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PROTEOLYTIC ACTIVITY OF DENTAL PLAQUE MATERIAL

VI. FRACTIONATION OF DENTAL PLAQUE EXTRACT BY GEL FILTRATION ON SEPHADEX AND BY ZONE ELECTROPHORESIS

by

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INTRODUCTION

In a series of papers (*Söder & Frostell,* 1966; *Söder,* in press) the proteolytic activity in crude suspensions of dental plaque material has been studied using casein and gelatin as substrates. The proteolytic activity in the supernatant after low speed centrifugation (*Söder,* in press) has been studied also using gelatin, hemoglobin, amino acid esters and poly-α-amino acids as substrates (*Söder,* in press). Recently *Söder, Lundblad & Lindqvist* (1966) showed that proteins from the dental plaque supernatant after low and high speed centrifugation can be fractionated by gel filtration through the porous dextran gel Sephadex (Pharmacia, Uppsala, Sweden). The proteins are retarded on the column roughly in the order of decreasing sedimentation coefficients or molecular sizes (*Porath & Flodin,* 1959; *Flodin,* 1962; *Whitaker,* 1963; *Gelotte,* 1964; *Andrews,* 1964, 1965; *Squire,* 1964). A separation into two main fractions was achieved. The first of these fractions contained a proteolytic enzyme fraction called Protease I

Between the two protein peaks another proteolytic enzyme fraction, Protease II, was found. Protease II was obtained in a relatively pure state when Sephadex G-100 or G-200 was used. The two proteases were both capable of breaking down gelatin and hemoglobin. Protease I seemed to have a higher molecular size than Protease II which was strongly activated by Ca^{++} ions in contrast to Protease I.

Regardless of whether dental plaque extract from many persons (pooled material) or from one person only was used the same two main protein peaks and the two proteases were found.

The gel filtration technique with Sephadex G-100 or G-200 offered possibilities as a preliminary fractionation procedure of dental plaque proteins and the fractions may be separated further by other techniques (*Porath & Flodin,* 1963; *Killander,* 1964).

Since Protease I was obtained together with high molecular proteins in the first protein peak, it was desirable to separate it further and thus one purpose of the present investigation was to use a combination of gel filtration and zone electrophoresis for the study of Protease I and the first protein peak.

Other experiments were made to characterize the two enzymes such as studies on the activating and inhibiting influence of various substances on pH stability and on the effect of incubation temperature. Determination of the absorption spectrum of the separated components, a recycling of the fractions and a determination of the activity on different substrates, natural (gelatin and hemoglobin) as well as synthetic (poly-α-amino acid) was performed also.

MATERIAL AND METHODS

Dental plaque material was collected from persons with normal and diseased gingiva and treated as described earlier (*Söder & Frostell,* 1966; *Söder,* in press). The plaque material was suspended in 0.1 M Tris(hydroxymethyl)aminomethan(TRIS)-HCl buffer pH 8.1.

In all, material from about 50 persons was studied. In some experiments pooled material was used whereas in others material

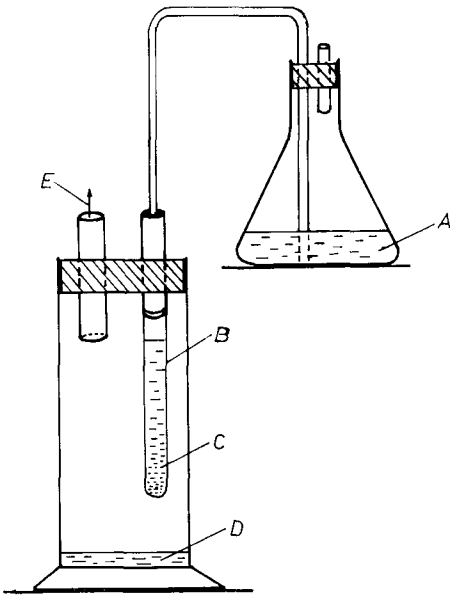


Fig. 1. Apparatus for continuous concentration of dental plaque extract. (A) The initial dental plaque extract. (B) Visking tube. (C) The concentrated dental plaque extract. (D) Buffer. (E) Vacuum outlet.

The dental plaque extract from (A) was drawn into the Visking tube as the buffer was pressed out in the partial vacuum and the proteins concentrated.

from only one person was tested. The suspended material was centrifuged routinely at 2500 × g for 15 minutes (original Wifug, Type XI, 4 × 100). The proteins in the supernatant were concentrated either in a Visking tube in crystal sucrose for different periods of time or by pervaporation from a Visking tube in vacuum (Fig. 1) at room temperature.

Gel filtration

The gel filtration experiments aiming at fractionating the dental plaque extract were performed on Sephadex G-25, G-100, G-100 super fine and G-200 (Pharmacia, Uppsala, Sweden) according to the description given by *Flodin* (1962). The gels were equilibrated with the medium and poured into the column. The medium contained 0.1 M TRIS-HCl buffer, pH 8.1 in 0.5 M NaCl and 1 per cent butanol as bactericidal agent. The gels were

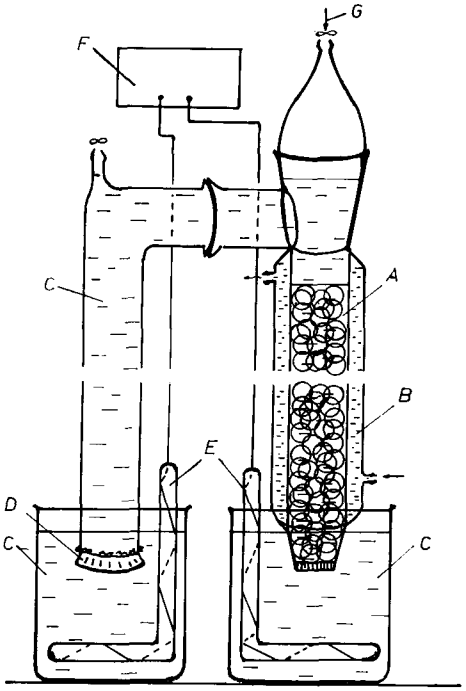


Fig. 2. Apparatus for zone electrophoresis. (A) The stabilizing medium (Sephadex G-25 or G-100). (B) The cooling jacket through which tap water was run. (C) The buffer solution. (D) A Visking tube membrane. (E) The electrodes. (F) The power supply. (G) Buffer solution to elute the proteins. The plaque extracts were applied at the top of the Sephadex gel between the gel bed and the buffer solution.

washed by a continuous flow of the medium for two or three days. Special care was taken to avoid excessive pressure from the overstanding buffer as this could cause too tight a packing of the gel resulting in a very slow flow rate.

