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RAPID PURIFICATION OF TONIN, ESTERASE B, ANTIGEN ψ AND KALLIKREIN FROM RAT SUBMANDIBULAR GLAND BY FAST PROTEIN LIQUID CHROMATOGRAPHY

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SUMMARY

Tonin, esterase B, antigen ψ and kallikrein from the rat submandibular gland were purified by fast protein liquid chromatography with Mono P or Mono Q columns. The purity of the separated proteins was evaluated by sodium dodecyl sulphate polyacrylamide gel electrophoresis and by isoelectrofocusing in flat-bed polyacrylamide gel. Tonin and esterase B were purified by DE-52 cellulose anion-exchange chromatography and chromatofocusing on Mono P in two and three steps, respectively. Antigen ψ and kallikrein were purified by a two-step procedure using DE-52 cellulose and Mono Q anion-exchange chromatography. The high resolution power of Mono Q revealed the different isoenzymes of kallikrein.

INTRODUCTION

Tonin, esterase B, antigen ψ and kallikrein are proteolytic enzymes belonging to the serine "kallikrein" family[1,2]. These enzymes share, to a great extent, substrate specificity and immunological determinants[2,3]. However, in spite of this close relationship, the physiological functions of the enzymes are probably very different, as indicated by the capacity of tonin to liberate the vasoconstrictor angiotensin II, whereas kallikrein will induce the release of bradykinin, a potent vasodilator. Therefore, to be able to study one of these enzymes without interference from any of the others, particularly when using immunological methods, it is of vital importance to obtain pure enzyme preparations.

Serine proteases have previously been purified from the rat submandibular gland by conventional methods[4–7], affinity chromatography[8–10] and chromatofocusing[11]. Recently, the purification of urinary kallikrein by high-performance liquid chromatography (HPLC)[12] and fractionation of cat submandibular gland saliva by

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fast protein liquid chromatography (FPLC)[13] have been described. Esterase B has been purified by a combination of ion-exchange chromatography, chromatofocusing, aprotinin-Sepharose affinity chromatography and HPLC[2]. Recent developments in gel materials for HPLC enable complex protein mixtures to be separated in an hour or less. HPLC offers advantages in resolution, speed and recovery, and is much used for the isolation and characterization of peptides and proteins[14]. However, excellent and rapid separation can also be obtained at only moderately increased pressures, for example by FPLC on resins such as Mono Q, a strong anion exchanger, and Mono P, a weak anion exchanger containing groups with various buffer capacities[15]. Separation on a Mono P column is based on the isoelectric point, p*I*, of the various proteins.

The purpose of this study was to investigate the utility of FPLC in the separation and purification of the serine proteases to a high level of purity. The purification was performed on the submandibular gland of the rat since all four enzymes are present in this organ and their behaviour could thus be observed simultaneously. Furthermore, due to the high concentration of all the serine kallikrein-like proteases, the rat submandibular gland is a rich source for enzyme preparation.

MATERIALS AND METHODS

Materials

DEAE-cellulose De-52 was obtained from Whatman (Maidstone, Kent, U.K.), Mono Q HR 5/5, Mono P HR 5/20 prepacked columns and Polybuffer™ 74 from Pharmacia Fine Chemicals (Uppsala, Sweden), S2266 from Kabi Diagnostica (Stockholm, Sweden), acrylamide, bis-acrylamide and ampholine (pH 3–5 and 6–10) from Bio-Rad Labs. (Richmond, CA, U.S.A.), agarose from Litex (Denmark), imidazole from Sigma Chemical Co. (St. Louis, MO, U.S.A.), silver nitrate from Koch-Light Lab. (Colnbrook, U.K.) and Aquacide® from Calbiochem (La Jolla, CA, U.S.A.).

Apparatus

A Pharmacia FPLC system was used, comprising two P-500 pumps, a V-7 valve injector, a GP-250 gradient programmer, an UV-1 monitor with HR 10 cell, an UV-2 monitor, a Frac-100 collector and a REC-482 recorder. In this system, the sample and eluent are not in contact with stainless steel, and no corrosion of the pump at high pH or in the presence of salts, with subsequent degradation of column performance, takes place.

Calibration parameters

The fraction collector peak cut-off was set at 10% of full scale deflection, the flow-rate at 120 (Mono Q) or 90 ml/h (Mono P) and the fraction size at 1.0 ml. The 100% full deflection of the UV monitor was usually set at an absorbance of 0.2, and the recorder chart speed at 0.5 cm/min.

Purification

DEAE-cellulose DE-52 ion-exchange chromatography. Submandibular glands were removed from 4-month-old male Wistar rats, weighed, cut into pieces and homogenized in a Potter Elvehjem homogenizer (1:10 wet weight:volume in 0.01 *M*

ammonium acetate buffer, pH 5.8; 20 strokes up and down; 4°C). The supernatant was applied to a Whatman DE-52 anion-exchange column (385 × 25 mm) equilibrated with 0.1 *M* ammonium acetate (pH 5.8) and eluted with a linear gradient from 0.1 to 0.5 *M* ammonium acetate (pH 5.8). The elution profile was followed by the absorbance at 280 nm and by the amidolytic activity with S2266 as substrate[16]. Tonin, antigen ψ and kallikrein was further identified by immunoelectrophoresis using an antikallikrein antiserum[17], and fractions containing the respective enzymes were pooled, dialyzed against phosphate-buffered saline (PBS; 0.01 *M* sodium phosphate, 0.15 *M* sodium chloride, pH 7.4) and concentrated with Aquacide®.

