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RAT SUBMANDIBULAR GLAND KALLIKREINS: PURIFICATION AND CELLULAR LOCALIZATION

P. BRANDTZAEG[1]
Institute of Pathology, University Hospital, Rikshospitalet, Oslo 1

K.M. GAUTVIK
Institute of Physiology, University of Oslo, Oslo 1

K. NUSTAD
Department of Clinical Chemistry, The Norwegian Radium Hospital, Oslo 3 and Institute for Surgical Research, Rikshospitalet, Oslo 1, Norway

J.V. PIERCE
Hypertension-Endocrine Branch, National Heart and Lung Institute, N.I.H., Bethesda, Md. 20014, U.S.A.

1 Four submandibular gland kallikreins (E.C. 3.4.21.8) were isolated by chromatography on DEAE-Sephadex A-50 and hydroxyapatite, followed by gel filtration and electrofocusing. The pI values were 3.87, 3.96, 4.07 and 4.16, and a common molecular weight of 34,000 was found.

2 The kallikreins were localized by direct immunofluorescence with an antibody to rat urinary kallikrein, to the granular tubules, striated duct cells and some main duct cells in the submandibular gland, and to striated duct cells in the sublingual gland. Kallikrein was not found in acini and stroma.

3 Several non-kallikrein esterases present in the submandibular gland reacted with the antibody to rat urinary kallikrein. The antibody was made monospecific for kallikrein by absorption with the crossreacting esterases.

4 We suggest that kallikrein is produced in striated duct cells. Granular tubules, which are differentiated from striated duct cells, have preserved the ability to produce kallikrein. These cells also store large quantities of kallikrein.

Introduction

Glandular kallikreins (E.C. 3.4.21.8) are serine proteinases. These enzymes show low affinity for substrates such as casein or haemoglobin, but liberate a biologically active peptide, lysyl-bradykinin (Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg), from a precursor protein, kininogen, which is found in most biological fluids. Lysyl-bradykinin has pronounced vasodilator effects, and the kallikrein-kinin system may play an important role in the local blood flow regulation in the salivary glands (Hilton, 1970; Gautvik, 1970).

Indirect evidence has suggested that kallikrein is localized in the acini of rat and mouse submandibular glands (Beraldo, Siqueira, Rodriques & Machado, 1972; Bhoola, Dorey & Jones, 1973). We now report the cellular localization of kallikrein in the rat submandibular and sublingual glands using a labelled antibody to rat urinary kallikrein (Nustad & Pierce, 1974).

[1] Present address: Department of Microbiology, Dental Faculty, University of Oslo, Blindern, Oslo, Norway.

Methods

Materials

The following substances were obtained commercially: cytochrome C, myoglobin, chymotrypsinogen A, and ovalbumin (Serva-Feinbiochemica, Heidelberg, W. Germany); bovine serum albumin (Behringwerke AG, Marburg, W. Germany); ultrapure sucrose (Mann Research Labs., U.S.A.); DEAE-Sephadex A-50 and Blue Dextran (Pharmacia Fine Chemicals, Uppsala, Sweden); Ampholines (LKB, Bromma, Sweden); acrylamide, *N,N'*-methylene-bis-acrylamide, ammonium persulphate, *N,N,N',N'*-tetra methylene diamine and Bio-Gel P-150 (Bio Rad, Labs., U.S.A.); agarose (Litex, Glostrup, Denmark); bradykinin (Sandoz, AG, Basel, Switzerland); tetramethylrhodamine isothiocyanate (Baltimore Biological Laboratory, U.S.A.); trypsin (TRL 2LA, Worthington Biochemical Corp., U.S.A.); yellow casein (nitrated, Calbiochem Inc., U.S.A.); α-

N-benzoyl-L-arginine ethyl ester HCl (Bz-Arg-OEt) and sodium dodecyl sulphate (Sigma Chemical Co., U.S.A.). The dodecyl sulphate was recrystallized after dissolution in absolute alcohol heated to 70°C, filtered and evaporated at room temperature.

Hydroxyapatite was prepared by a new method (Pierce & Nustad, unpublished). Rat urinary kallikrein and the corresponding antiserum were prepared as described by Nustad & Pierce (1974).

