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FRACTIONATION OF HYALURONIDASES FROM DENTAL PLAQUE MATERIAL BY ION-EXCHANGE CHROMATOGRAPHY AND GELFILTRATION

by

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INTRODUCTION

In an earlier article (*Nord et al.*, 1969) hyaluronidases from dental plaque material were separated on Sephadex G-200, Bio-Gel A-1.5m and Bio-Gel A-5m columns. Two fractions with hyaluronidase activity were often demonstrated after gelfiltration through Sephadex G-200. The optimal pH and thermostability of the hyaluronidase activity in the two fractions were studied also.

The object of the present study was to attempt to obtain further separation of the hyaluronidase activity from dental plaque material through ion-exchange chromatography and gelfiltration.

MATERIAL AND METHODS

Dental plaque material was collected from 50 healthy patients (25 men and 25 women) according to a method described by *Söder* and *Frostell* (1966). In the experiments pooled material from 4—6 patients was used. The wet weight of the pooled material was determined on a Sartorium Selecta balance. The material was then suspended in 0.005 M imidazole-HCl buffer (pH 6.5) and homogenized in a Virchow glass mortar ten times with one turn of the pestle each time. The suspension was centrifuged at 20,000 × G (type IEC

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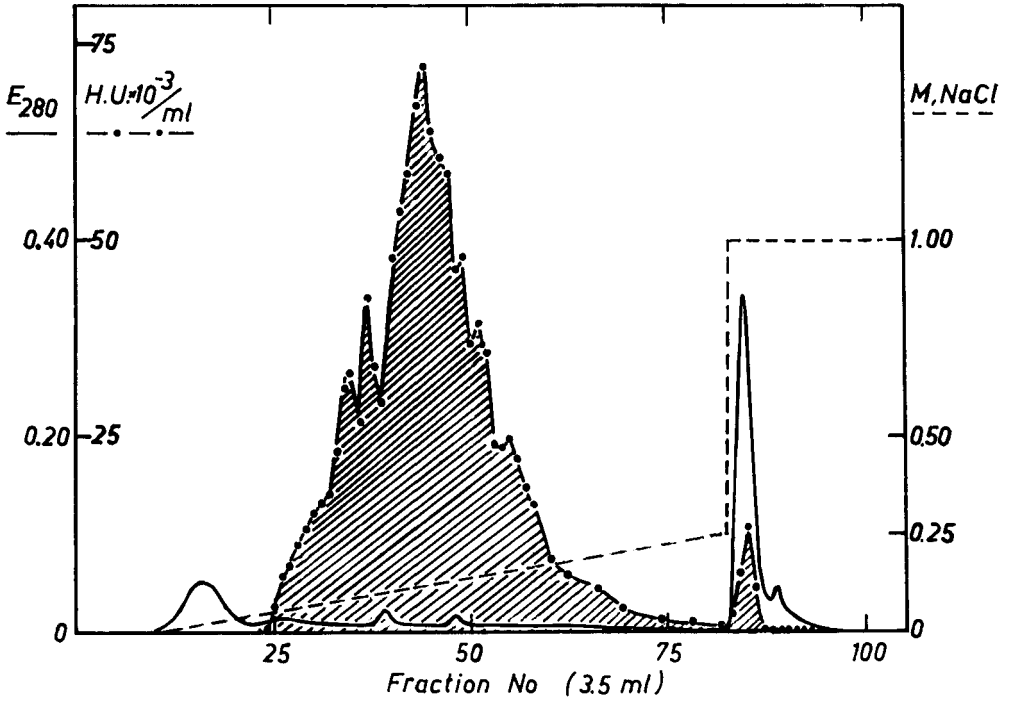


Fig. 1. Chromatography of dental plaque supernatant (3.7 ml) on a 1.8×28 cm DEAE-Sephadex A-50 column. The chromatogram was developed with 0.005 M imidazole-HCl (pH 6.5) plus continuously increasing NaCl concentrations. The flow rate was 66 ml/hour at 21—23°C.

centrifuge) for 15 minutes at 4°C. The supernatant was dialyzed against 0.005 M imidazole-HCl buffer (pH 6.5) for 20 hours at 4°C and used for determination of the hyaluronidase activity and the chromatography experiments.