Chromatofocusing of tonin. A 500-µl sample of the void volume from the Whatman DE-52 column (fractions 20–40, 1.7 mg protein/ml, Fig. 1), containing tonin and a different esterase that was initially labelled esterase 1, was applied on the Mono P column (200 × 5 mm), equilibrated with starting buffer (0.025 *M* imidazole, pH 7.4) and eluted with Polybuffer 74®, diluted 1:10, pH 4.0. Since the esterase 1 fraction obtained by this procedure was found to contain tonin, a second Whatman DE-52 ion-exchange column (385 × 25 mm) was included before FPLC. This ion-exchange column was equilibrated with 0.02 *M* Tris–HCl buffer (pH 8.0, 0.9 S) and eluted with a gradient of 0–0.3 *M* sodium chloride.

Anion-exchange chromatography of antigen ψ and kallikrein. A 500-µl sample of fractions 130–140 from the DEAE ion-exchange column, containing both antigen ψ and kallikrein (Fig. 1; 1.2 mg protein/ml), was applied to the Mono Q column (50 × 5 mm), equilibrated with 0.1 *M* ammonium acetate (pH 4.8) and washed with 8 ml of the same buffer (4 min) before applying a linear gradient up to 1.0 *M* ammonium acetate (pH 4.8). In initial experiments, the pH and ionic strength were varied to determine the optimum separation conditions.

Anion-exchange chromatography of kallikrein. A 500-µl sample of fractions 140–165 from the DEAE ion-exchange column, containing kallikrein (Fig. 1, 1.11 mg protein/ml), was applied to the Mono Q column (50 × 5 mm). The buffers were the same as described above. The gradient applied was chosen to give an optimum resolution (see Results).

Electrophoretic techniques

Immunoelectrophoresis was performed as previously described using a rabbit antiserum against rat submandibular gland kallikrein[17]. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli[18]. The gels were stained for protein using the silver technique described by Wray *et al.*[19]. The marker kit of low-molecular-weight proteins consisted of phosphorylase *b* (molecular weight, MW = 94 000), albumin (MW 67 000), ovalbumin (MW 43 000), carbonic anhydrase (MW 30 000), soybean trypsin inhibitor (MW 20 000) and α-lactalbumin (MW 14 100). Thin-layer isoelectrofocusing (IEF) was performed as described in the manufacturer's manual (Bio-Rad). The gels were stained for proteins with Coomassie blue[20].

Determination of enzymes

Tonin enzyme activity was measured by the fluorometric assay described by Boucher *et al.*[4]. Immunoreactive tonin[21] and kallikrein[22] were determined by specific immunoradiometric assays (IRMAs). The amidolytic activity was determined using

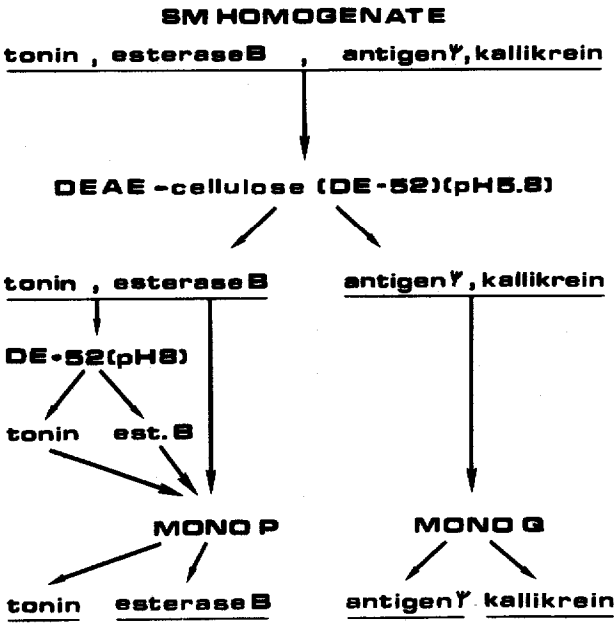

Fig. 1. Flow chart of the purification procedures used for purification of tonin, esterase B (before identification, called esterase 1), antigen ψ and kallikrein.