When the column was considered completely equilibrated with the buffer a thin layer of Sephadex G-25 was applied on top to prevent particles from entering the gel.

Application of the samples were made on top of the gel after the buffer had been removed or between the gel-bed and the overstanding buffer (*Flodin*, 1962).

The elution was made at room temperature in the buffer mentioned above and was continuously followed using a UV absorp-

tion monitor (Ultraviolet absortiometer, Uvicord, L.K.B. produkter, Stockholm, Sweden) and a recording meter. The effluent was collected in fractions of a desired volume in a fraction collector furnished with a special device for obtaining constant volumes.*)

The optical density of the various fractions were measured in a Beckman spectrophotometer model DU, at 220, 260 and 280 mμ with 1 cm quartz cuvettes. Generally the absorption (E) measured at 280 mμ is given in the figures. The protein content was determined by the method of *Warburg & Christian* (1942).

Zone electrophoresis

Zone electrophoresis was carried out in a vertical column (Fig. 2) as described by *Gelotte, Flodin & Killander,* 1962. Sephadex G-25 or G-100 were used as stabilizing media. In some experiments a 2.2 × 50 cm column was used, in others a 0.60 × 80 cm column. The medium was buffered with 0.27 M TRIS-HCl buffer (pH 8.6 and I = 0.15) containing 1 per cent butanol.

The columns were equipped with cooling jackets through which tap water was run at a temperature of + 10° C.

In some experiments the dental plaque supernatant was treated directly with zone electrophoresis and in other experiments the fractions from Sephadex G-100 separations corresponding to the first protein peak were pooled and concentrated before the electrophoresis. The concentrated sample was added to the top of the gel and different voltages were applied for various periods of time. At the end of the experimental period the fractions were eluted and the proteolytic activity was determined. In some experiments the concentrated fractions were partly developed through the column before the electrophoresis began.

In the various experiments voltages from 250 to 980 V were applied giving a current of 30 to 50 mA. The time of the electrophoresis varied from two to seventeen hours. The elution after electrophoresis was made at a flow rate of 4—20 ml/hour in different experiments.

Other data for the various columns in Sephadex separation and zone electrophoresis experiments are given in the legends of the figures. Effluent volumes are given in ml.

*) constructed by Eng. L.-G. Falksveden, chemistry department, Natl. Bact. Lab. Stockholm, Sweden.

Assay of the proteolytic activity

Determination of the proteolytic activity of the two proteases was carried out with gelatin, hemoglobin and poly-L-lysine hydrobromide (PLL).

Gelatin (U.S.P. gran., Fisher Sc. Co., N.J., U.S.A.) splitting activity was assayed by the change in viscosity of gelatin solution according to the method of *Hultin* (1946, 1948) and *Lundblad* (1952, 1962), *Lundblad et al.* (1966) and *Lundblad & Hultin* (1966) and described in a previous paper (*Söder,* in press): 1.0 ml of the enzyme fraction was mixed with 3.0 ml of a 4 per cent gelatin solution in 0.1 M TRIS-HCl buffer, pH 7.5 or 8.5, containing 0.01 per cent merthiolate as bactericidal agent. The outflow times of the substrate-extract mixtures were measured in Ostwald viscosimeters at 37° C for 20 hours and the proteolytic activity was calculated according to *Hultin*'s formula. The values so obtained were multiplied by 10^9 and called *Hultin* units (H.U.).

Hemoglobin splitting activity was measured by the method of *Anson* (1938), *Ruyssen & Lauwers* (1963), using urea denatured bovine hemoglobin (Difco lab., Michigan, U.S.A.). The reaction mixture consisted of 1.0 ml of the dental plaque extract, 2.0 ml 0.2 M TRIS-HCl buffer, pH 7.5 or 8.5 and 1.0 ml of a 4 per cent hemoglobin solution. The reaction mixture was incubated in a water bath at 37° C for 20 hours.

The enzymatic reaction was stopped by adding 5.0 ml 0.3 M trichloroacetic acid (TCA). The amount of amino acids released was determined spectrophotometrically at 280 mμ using tyrosine as a standard.

Poly-L-lysine hydrobromide (PLL) (Pilot Chemical Inc., Watertown, Mass., U.S.A.) (*Fasman et al.,* 1961; *Katchalski et al.,* 1954, 1961) with a molecular weight of 115,000 was made into a 2 per cent stock solution by dissolving 0.2 g substrate in 10.0 ml 0.20 M NaCl. The reaction mixture consisted of 1.50 ml stock substrate solution, 1.50 ml 0.2 M glycine-NaOH buffer, pH 9.1 and either 1.0 ml of the dental plaque extract or 1.0 ml corresponding buffer. A viscosimetric method was used to measure the proteolytic activity (*Hultin,* 1948) at a temperature of 37° C for various periods of time as described earlier (*Söder,* in press).

Other information is given in the figure legends.

EXPERIMENTS AND RESULTS

Figure 3 shows a typical example of gel filtration separation representative of more than 30 separations of pooled material or material from one person. There were two main protein peaks demonstrable. The first of high molecular weight and containing