Ion-exchange chromatography

DEAE-Sephadex A-50 (Pharmacia, Uppsala, Sweden) was used for the ion-exchange chromatography of hyaluronidases from dental plaque material. Before packing the column DEAE-Sephadex A-50 was suspended in 0.5 M imidazole-HCl buffer at pH 6.5. The adsorbent was allowed to settle for 24 hours and the smallest particles were removed. The adsorbent was washed several times on a Büchner funnel with 0.005 M imidazole-HCl buffer (pH 6.5) and was then resuspended in the same buffer. The column was packed with the adsorbent and equilibrated with 0.005 M imidazole-HCl buffer (pH 6.5).

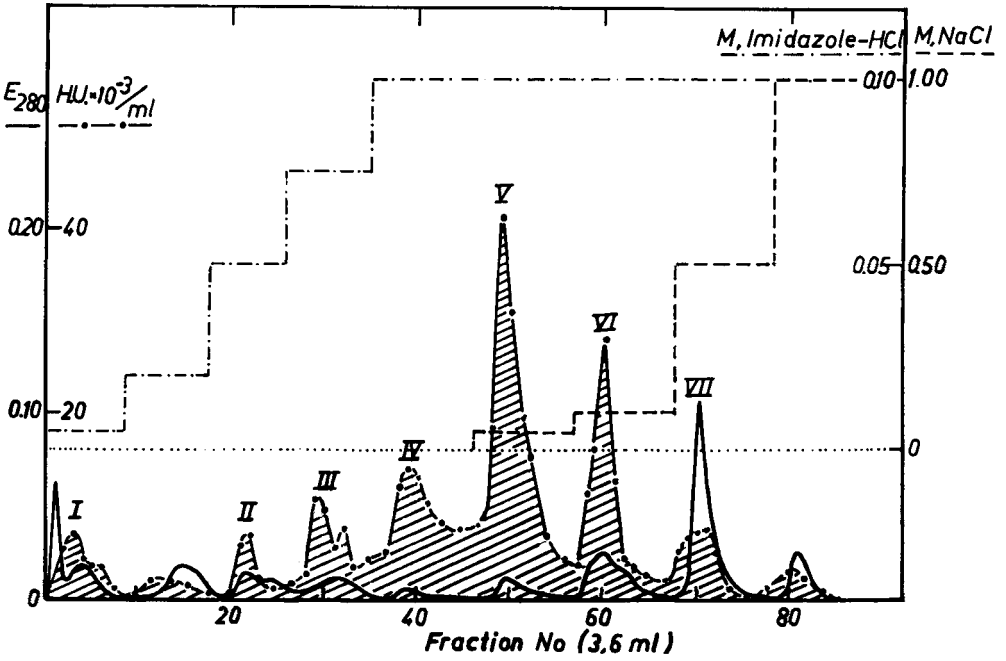


Fig. 2. Chromatography of dental plaque supernatant (10.0 ml) on a 1.2×3 cm DEAE-Sephadex A-50 column. The chromatogram was developed with stepwise increasing concentrations of imidazole buffer and NaCl. The flow rate was 84 ml/hour at 21—23°C.

The dimensions of the columns and other data are given in the figure legends. In one experiment (Fig. 1) with DEAE-Sephadex A-50 the chromatogram was developed with an elutant of continuously increasing ionic strength. First, 0.005 M imidazole-HCl (pH 6.5) was used and after 40 ml NaCl was added in such a way that a continuously increasing ionic strength of NaCl from 0—0.25 M was obtained and after a total volume of 290 ml, 40 ml of 0.100 M imidazole-HCl (pH 6.5) with 1.00 M NaCl was passed through the column.

In two experiments (Fig. 2 and 3) the elution was performed with a stepwise increasing gradient of 1) 0.005, 2) 0.020, 3) 0.050, 4) 0.075, 5) 0.100 M imidazole-HCl buffer (pH 6.5), 6) 0.100 M imidazole HCl buffer with 0.050 M NaCl, 7) 0.100 M imidazole-HCl buffer with 0.100 M NaCl, 8) 0.100 M imidazole-HCl buffer with 0.500 M NaCl and 9) 0.100 M imidazole-HCl buffer with 1.00 M NaCl. The effluent was collected in a fraction collector (LKB, Stockholm, Sweden) in fractions of equal volumes.