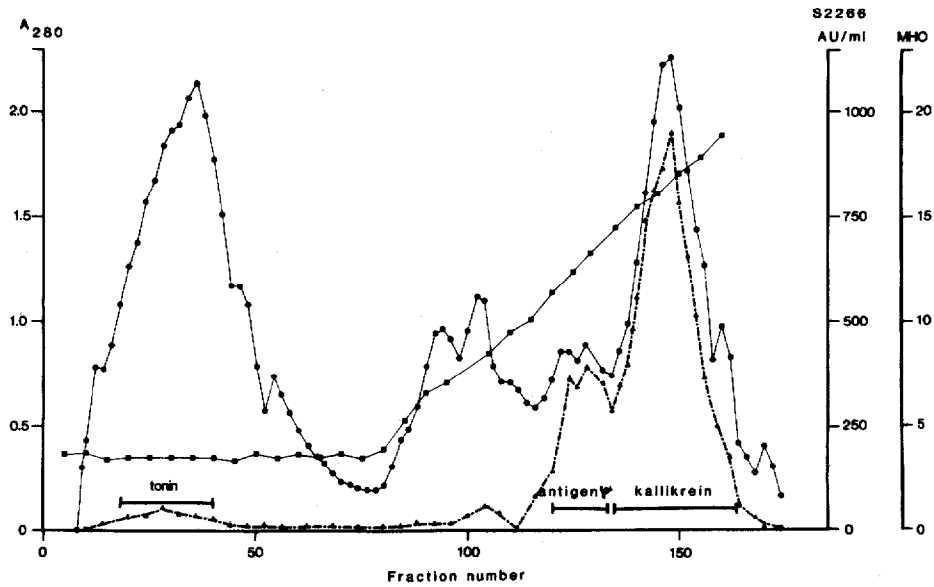

Fig. 2. Anion-exchange chromatography on a Whatman DE-52 column of submandibular gland homogenate with a gradient from 0.1 to 0.5 *M* ammonium acetate (pH 5.8). Fractions containing tonin, antigen ψ and kallikrein are indicated. ●, Absorbance at 280 nm; ▲, amidolytic activity (S2266); ■, ionic strength. MHO = Ω^{-1}.

the chromogenic substrate for glandular kallikrein, S2266[16]. The protein concentration was measured with the method of Lowry *et al.*[23], using bovine serum albumin as a standard.

Identification of esterase 1

This enzyme was compared with the submandibular gland esterase B purified by Khullar *et al.*[2] by double immunodiffusion using antisera against esterase B[2] and by isoelectrofocusing as described above.

RESULTS

An overview of the purification procedures applied for separation of the various serine proteases from the rat submandibular gland is shown in Fig. 1. In the first purification step, ion-exchange chromatography on the Whatman DE-52 column (Fig. 2), tonin was eluted in the void volume and antigen ψ and kallikrein as two separate peaks. The enzymes were identified by immunoelectrophoresis, and the pooled fractions were used for further purification.

Chromatofocusing of tonin and esterase B

The elution of tonin from the Mono P chromatofocusing column occurred at

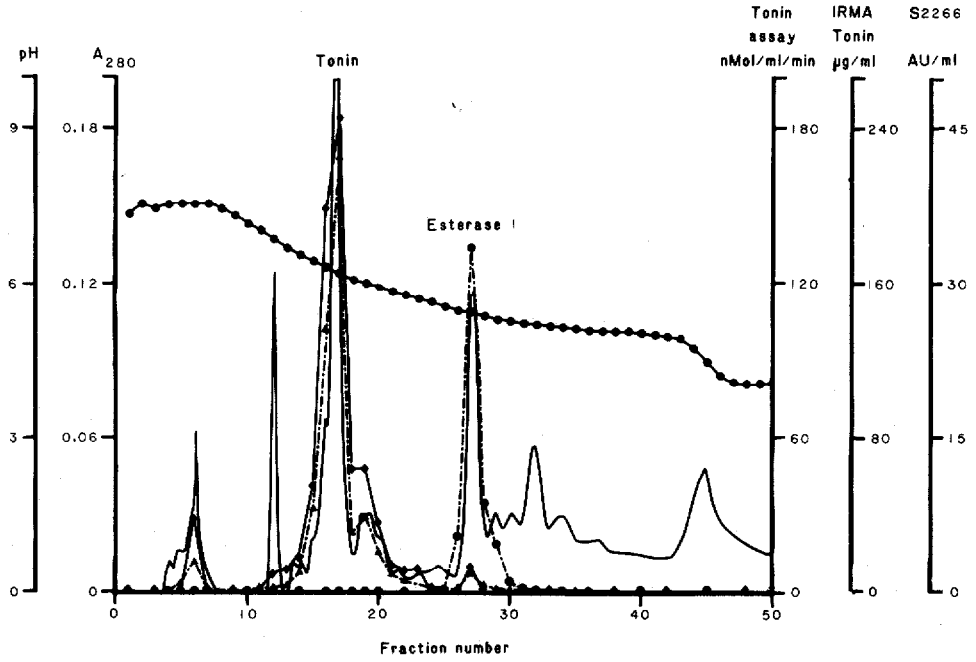

Fig. 3. Chromatofocusing profile of tonin from Mono P. Conditions for chromatography: starting buffer, 0.025 *M* imidazole (pH 7.4); eluent, Polybuffer 74 (1:10, pH 4.0); flow-rate, 1.5 ml/min. Fractions containing tonin (17) and esterase 1 (27) are indicated. ———, Absorbance at 280 nm; ▲– · – · –▲, tonin enzyme activity; ◆—◆, immunoreactive tonin (IRMA); ●– · – · –●, amidolytic activity (S2266); ●—●, pH.