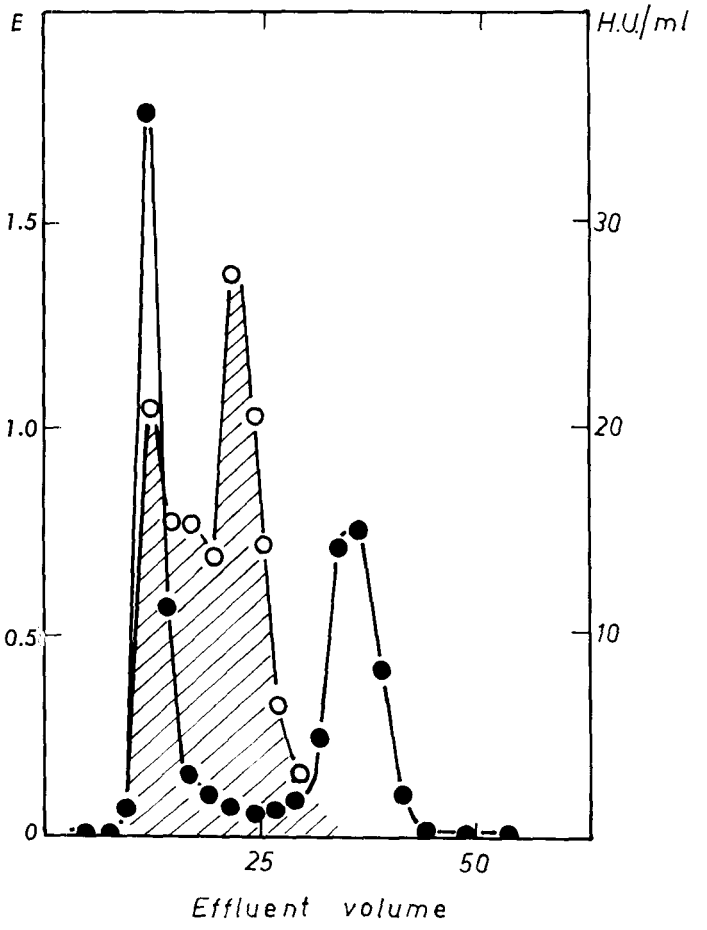


Fig. 3. The fractionation of 1.7 ml dental plaque supernatant pooled from 3 persons (16.0 mg protein) through a 0.60×165 cm Sephadex G-100 column in 0.1 M TRIS-HCl buffer (pH 9.0), 0.5 M NaCl and 1 per cent butanol as bactericidal agent. The flow rate was 4.5 ml/hour and the fractions were collected in 2.4 ml volumes. ●——● = E_{280}; o///o = H.U./ml.

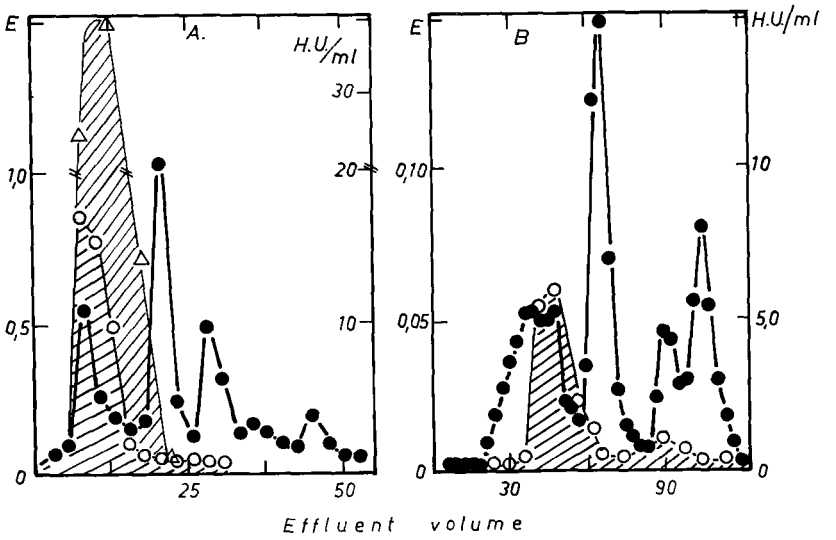


Fig. 4. a) Fractionation of 1.5 ml dental plaque supernatant pooled from three different patients by zone electrophoresis on a 0.60×80 cm column. The electrophoresis was carried out for 2 hours at 770 V, 43 mA. The stabilizing medium was Sephadex G-25 in a 0.27 M TRIS-HCl buffer (pH 8.6). The elution was made with the same buffer at a flow rate of 5.5 ml/hour.
●——● = E_{280}, o///o = unactivated protease, (H.U./ml),
Δ///Δ = protease activated with 0.125 M Ca++.

b) The fractionation of pooled dental plaque supernatant from 4 persons (1.5 ml) by zone electrophoresis through a 1.9×70 cm column of Sephadex G-25 as stabilizing medium. The electrophoresis was carried out for 17.5 hours at 980 V, 32 mA. in 0.1 M boric acid-NaOH buffer (pH 9.5). The elution was made in the same buffer at a flow rate of 4.7 ml/hour.
●——● = E_{280}, o///o = H.U./ml.

a proteolytic enzyme active against gelatine and hemoglobin. The second protein peak was of lower molecular weight. The second protease was found between the protein peaks. Further studies on those enzyme fractions usually were made after this basic fractionation and the enzyme fractions were studied separately.

When zone electrophoresis was carried out on the dental plaque supernatant only one main enzyme peak was obtained (Fig. 4). The zone electrophoresis were carried out for 2 hours at 770 V, 43 mA. or for 17.5 hours at 980 V, 32 mA. If the pro-

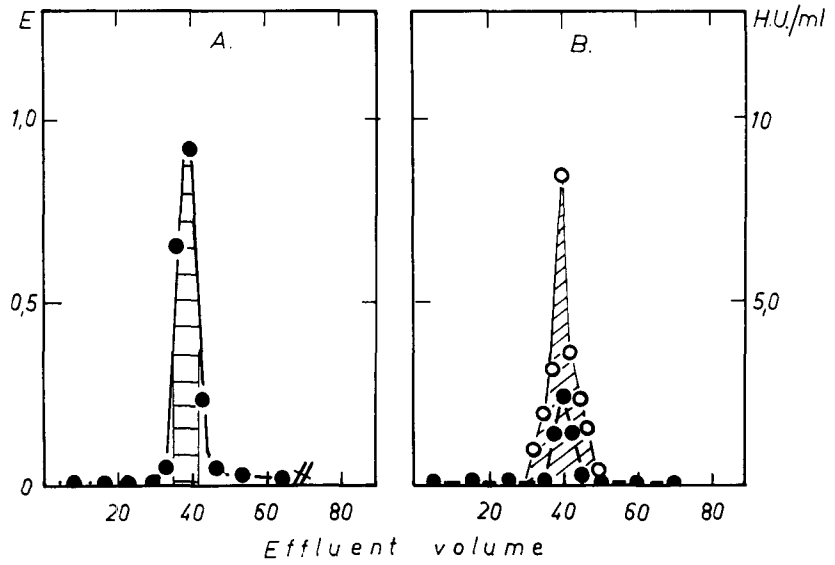


Fig. 5 a) Pooled dental plaque material from three persons (12.0 mg protein) was filtered through a 2.2×60 cm Sephadex G-100 column with 0.27 M TRIS-HCl buffer (pH 8.6), and 1 per cent butanol as bactericidal agent. Flow rate = 20 ml/hour. The diagram shows only the first protein peak. ●——● = E_{280}. The shaded area was taken for zone electrophoresis in b.

b) Zone electrophoresis of the two fractions from the first protein peak, containing 3.8 mg protein, was run for 2 hours at 300 V and 60 mA. in the same buffer and Sephadex supporting medium as in (a). The flow rate was about 20 ml/hour. ●——● = E_{280}, o///o = H.U./ml.

tease was activated with Ca^{++} the base of the gelatinolytic peak became a little broader (fraction 6 to 19 ml Fig. 4 a). However, the activated peak was not symmetrical with the unactivated enzyme which suggests the presence of two enzymes in these fractions. The proteins, however, were well separated into many more components than observed in gel filtration.