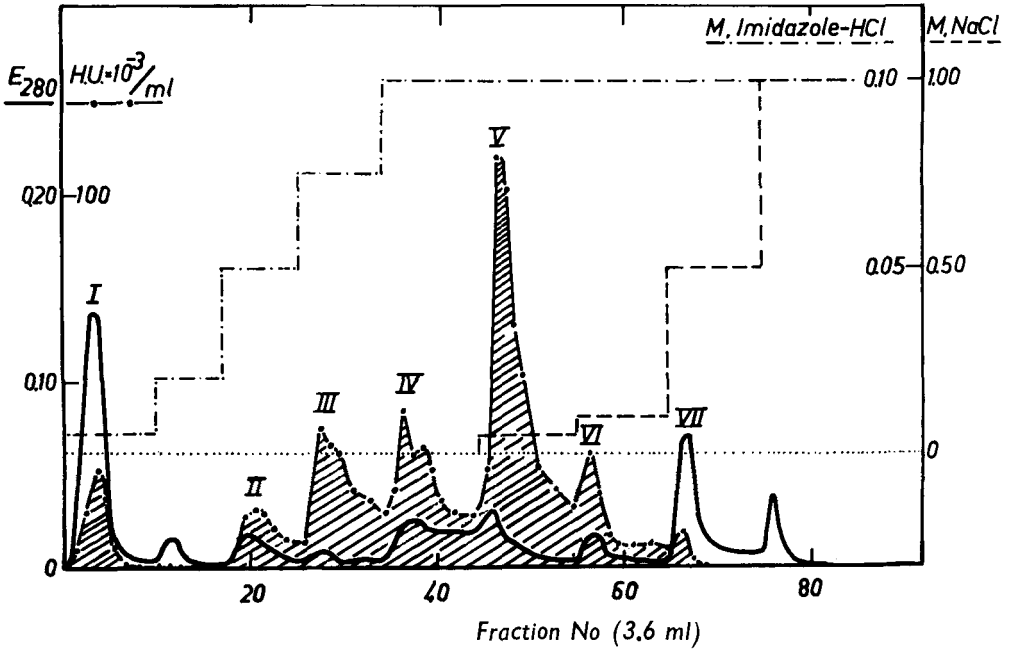


Fig. 3. Chromatography of dental plaque supernatant (9.5 ml) on a 1.2×5 cm DEAE-Sephadex A-50 column. The chromatogram was developed with stepwise increasing concentrations of imidazole buffer and NaCl. The flow rate was 75 ml/hour at 21—23°C.

Gelfiltration

Bio-Gel A-0.5m (Bio-Rad, Richmond, California, USA) was used for rechromatography of the hyaluronidase activity peaks.

The gel was treated according to the manufacturer's instructions. The size of the column and other data are given in the figure legends. The elution was made at 21—23°C with 0.005 M Tris-HCl buffer in 0.5 M NaCl, pH 8.1. The medium contained 2 per cent butanol as bactericidal agent. The effluent was collected in a fraction collector (LKB, Stockholm, Sweden) in fractions of equal volumes. The fractions were specified by the ratios between the elution volume (V_e) and the void volume (V_o) (*Flodin*, 1962). The column void volume (V_o) was determined by using Blue dextran 2000 (Pharmacia, Uppsala, Sweden).

Protein determination

The optical density of the fractions was measured at 260 and 280 nm in a Beckman spectrophotometer model DU with a 1 cm light path cell.

Assay of hyaluronidase activity

Hyaluronidase activity was determined in Ostwald viscosimeters at 37°C according to a method elaborated by *Hultin* (1946, 1947, 1948) and used in studies by *Söder* and *Nord* (1969). The enzyme activity was calculated from the formula of *Hultin;* the values were multiplied by 10^9 and called Hultin units (H.U.).

EXPERIMENTS AND RESULTS

Figure 1 shows the fractionation of hyaluronidase activity in a pooled dental plaque supernatant by ion-exchange chromatography on DEAE-Sephadex A-50 with an eluant continuously increasing in ionic strength. The main part of the hyaluronidase activity was found between 0.06—0.25 M NaCl in 0.005 M imidazole-HCl and a small part at 0.100 M imidazole-HCl with 1.00 M NaCl. The maximum activity peak was found at about 0.13 M NaCl in 0.005 M imidazole-HCl.