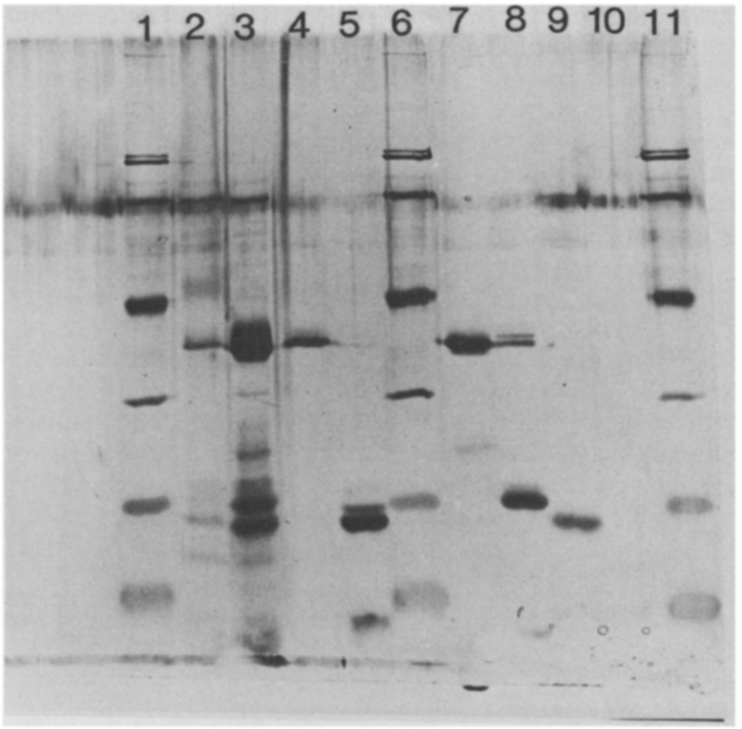

Fig. 4. SDS-PAGE of protein peaks from the Mono P column. The rows: 1, 6 and 11 = standard proteins; 2 = SM gland homogenate, crude preparation; 3 = void volume from the DE-52 (pH 5.8) column; 4, 5, 7–10, Mono P fractions 6, 12, 17 (tonin), 27 (esterase 1), 32 and 45 respectively. The gel was stained with the silver technique.

a position equivalent to pH 6.2 (Fig. 3), identical to the p*I* value previously reported for tonin[7]. The peaks of tonin activity and tonin immunoreactivity coincided with the protein ($A_{280\ nm}$) peak in this position. The main peak of amidolytic activity was eluted at pH 5.6, and represents a so far unidentified esterase which we named esterase 1. Kallikrein immunoreactivity was not present in either the crude or separated fractions. SDS-PAGE of the protein peaks collected from the Mono P column showed the various peaks to contain one major protein (Fig. 4). With the sensitive silver-staining technique, trace amounts of one contaminating protein with a lower molecular weight could be observed in the tonin fraction. The esterase 1 preparation gave one major and two minor bands, the latter in the same position as tonin despite their being well separated on the chromatofocusing column. Thin-layer IEF of these fractions is shown in Fig. 5. The results are in accord with those obtained by SDS-PAGE. A later fraction with no esterase activity (Fig. 3, fraction 32) showed on IEF one band at the same position to that of esterase 1, but having a slightly lower molecular weight according to SDS-PAGE. This fraction may contain a degradation product of esterase 1 but was not further characterized. To separate tonin from esterase 1, a second ion-exchange step was included using the Whatman DE-52 column and a buffer that made both enzymes adhere to the column material. The eluted

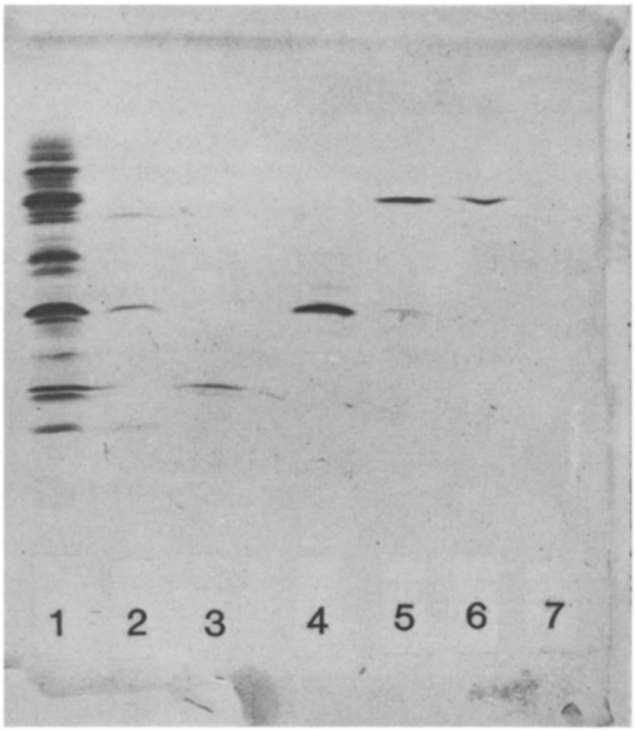

Fig. 5. Thin-layer IEF of protein peaks from the Mono P column. Rows: 1 = void volume from the DE-52 (pH 5.8) column; 2–7 = Mono P fractions 6, 12, 17 (tonin), 27 (esterase 1), 32 and 45. The gel was stained with Coomassie blue.