Alternatively the dental plaque extracts were fractionated first on Sephadex G-100 and the fractions containing the first protein peak and Protease I were pooled, concentrated as described earlier and transferred to the electrophoresis column. The zone electrophoresis were run for shorter or longer periods of time (Fig. 5 and 6 respectively).

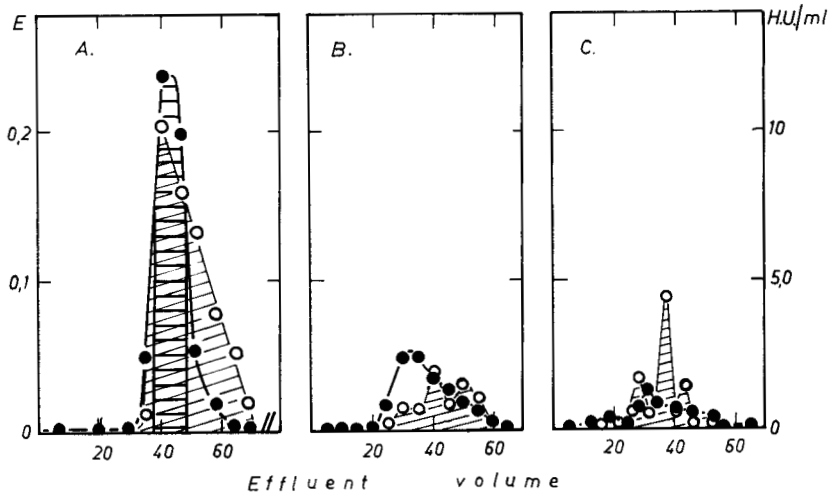


Fig. 6 a) Pooled dental plaque supernatant from 4 different patients (14.0 mg protein) separated on a 2.2×60 cm Sephadex G-100 column in 0.27 M TRIS-HCl buffer (pH 8.6) and 1 per cent butanol. Flow rate about 20 ml/hour. 1.0 ml of each fraction was used for gelatinolytic activity determination. ●——● = E_{280}, o///o = H.U./ml. The shaded area (marked ▤) was taken for zone electrophoresis.

b) The half fraction from 6 a containing 2.4 mg protein was used for zone electrophoresis in the same gel and buffer for 19 hours at 300 V and 60 mA. The flow rate was about 20 ml/hour.

c) A ¼ fraction from 6 a containing 1.2 mg protein was used for zone electrophoresis in the same gel and buffer for 17 hours at 250 V and 60 mA. The elution flow rate was about 20 ml/hour.

The short time experiments for 2 hours at 300 V and 60 mA. revealed almost no movement of either the proteins or Protease I (Fig. 5 a and b).

However, when the electrophoresis was run for 17 or 19 hours a better separation of the proteins was obtained (Fig. 6). Figure 6 a shows the initial activity of the first protease located in Sephadex fractions. After pooling and concentrating these fractions they were fractionated further by zone electrophoresis. The protein and gelatinolytic distribution is shown in Fig. 6 b and c. The proteins were distributed in small fractions not quite separated from each other, while the enzyme activity was divided in three main fractions approximately corresponding to the proteins in

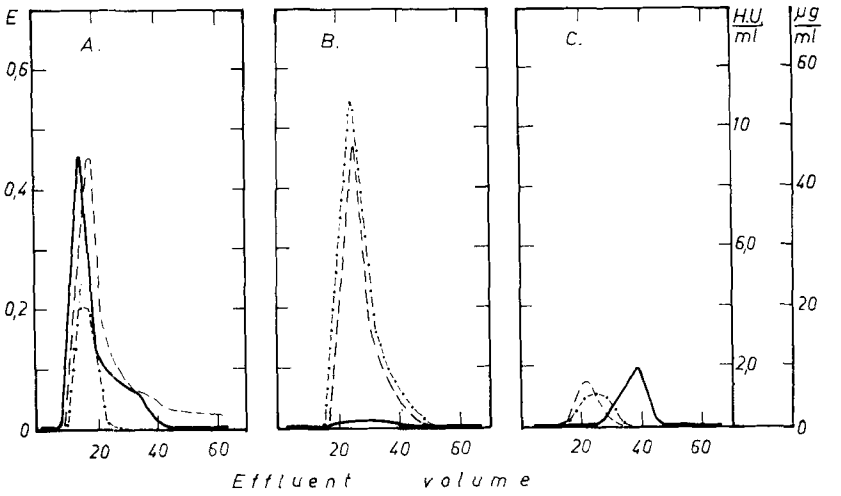


Fig. 7. Recycling experiments of the various fractions from the separation of pooled dental plaque supernatant shown in Fig. 3. The fractions were collected in 3 ml volumes. ———. = E_{280}. — — — = H.U./ml, —.—.— = µg tyrosine/ml (hemoglobinolytic activity).

a) The fractions 9 to 17 ml from Fig. 3 were pooled, concentrated and applied on a 0.60×165 cm Sephadex G-100 column after determination of the initial activity.