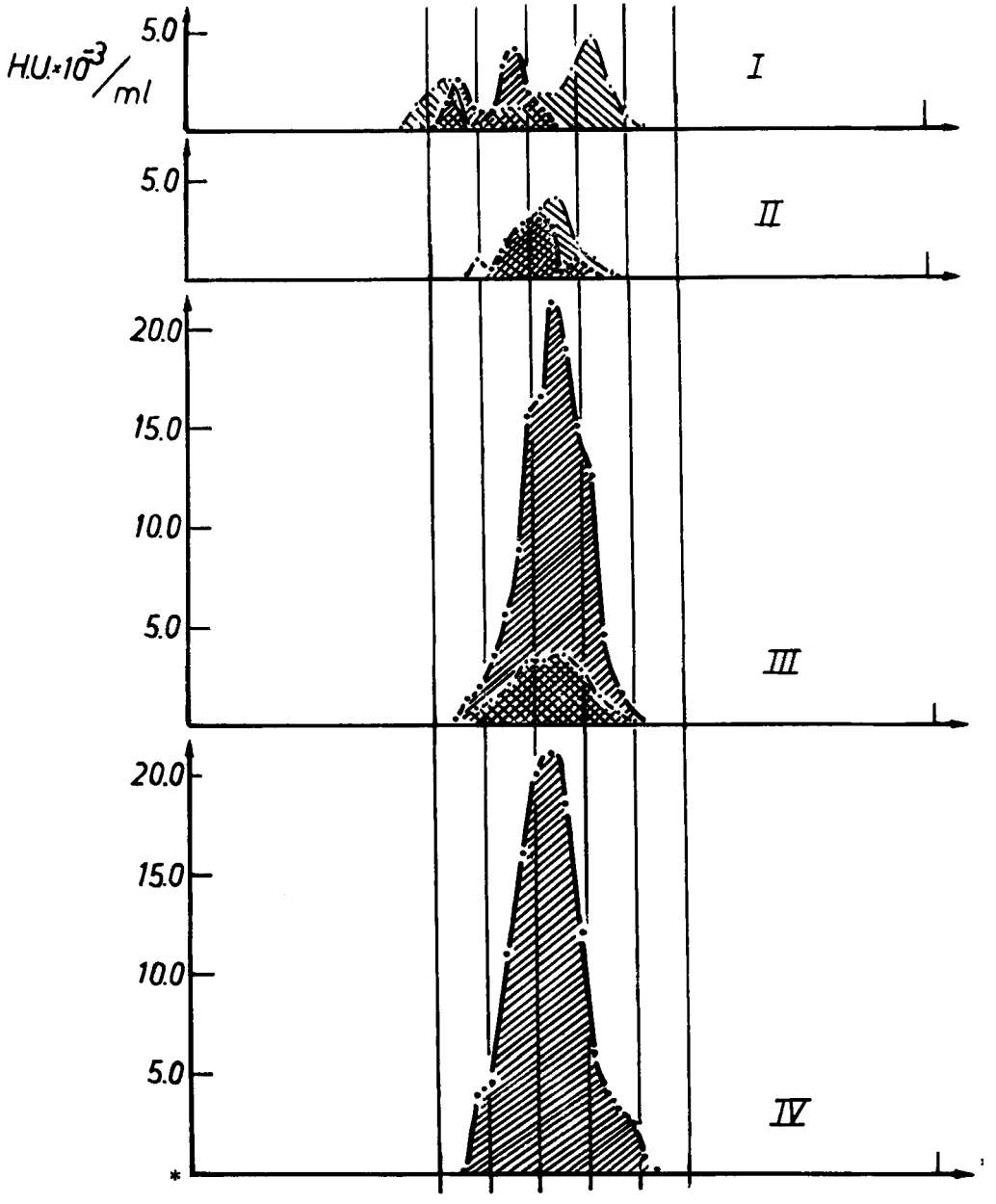
A fractionation with a stepwise increasing gradient of imidazole buffer and NaCl concentration is shown in Figure 2. The hyaluronidase activity in peak I was found at 0.005 M imidazole-HCl, peak II at 0.050 M imidazole-HCl, peak III at 0.075 M and peak IV at 0.100 M imidazole-HCl. The highest hyaluronidase activity peak (V) appeared at 0.100 M imidazole-HCl with 0.050 M NaCl. Finally, peak VI at 0.100 M imidazole-HCl with 0.100 M NaCl and peak VII at 0.100 M imidazole-HCl with 0.500 M NaCl were obtained.