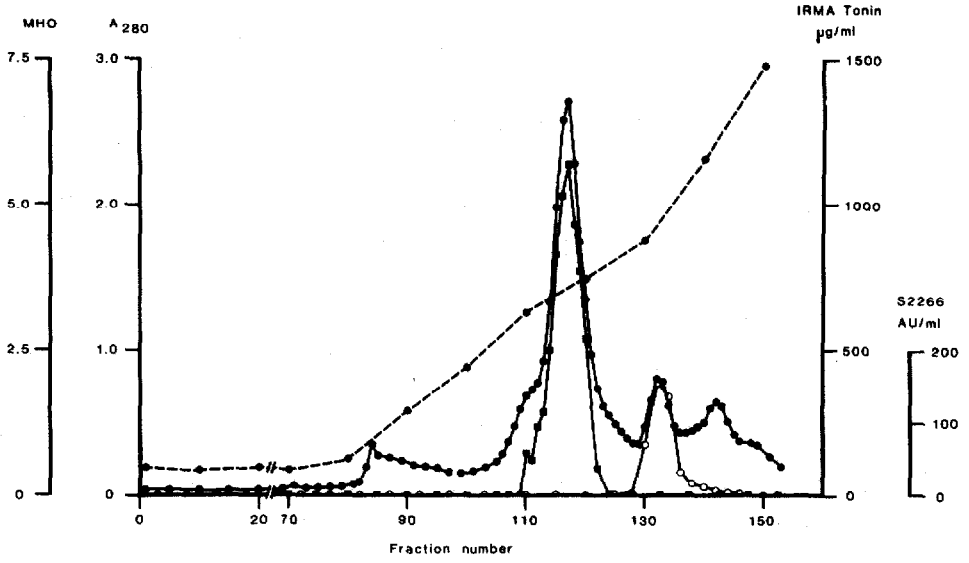

Fig. 6. Anion-exchange chromatography on Whatman DE-52 of tonin and esterase 1 (later identified as esterase B). Conditions for chromatography: starting buffer, 0.02 *M* Tris (pH 8.0); gradient, 0–0.3 *M* sodium chloride. Fractions containing tonin and esterase 1 are indicated. ●—●, Absorbance at 280 nm; ■—■, tonin enzyme activity; ○—○, amidolytic activity; ●- - -●, ionic strength.

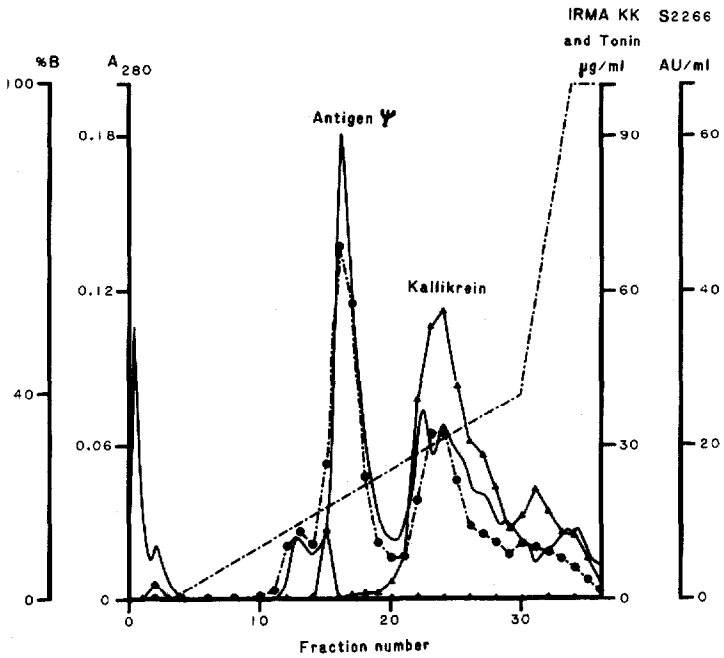

Fig. 7. Anion-exchange chromatography on Mono Q of antigen ψ and kallikrein. Conditions for chromatography: starting buffer, 0.1 *M* ammonium acetate (pH 4.8); gradient, 0.1–1.0 *M* ammonium acetate (pH 4.8); flow-rate, 2.0 ml/min. Fractions containing antigen ψ and kallikrein are indicated. ———, Absorbance at 280 nm; ▲—▲, immunoreactive kallikrein (IRMA); ◆—◆, immunoreactive tonin (IRMA); ●– · – · –●, amidolytic activity (S2266); – · – · –, ionic strength.

tonin and esterase 1 fractions (Fig. 6) were further purified by FPLC as described above and gave an esterase 1 preparation free from tonin, *i.e.*, giving only one band on IEF. In IEF, esterase 1 was found to behave similarly to esterase B supplied by Khullar *et al.*[2]. In double immunodiffusion using an antiserum against esterase B, immunological identity was seen between esterase B and esterase 1. Both enzymes showed partial identity to the other submandibular gland esterases. It was therefore concluded that esterase 1 was identical to esterase B.