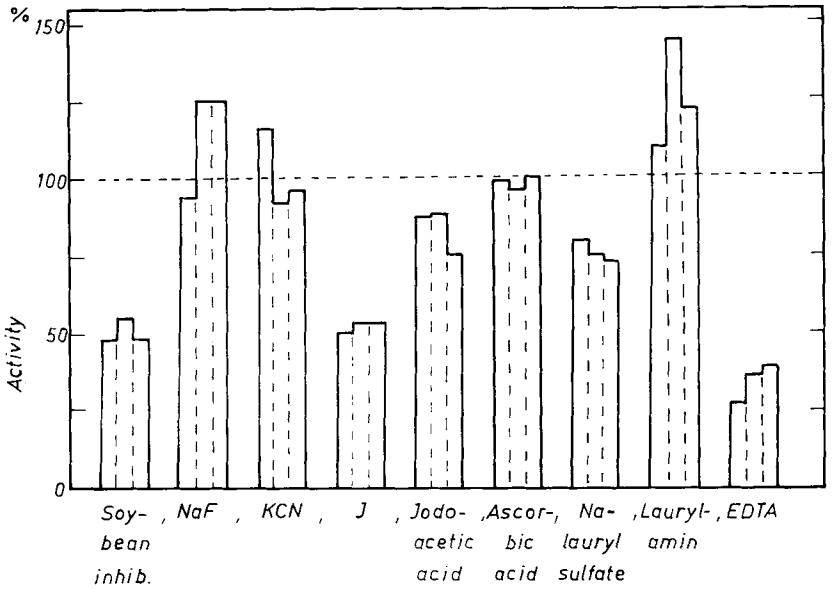
b) The fractions 18 to 25 ml from Fig. 3 were pooled, concentrated and applied to the same column as (a), after determination of the initial activity.

c) The fractions 26 to 34 ml from Fig. 3 were treated in the same way as (a) and (b).

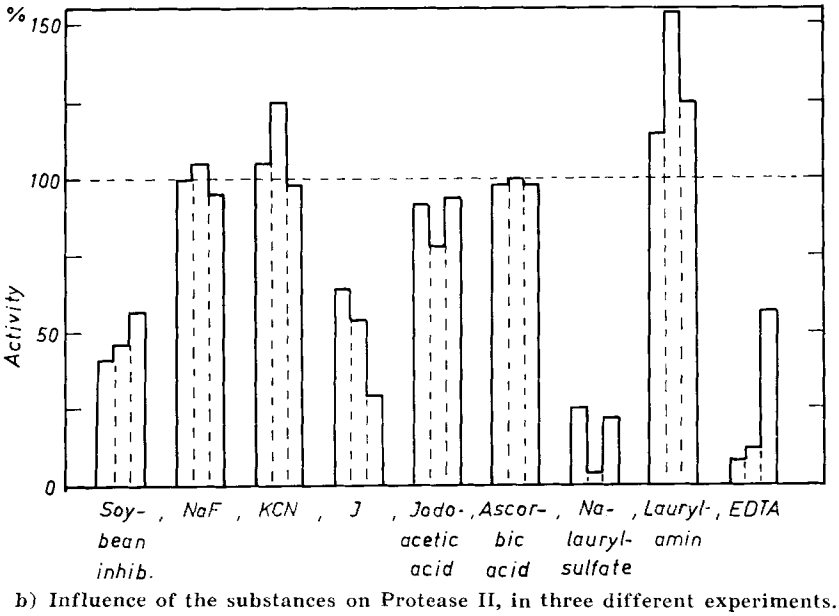
the different fractions. The gelatinolytic activity of the different fractions varied between 2 and 3 H.U./ml and was low compared to the initial activity (11 H.U./ml). The gelatinolytic activity was divided into fractions which all contained relatively large amount of proteins (Fig. 6 c).

The results of the recycling experiments are shown in Fig. 7. After separation of the two protein peaks and the Protease II on Sephadex G-100 the fractions containing protein or enzyme peaks were pooled and concentrated and a second filtration was carried out. The optical density of the fractions obtained was measured and the proteolytic activity determined. Figure 7 a shows that the first protein peak was separated into a main peak which

Diagram I. Influence of different substances on the proteolytic activity of the purified enzyme fractions. The enzymes were incubated with the substance for 15 minutes before assay. The gelatinolytic activity was then determined for 20 hours at 37°C at pH 8.1 with the substances present during the enzyme assay. The substances were tested in a final concentration of 10^{-2} M except soy bean trypsin inhibitor which was tested in final concentration of 0.02 per cent.



a) Influence of the substances on Protease I, in three different experiments.



b) Influence of the substances on Protease II, in three different experiments.

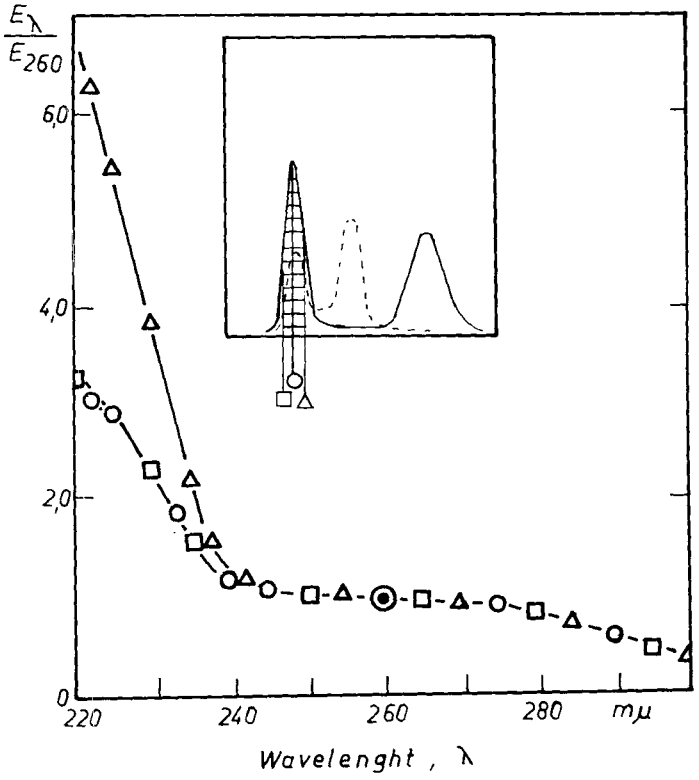


Fig. 8. Ultraviolet absorption spectra of the different fractions after separation on a 0.60×165 cm Sephadex G-100 column in 0.1 M TRIS-HCl buffer pH 8.1.