Another fractionation on DEAE-Sephadex A-50 with stepwise increasing ionic strength (Fig. 3) showed that similar peaks of hyaluronidase activity were achieved. In this experiment peak V represented about 48 per cent of the total enzyme activity, peak I, II, III and IV about 45 per cent together and peak VI and VII about 7 per cent together.

Before rechromatography on Bio-Gel A-0.5m the material in the peaks marked in Figures 2 and 3 were concentrated in a Visking tube against sucrose at 21—23°C. The concentrated peaks were called fractions I—VII.

After gelfiltration of fraction I activity peaks were obtained between V_e/V_o =1.00—1.20, 1.30—1.40 and 1.60—1.70. Fraction II gave one activity peak around 1.45—1.50. One activity peak between 1.40—1.50 was obtained from fraction III as well as fraction IV. Fraction V showed a maximum activity peak around 1.47—1.49. One broad activity peak was found between 1.30—1.60 in fraction VI and the maximum activity in fraction VII appeared around 1.46—1.48. (Fig. 4).

Table I shows the gelfiltration V_e/V_o values of the maximum activities in the different fractions.


H.U.×10⁻³/ml
5.0
I
5.0
II
20.0
15.0
10.0
5.0
III
20.0
15.0
10.0
5.0
IV
*

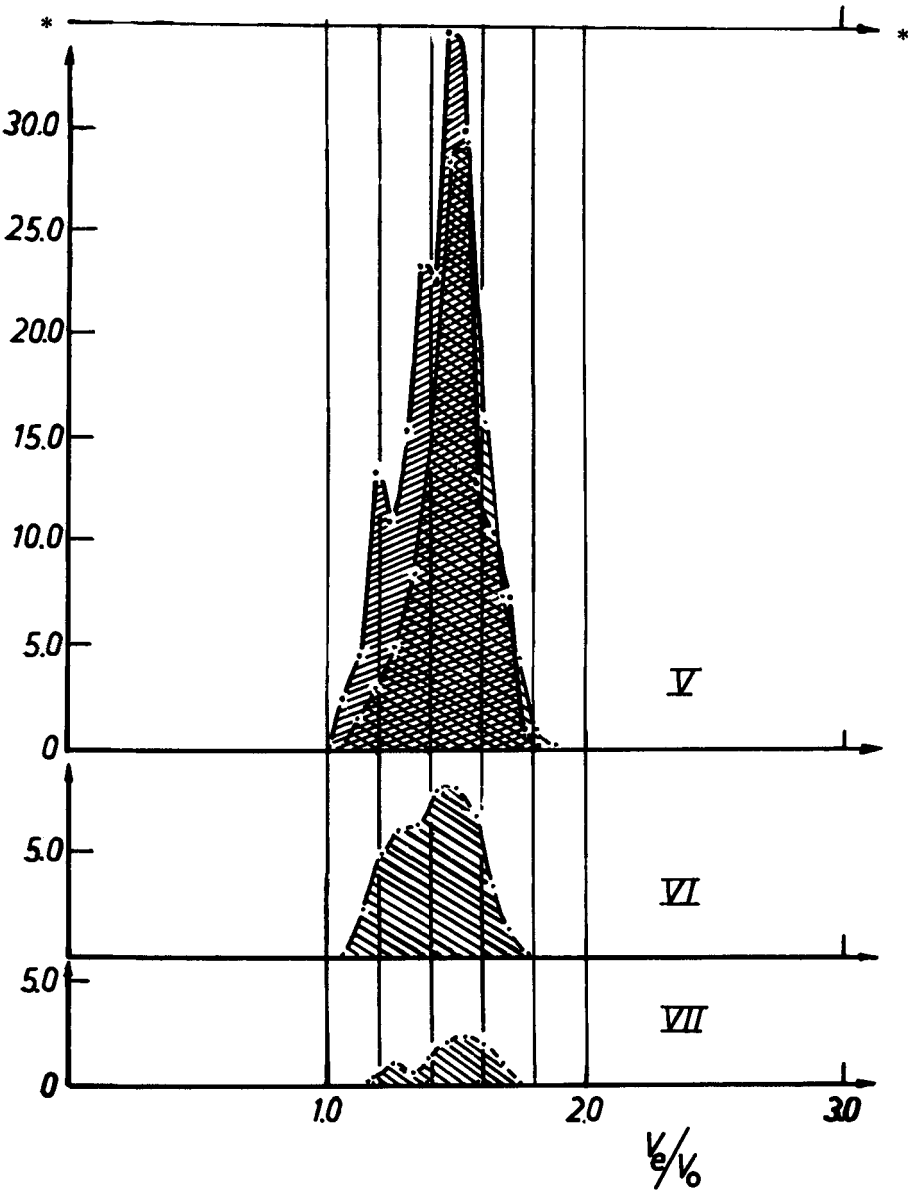


Fig. 4. Gelfiltration of the peaks marked in Figs. 2 and 3 on 1.0×165 cm Bio-Gel A-0.5m columns. The eluant was 0.005 M Tris-HCl buffer in 0.5 M NaCl, pH 8.1, and 2 per cent butanol. Hyaluronidase activity of fractions from the first ion-exchange separation = ·▨· Hyaluronidase activity of fractions from the second ion-exchange separation = ·////· Elution volume of BD (V_o) was 48.0 ml.

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Table I.

V_e/V_o values for maximum activities after gelfiltration of the different fractions from Figs. 2 and 3

Ion-exchange separation	Fraction number in ion-exchange	I	II	III	IV	V	VI	VII
First stepwise (Figure 2)	V_e/V_o in gel-filtration experiment	1.10 1.64	1.49	1.50	*)	1.48	1.48	1.47
Second stepwise (Figure 3)		1.11 1.33	1.45	1.48	1.44	1.48	*)	*)

*) These fractions were not gelfiltered.

DISCUSSION

Two different ion-exchange procedures have been used in order to separate hyaluronidases of dental plaque supernatants.