Anion-exchange chromatography of antigen ψ and kallikrein

Antigen ψ in the antigen ψ- and kallikrein-containing fraction from the Whatman DE-52 column (pH 5.8) was separated from kallikrein by Mono Q anion-exchange chromatography (Fig. 7). Antigen ψ was eluted at 0.20 *M* and kallikrein at 0.36 *M* ammonium acetate, respectively. Immunoreactive kallikrein was not found in the protein peak corresponding to antigen ψ. A minor peak of immunoreactive tonin was eluted in the front of this peak. SDS-PAGE and thin-layer IEF showed that the antigen ψ and kallikrein were completely separated (Figs. 8 and 9).

Anion-exchange chromatography of kallikrein

The kallikrein-containing fraction from the Whatman DE-52 column (pH 5.8)

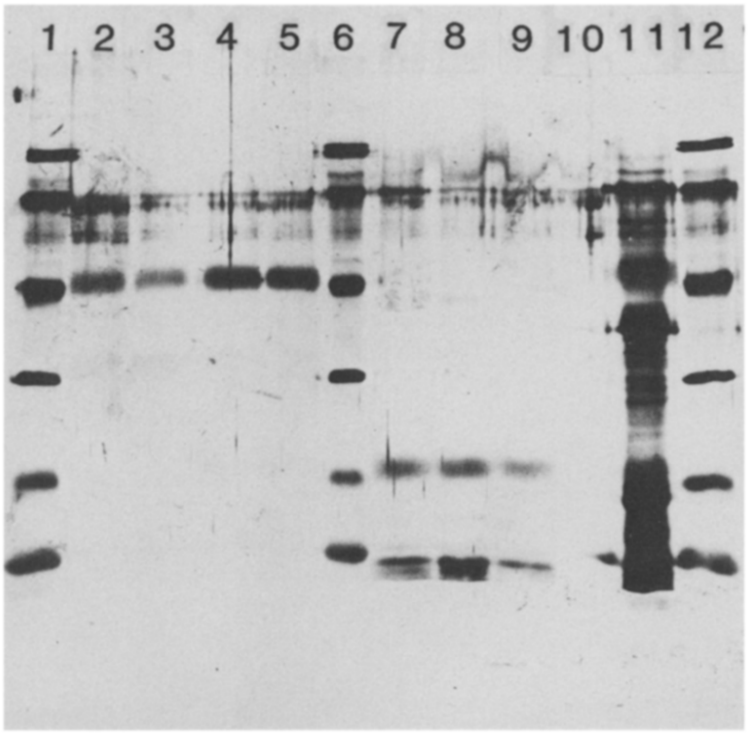

Fig. 8. SDS-PAGE of protein peaks from the Mono Q column. Rows: 1, 6 and 12 = standard proteins; 2–5 = kallikrein fractions 25–22, respectively; 7–9 = antigen fractions 17–15, respectively; 11 = SM gland homogenate (row 10 is empty). The gel was stained by the silver technique.

was applied on the Mono Q anion-exchange column (Fig. 10). Kallikrein was eluted in seven distinct peaks at ammonium acetate concentrations ranging from 0.25 to 0.38 *M*. The curves of enzyme activity and immunoreactivity did not show the same degree of resolution, but indicated that all peaks represented kallikrein. SDS-PAGE of the fractions showed one major protein band at identical position and minor amounts of possible degradation products as contaminants (Fig. 11). Small differences in the p*I* values of these different Mono Q fractions were revealed by thin-layer IEF (Fig. 12).

One experiment on Mono P gave approximately 0.2 mg and 0.1 mg pure tonin and esterase B, and another on Mono Q gave approximately 0.3 mg and 0.2 mg pure antigen ψ and kallikrein, respectively. Tonin and kallikrein were purified 17- and 12-fold, respectively, from the submandibular gland homogenate as calculated from the IRMA values and tonin enzyme activity. Similar calculations could not be done for esterase B and antigen ψ since the amidolytic activity is not specific for these enzymes alone.