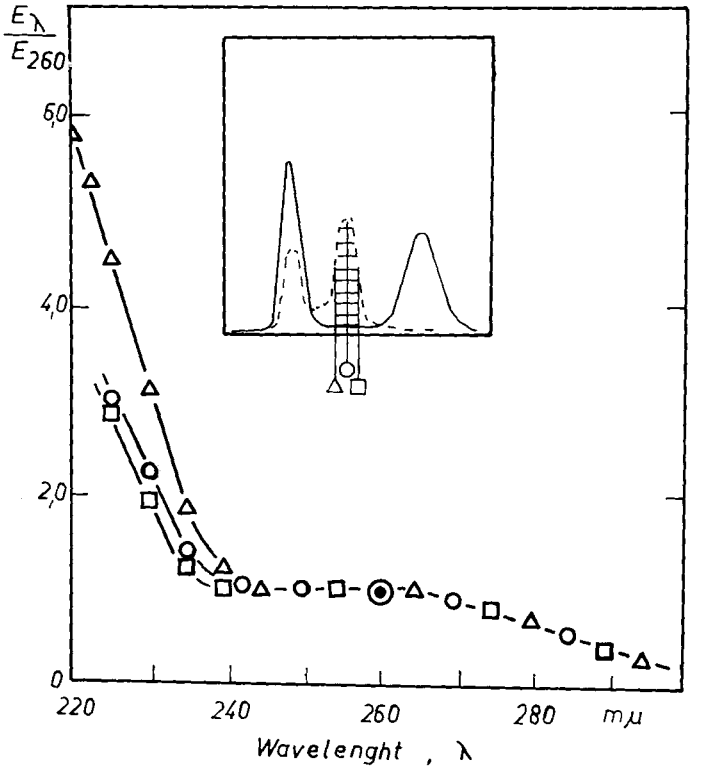
a) Protein and Protease I fractions, according to the inserted separation figure.

trailed off into a second smaller one; however, maximum protease activity was no longer situated in the fraction containing the protein maximum.

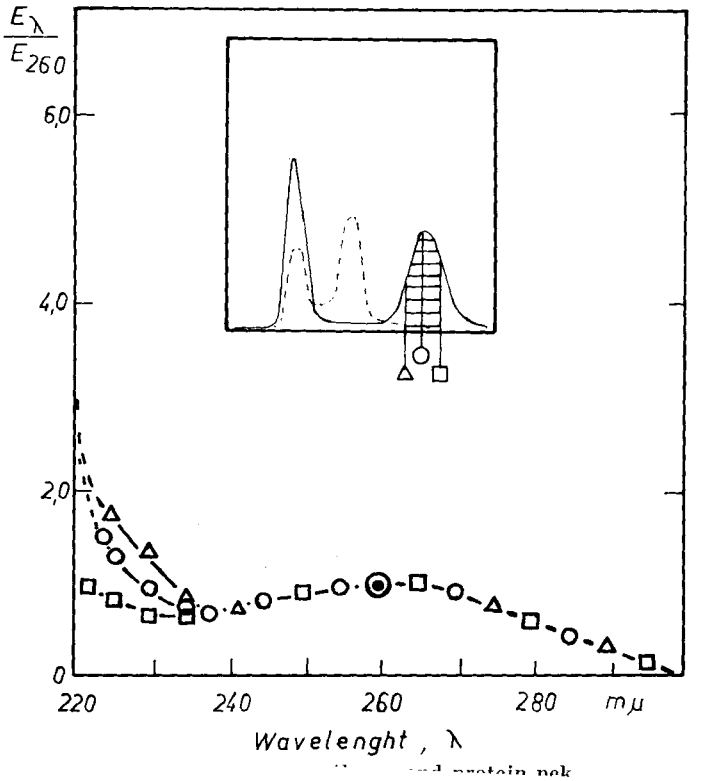
As can be seen from figure 7 b Protease II was obtained relatively pure and the activity against gelatin and hemoglobin was high.

The second protein peak showed almost no activity against hemoglobin and gelatin (Fig. 7 c).

The effect of various substances on the proteolytic activity of Protease I and II are shown in diagram I. Soy bean trypsin inhibi-



8 b) Protease II fractions.



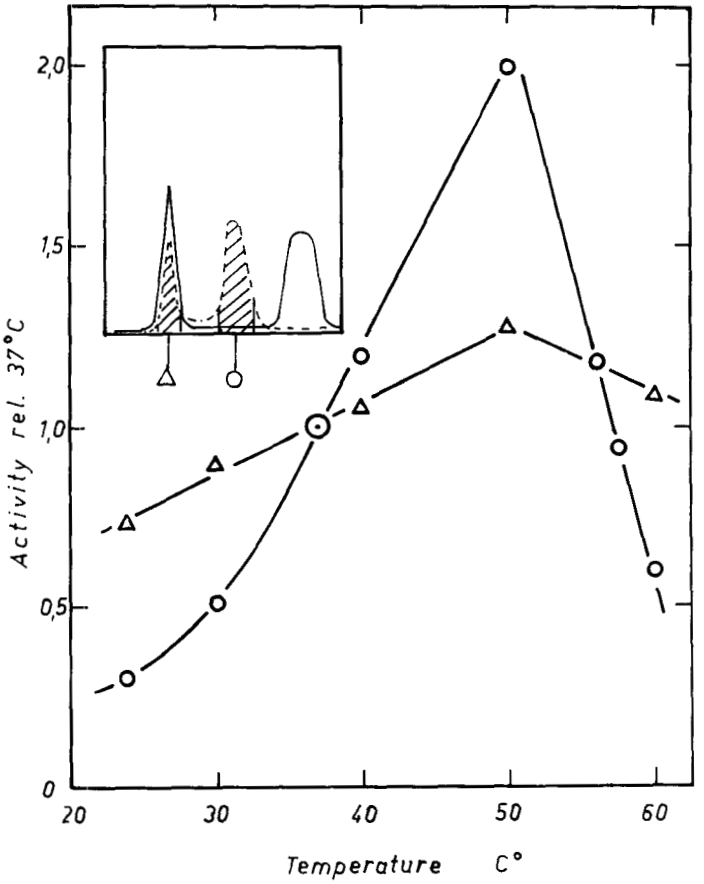


Fig. 9. Effect of incubation temperature on hemoglobinolytic activity over a period of 18 hours at pH 8.0. Δ——Δ = Protease I, o——o = Protease II.

tor, iodine, Na-lauryl sulfate and EDTA, had an inhibiting effect on both enzymes. EDTA and Na-lauryl sulfate had a greater inhibiting effect on Protease II. Laurylamin had an activating effect on Protease I and II. Ascorbic acid, KCN and NaF had a low varying effect. Iodoacetic acid had a slight inhibiting effect on Protease I and II.

