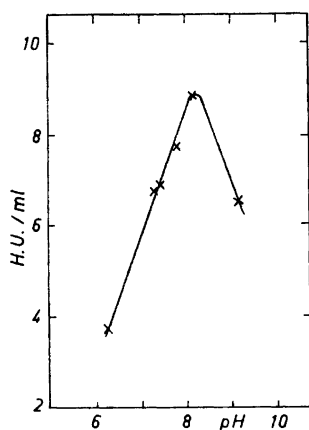


Fig. 10. The influence of pH on the gelatinase activity of protease I in pooled fractions. The enzyme reaction was run for 18 h and the activity is expressed in H.U.

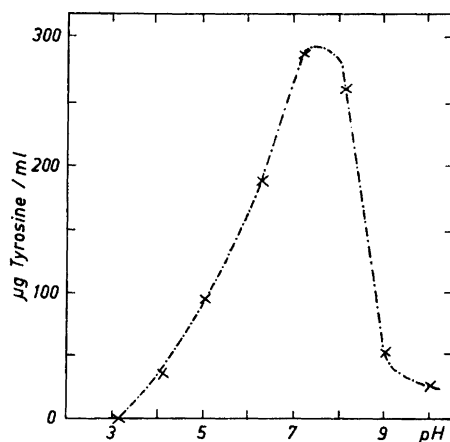


Fig. 11. The influence of pH on the hemo-globinase activity of protease II in pooled fractions. The activity is expressed in μg tyrosine per ml after 18 h reaction time.

The influence of pH on the gelatinase activity of protease I is given in Fig. 10 and shows a maximum at pH 8.5. The influence of pH on the proteolytic activity of protease II was studied with hemoglobin as substrate since the results with gelatin varied considerably (Fig. 11). Protease II had a maximum at pH 7.8.

DISCUSSION

These results reveal that two different major protein fractions always were present in dental plaque material obtained from many patients and that they could be well separated by filtration through Sephadex gels. These fractions were found in material from either single individuals or in pooled samples from several persons. Two proteolytic enzyme fractions were found; one coin-

cident with the first protein peak (protease I) and the other located between the two protein peaks (protease II). The occurrence of a peak at 220 $m\mu$ in fractions 45—50 (Fig. 1) seems to indicate the presence of non-aromatic amino acids in these fractions.

The purity of protease I is doubtful partly because of the high protein content of the fractions containing it. Its molecular weight is probably over 200 000 because it flowed in the void volume when Sephadex G-200 was used.²⁴⁻²⁶ When the gelatin and hemoglobin splitting effects of protease I were studied a much lower hemoglobinase activity relative to its gelatinase activity was found in contrast to protease II, as can be seen in Fig. 7. These facts suggest that there could be more than one enzyme in this fraction, *i.e.* one hydrolyzing only gelatin (fraction 5 in Fig. 7) and the other both gelatin and hemoglobin (fraction 6 in Fig. 7). These high molecular weight enzymes were not influenced by Ca^{2+} and had a different pH optimum than protease II. The increasing activity of protease I in Fig. 3 when Ca^{2+} was added was very probably due to a change in pH.

Protease II had a high specific gelatinase activity of 500—850 H.U./mg protein and is believed to be a much purer fraction than protease I. Its molecular weight appears to be lower than 100 000, probably in the range 40 000—50 000. This enzyme had a definite requirement for Ca^{2+} , as opposed to protease I.

Both enzymes had pH optima in the alkaline range. The effect of pH on the proteolytic activity of protease II with gelatin as substrate was difficult to determine since the results varied considerably; however, there was a definite increased activity in the alkaline range, which was confirmed when a pH curve was obtained with hemoglobin as substrate.

The two enzymes were moderately inactivated during the first 10 min of heating at 50° and 60°C but showed partial reactivation after additional 5 min heating. These results, shown in Figs. 8 and 9, may indicate the presence of an inhibitor which was destroyed after 15 min of heating. After prolonged heating the enzymatic activity decreased because of thermal inactivation.

Further investigations of these enzymes are in progress.

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