DISCUSSION

The present results indicate that the FPLC technique with chromatofocusing

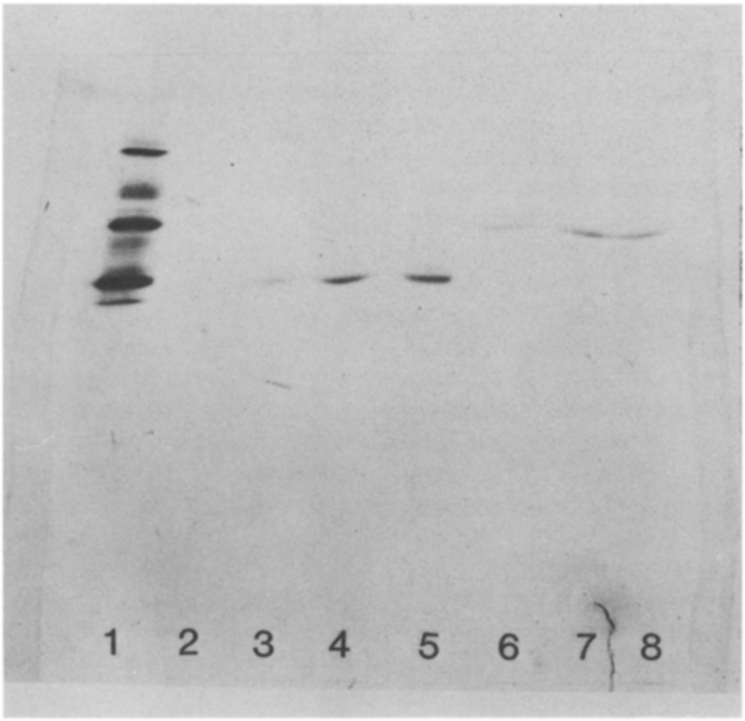

Fig. 9. Thin-layer IEF of protein peaks from the Mono Q column. Rows: 1 = fractions 130–140 from the DE-52 (pH 5.8) column; 2–8 = antigen ψ fractions (from the Mono Q column) 2, 15, 16, 17 and kallikrein fractions 22, 23, 25. The gel was stained with Coomassie blue.

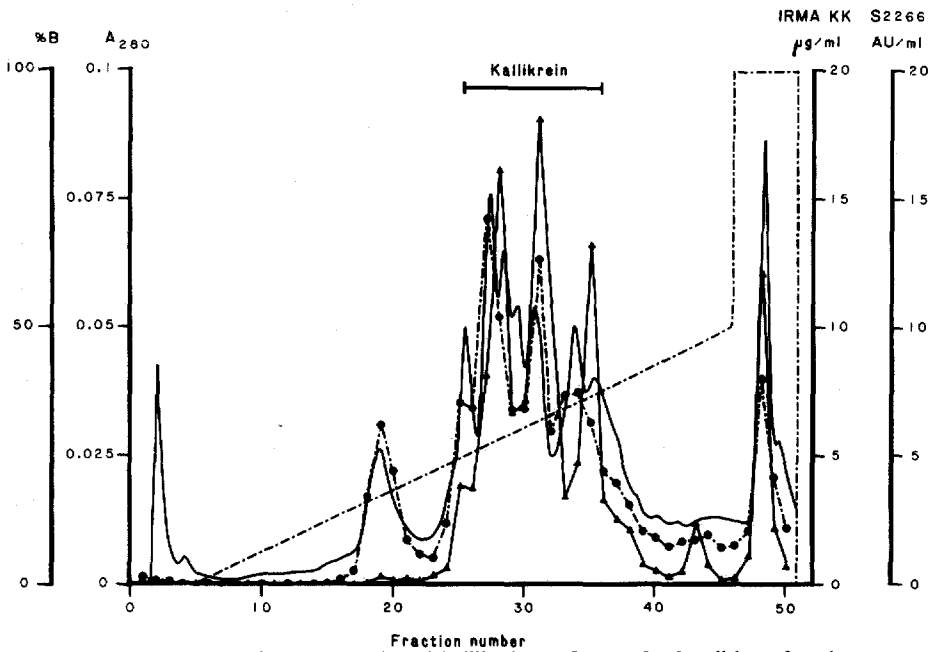

Fig. 10. Anion-exchange chromatography of kallikrein on Mono Q. Conditions for chromatography: starting buffer, 0.1 *M* ammonium acetate (pH 4.8); gradient, 0.1–1.0 *M* ammonium acetate (pH 4.8); flow-rate, 2.0 ml/min. Fractions containing kallikrein are indicated. ———, Absorbance at 280 nm; ▲—▲, immunoreactive kallikrein (IRMA); ●- - - -●, amidolytic activity (S2266); – · – · –, gradient profile.

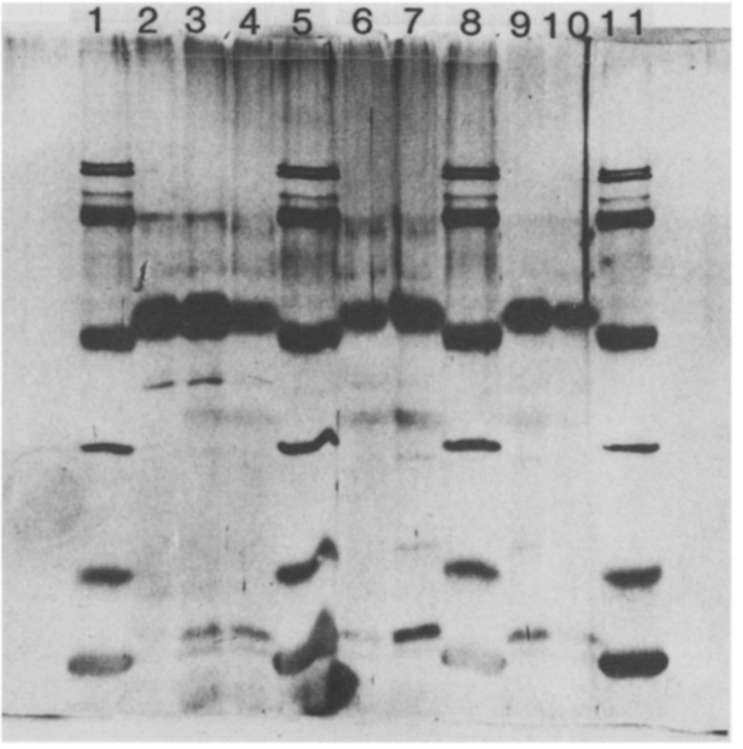

Fig. 11. SDS-PAGE of protein peaks from the Mono Q column. Rows: 1, 5, 8 and 11 = standard proteins; 2–4, 6, 7, 9, 10 = fractions 25, 26, 28, 30, 31, 33 and 35. The gel was stained by the silver technique.

and ion-exchange columns can be useful in isolating closely related proteins such as the serine proteases of the kallikrein family.