Ultraviolet absorption spectra of different parts of the separation curve from a Sephadex G-100 fractionation are shown in Fig. 8 The absorption at 260 and 280 mμ for the proteases and

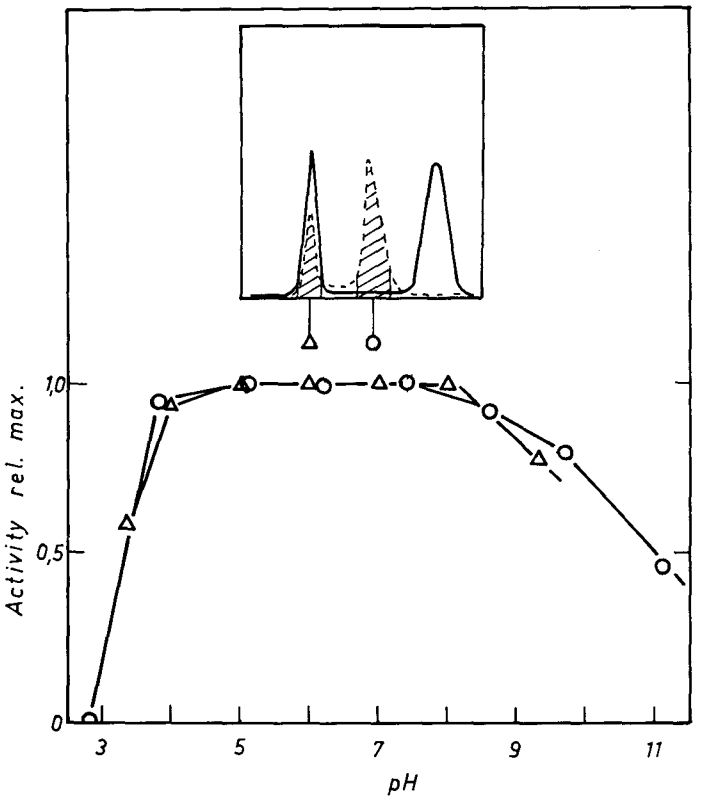


Fig. 10. Effect of pH on the stability of proteases. The enzymes were incubated for 30 minutes at 37°C in different buffers at the desired pH. The initial activity was determined at pH 7.8. The remaining hemoglobinolytic activity was determined after 20 hours incubation at 37°C at pH 7.8. △——△ = Protease I, o——o = Protease II.

the protein peaks showed that these fractions contained not only proteins but nucleic acids as well (*Chargaff & Davidsson*, 1955).

The effect of incubation temperature on the enzymatic activity of the two proteases are shown in Fig. 9. Protease I activity showed relatively small changes with temperature and at the maximum activity temperature (about 50° C) only a 25 per cent rise over the 37° C activity was noted. At 22° C the activity decreased about 25 per cent. There was still activity at 60° C.

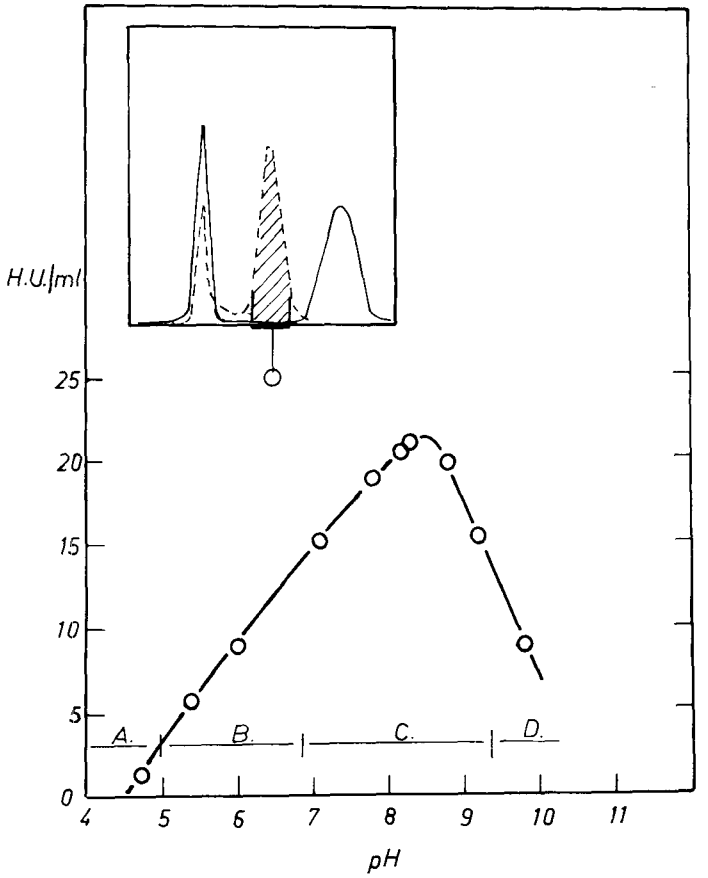


Fig. 11. Effect of pH on the proteolytic activity of Protease II using gelatin as substrate. The activity was determined after 20 hours incubation at 37°C as H.U./ml. The pH's were determined at the end of the experimental period. A = glycine-NaOH, B = succinic acid-NaOH, C = TRIS-HCl, D = glycine-NaOH.

Protease II was much more sensitive to changes in temperature. At 50° C the activity was twice as high as at 37° C. Only 50 per cent of the activity was left at 60° C and about 25 per cent at 22° C. As can be seen from Fig. 9, the optimum temperature for both proteases was found close to 50° C.

The effect of pH on the stability of the two proteases is shown in Fig. 10. pH inactivation of the proteases was tested by ex-