The procedure employing an eluant with continuously increasing ionic strength (Fig. 1) gave one broad and one small hyaluronidase activity peak. The broad peak appeared to consist of several components. In the separations using eluants increasing in ionic strength in a stepwise manner (Fig. 2 and 3) seven activity peaks were obtained. Although this result suggests the existence of different hyaluronidases, with the stepwise technique a substance may appear in more than one fraction.

The two stepwise chromatograms showed the greatest activity peak in fraction V.

The protein distribution in the chromatogram with continuously increasing ionic strength was different than that obtained by increasing the ionic strength in a stepwise manner.

The reseparation of the ion-exchange fractions on Bio-Gel columns showed that the first fraction contained three activity peaks that differed from the peaks of the other fractions.

These results confirm the existence of different hyaluronidases in dental plaque supernatants (*Nord et al.*, 1969).

SUMMARY

Ion-exchange chromatography fractionations of dental plaque supernatants on DEAE-Sephadex A-50 are described. The fractionations were developed

with eluants increasing both continuously and stepwise in ionic strength. Gelfiltration of the ion-exchange fractions on Bio-Gel A-0.5m showed that dental plaque supernatants contain a number of closely related hyaluronidase enzymes.

RÉSUMÉ

FRACTIONNEMENT DES HYALURONIDASES PROVENANT DE PLAQUES MICROBIENNES DENTAIRES PAR CHROMATOGRAPHIE SUR ÉCHANGEURS D'IONS ET FILTRATION SUR GEL

L'auteur décrit des expériences de fractionnement de surnageants de plaque par chromatographie sur échangeurs d'ions sur DEAE-Sephadex A-50. Les fractionnements ont été obtenus avec des éluants dont on augmentait la force ionique tant de façon progressive que de façon discontinue. La filtration sur Bio-Gel A-0,5m des fractions séparées par échange d'ions a montré que les surnageants de la plaque contenaient plusieurs hyaluronidases très proches les unes des autres.

ZUSAMMENFASSUNG

HYALURONIDASE AKTIVITÄT IN ZAHNBELAGMATERIAL

Chromatografischer Ionenauswechslungs fraktionierungen von der Zahnbelags-supernatanten auf DEAE-Sephadex A-50 wurden beschrieben. Fraktionierungsmetoden mit linear und schrittweise Eluanten wurden entwickelt.

Gelfiltrierung der Ionenauswechslungsfraktionen auf Bio-Gel A-0.5m zeigte, dass die Zahnbelag-supernatanten eine Reihe von nahe verwandten Hyaluronidasen enthielten.

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