Tonin and esterase B are easily purified by a two-step chromatographic procedure; anion-exchange chromatography followed by FPLC chromatofocusing (Mono P). Immunoradiometric analysis, SDS-PAGE and thin-layer IEF showed that the tonin preparation contained predominantly tonin. Minor impurities were only observed with the sensitive silver-staining method. However, to obtain a pure esterase B preparation on Mono P, tonin and esterase B had to be separated by a second ion-exchange chromatography prior to application on the Mono P column. Tonin was eluted from the chromatofocusing column at a pH corresponding to its p*I*, whereas esterase B was eluted at a pH higher than its p*I*. This phenomenon was also described for esterase B by Khullar *et al.*[2]. For antigen ψ and kallikrein, chromatofocusing was not superior to FPLC anion-exchange chromatography, since the p*I* of these enzymes (4.1–4.5[6] and 3.8–4.2[3]) were in the lower limit of the Polybuffer 74 (pH 4.0). The separation of antigen ψ and kallikrein was better performed on Mono Q with a low pH buffer. These proteins were completely separated by a two-step anion-exchange chromatography, *i.e.*, cellulose anion-exchange and Mono Q chromatography. Immunoradiometric analysis, SDS-PAGE and IEF verified this conclusion.

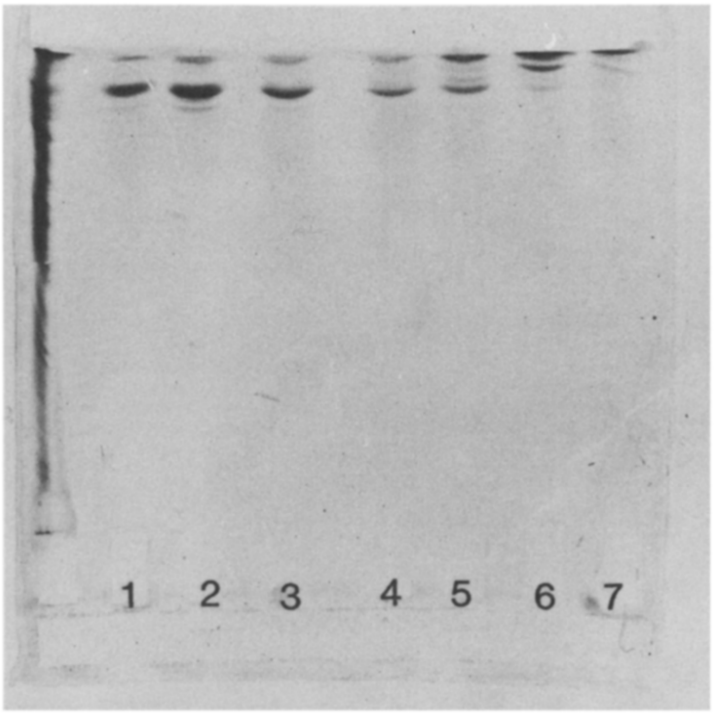

Fig. 12. Thin-layer IEF of protein peaks from the Mono Q column. Rows: 1–7 = fractions 25, 26, 28, 30, 31, 33 and 35. The gel was stained with Coomassie blue.

Kallikrein can be separated into several different isoenzymes by a two-step ion-exchange chromatography. No differences in molecular weight were seen by SDS-PAGE, however minor differences in p*I* were observed by IEF. At a higher pH (> 6.8), kallikrein tends to elute as a broad peak with lower separation efficiency.

Recently, Funae *et al.*[12] have described an HPLC system with a continuous-flow enzyme detector for rapid separation and measurement of rat urinary kallikrein. The FPLC system described in the present paper competes with HPLC in speed and reproducibility of the chromatograms obtained. Tonin, esterase B, antigen ψ and kallikrein were eluted within 20, 30, 20 and 40 min, respectively. Due to the sensitivity of the ultraviolet detectors and the efficiency of the columns, a protein profile can be obtained in volumes as small as 100 μl, especially when the total protein concentration is greater than 0.5 mg/ml. The columns used to separate tonin, esterase B, antigen ψ and kallikrein are not designed for preparative separation of proteins. However, the rapid separation procedure allowed several analyses to be performed each day. Columns with higher capacities are now available from Pharmacia.

Kallikrein[3], tonin[4], esterase B[2] and antigen ψ[6] have previously been purified by conventional methods to an high level of purity and with a purification factor comparable to that obtained in the present study. However, the great advantage of FPLC is that the high level of purity could be achieved by a two- or three-step purification procedure and in a relatively short time.

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