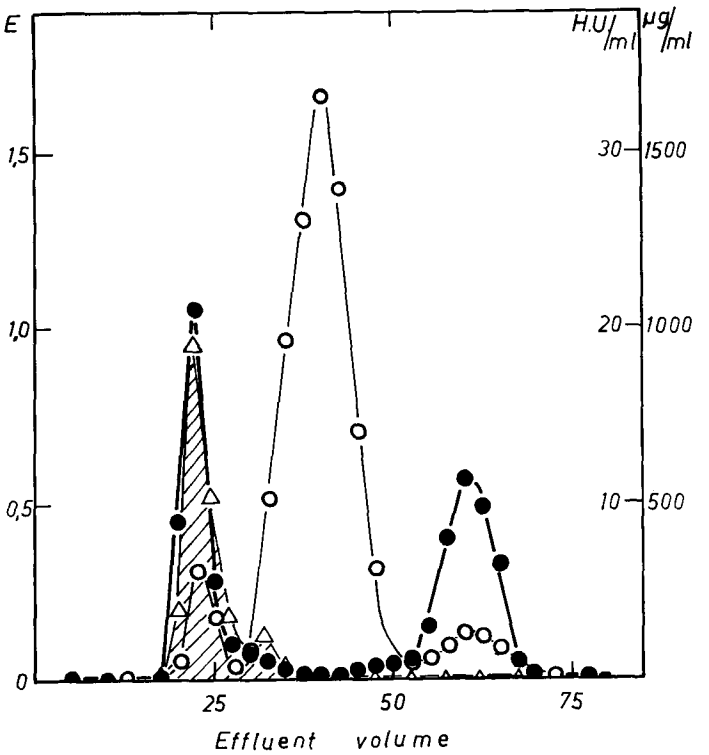


Fig. 12. Separation of 2.0 ml pooled plaque supernatant containing 10.0 mg protein on a 0.64×170 cm Sephadex G-100 superfine column in 0.1 M TRIS-HCl buffer (pH 8.1), 0.5 M NaCl and 1 per cent butanol as bactericidal agent. The fractions were collected in 2.6 ml volumes. The hydrolysis of the poly-L-lysine hydrobromide was carried out in 0.2 M glycine-NaOH buffer (pH 9.1) for 5 hours at 37°C. ●——● = E_{280}, △///△ = hydrolysis of poly-L-lysine expressed in Hultin units, o——o = µg tyrosine (hemoglobinolytic activity).

posing the enzymes to a desired pH for 30 minutes at 37° C and then determining the activity remaining at a pH where they were stable (pH 7.8). The two proteases were found to be stable over a very wide pH range. The activity remaining of both proteases after exposure to a pH ranging from 3.5 to 8.5 were almost as high as the initial. The activity decreased very rapidly on the acid side and none was observed at pH 2.8. On the alkaline side the activity decreased less rapidly.

The influence of pH on the gelatinolytic activity of Protease II is shown in figure 11. The maximum of activity was at pH 8.5.

Action on a synthetic substrate. The proteolytic activity of the two proteases on the synthetic substrate poly-L-lysine hydrobromide (pH 9.1) was studied (Fig. 12). Only Protease I had hydrolysing effect on poly-L-lysine; Protease II had no effect at all.

DISCUSSION

These gel filtration experiments confirmed earlier work by *Söder, Lundblad & Lindqvist* (1966) that dental plaque supernatant after low speed centrifugation contained two main protein peaks after separation by gel-filtration, one of high and the other of low molecular weight (Fig. 3). Two well separated proteolytic enzyme fractions were found also.

Attempts to separate the high molecular proteins from Protease I with zone electrophoresis (Fig. 4—6) suggests that the enzyme was either closely coupled to or a part of these proteins. Some of the activity was lost during the electrophoresis experiments (Fig. 6), which may represent a component labile to such treatment.

The ultraviolet absorption spectra of the high molecular peak suggests the presence of both proteins and nucleic acids. It is possible that these components are combined in some form of macromolecular particle.

On the other hand recycling the high molecular peak showed that the maximum activity of Protease I was displaced slightly from the maximum protein content, which suggests that the proteolytic activity may be separated from the high molecular protein component with the proper techniques.

Another difference between the two proteases was shown by the effect the substances listed in Diagram I had on their activity.

Furthermore, Fig. 9 demonstrated that Protease II was more sensitive than Protease I to changes in incubation temperature.

The two proteases, however, reacted similarly in their stability at various pH's (Fig. 10). They were stable over the pH range of 3.5 to 8.5 with a gradual decrease in stability at higher pH's.

When the possible rôle of proteolysis in dentin caries and periodontal disease is discussed, Protease II must be considered more likely to be involved due to its molecular weight, its activation by calcium and its stability over a great part of the pH scale.

Interesting results, however, were obtained with the synthetic substrate poly-L-lysine. This substrate had been used earlier (*Söder,* Part IV, in press) and was shown to be a very good substrate for studying proteolytic enzymes in dental plaque extract. The present results, however, demonstrated that only Protease I could hydrolyse this substrate (Fig. 12). This is perhaps the most specific difference between the two enzymes.

Further investigations of these enzymes are in progress.

This work was supported by the Swedish medical Research Council (Project No 24 ×-60-02).

SUMMARY

Proteolytic activity of dental plaque supernatant after low speed centrifugation has been studied after separation by gel-filtration on Sephadex. Two main protein fractions and two different proteases (I and II) were obtained. Further separations of the enzyme fractions were carried out by zone electrophoresis and recycling. The ultra-violet absorption spectra of the proteases revealed that nucleo proteins were present. The effect on temperature and pH on the two proteases as well as the activating and inhibiting influences of various substances were studied. The high molecular Protease I was active against the synthetic substrate poly-L-lysine hydrobromide which Protease II could not hydrolyse.

RÉSUMÉ

ACTIVITÉ PROTÉOLYTIQUE DE LA PLAQUE DENTAIRE

Le surnageant de substance de plaques dentaires centrifugée à une vitesse réduite (2.500 g) séparé par filtration sur gel Sephadex a été étudié en ce qui concerne la présence d'une activité protéolytique. On a obtenu deux fractions protéiniques essentielles et deux différentes protéases (I et II). De plus on accompli la

séparation de ces enzymes par électrophorèse de zone réitérée. Le spectre d'absorption UV de ces protéases a révélé la présence de nucléoproteines.

En outre on a étudié l'action de la température et du pH sur les deux protéases ainsi que l'activation ou l'inhibition éventuellement produite par différentes substances. La protéase I, de poids moléculaire élevé était active envers le substrat synthétique poly-L-lysine-hydrobromide que la protéase II ne pouvait hydrolyser.

ZUSAMMENFASSUNG

PROTEOLYTISCHE AKTIVITÄT IN ZAHNBELAGMATERIAL

Der Supernatteil von bei niedriger Geschwindigkeit zentrifugiertem Zahnbelagmaterial wurde nach Separation mittels Sephadex-Gelfiltration auf proteolytische Aktivität geprüft. Zwei proteine Hauptfraktionen und zwei verschiedene Proteasen (I und II) wurden erhalten. Weitere Separationen dieser Enzyme wurden mit Hilfe von wiederholter Zonenelektrophorese erarbeitet. Die Ultraviolett-Absorptionsspektra der Proteasen ergaben die Gegenwart von Nukleoproteinen.

Der Effekt von Temperatur und pH auf beide Proteasen wie auch der aktivierende und/oder inhibierende Einfluss verschiedener Substanzen wurden untersucht. Die hochmolekulare Protease I erwies sich als aktiv gegenüber dem synthetischen Substrat Poly-L-Lysin-hydrobromid, welches die Protease II nicht zu hydrolisieren vermochte.

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