Preparation of submandibular gland extract

Twenty female Sprague-Dawley rats (200–300 g) were killed by a blow on the neck. The excised submandibular glands were cut in small pieces and a 30% (w/v) homogenate was prepared in 0.10 M NaCl–0.01M Na-phosphate (pH 7.0) using a Potter-Elvehjem homogenizer (20 strokes, 4°C). The homogenate was centrifuged at 660 × *g* for 5 minutes. The supernatant fluid was treated with Na-desoxycholate (0.5 w/v), dialysed against 0.10 M NaCl–0.01M Na-phosphate (pH 6.0) at 4°C for 30 h, and centrifuged at 20,000 × *g* for 60 minutes. The precipitate was washed and the supernatants combined to give Fraction 1 in 70 ml (Table 1).

Immunoelectrophoresis

Electrophoresis of 2 to 5 µl samples was followed by diffusion against antiserum for 48 h (Laurell, 1966). The plates were pressed, washed and then stained with Coomassie Brilliant Blue (Weeke, 1973).

Immunodiffusion

Micro double diffusion analysis was carried out as described by Brandtzaeg (1970).

Crossed immunoelectrophoresis

Glass plates of 10 × 10 cm were covered with 16 ml of 1% agarose in 0.05 M Tris HCl (pH 8.6) containing 0.2 ml antiserum to rat urinary kallikrein (Nustad & Pierce, 1974). A 2 cm broad strip of solidified agarose was removed along one side and a polyacrylamide gel used for electrofocusing was placed along the cutting edge and fastened with agarose at 50°C without antiserum. Electrophoresis into the antibody-containing agarose was performed with 2 V/cm at 15°C for 16 h with 0.05 M Tris HCl (pH 8.6) in the electrode vessels. The plates were washed, pressed and stained with Coomassie Brilliant Blue or 0.5% Amido Black.

Molecular weight determinations

Rat submandibular esterases were subjected to gel filtration in a Bio-Gel P-150 column at 4°C, with Blue dextran, bovine serum albumin, ovalbumin and myoglobin as standards (Andrews, 1965). Some preparations were also examined by polyacrylamide gel electrophoresis in the presence of dodecyl sulphate. The samples were treated according to Dunker & Rueckert (1969) or by method one of Weber, Pringle & Osborn (1972). Electrophoresis and calculations were carried out as described by Weber *et al.* (1972). Reference proteins were cytochrome C, myoglobin, chymotrypsinogen A, ovalbumin, and bovine serum albumin.

Protein determination

The protein content of main fractions was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Bovine serum albumin was used as standard (absorbance $A_{280\,nm}^{0.1\%,1\,cm} = 0.64$ in 0.15 M NaCl—0.01 M Na-phosphate, pH 7.4). Protein concentrations in column effluents were measured in a 1 cm cuvette at 280 nm.

Biological assay of kallikrein

Kinin formation was measured on the rat isolated uterus by incubating enzyme fractions with rat kininogen 2, and is expressed as bradykinin equivalents formed per min (Gautvik, Kriz & Lund-Larsen, 1972). The submandibular gland kallikreins contracted the rat isolated uterus when added directly to the organ bath without preincubation with kininogen. This oxytocic activity probably reflects release of kinins from kininogen in the uterus (Nustad & Pierce, 1974), and is specific for the kallikreins (see Results section).

The hypotensive effect of the esterases was measured in rats anaesthetized with Nembutal (40 mg/kg body weight i.p.). Samples of 50 µl were injected into the external jugular vein and the blood pressure recorded through a catheter in the common carotid artery.

Esterase activity

The hydrolysis of α-*N*-benzoyl-L-arginine ethyl-ester (Bz-Arg-OEt) during 5 min at 37°C and at pH 8.5 was determined (Nustad, Pierce & Vaaje, 1975). One esterase unit (EU) was defined as the amount of enzyme that hydrolysed 1 µmole of Bz-Arg-OEt per minute.

Caseinolytic activity

Nitrated casein (yellow casein) 1.5% was incubated with enzyme fractions in 1 ml 0.10 M Na-phosphate (pH 7.4). Samples of 0.4 ml were removed at zero time and after 60 min incubation at 37°C, precipitated with 0.6 ml 1.7 M perchloric acid, stored at 4°C for 1 h, filtered on sintered glass filters (Pyrex G3) and read at 280 nm in a microcuvette (Laake & Vennerød, 1974).

One caseinolytic unit (CU) was defined as the amount of enzyme that increased the A_{280} absorbance of the acidic filtrate by 1 in 1 minute.

Electrofocusing

The method of Nustad & Pierce (1974) was used, except that the pH values of fractions were measured at 4°C rather than at room temperature (Haglund, 1971).

Gel electrofocusing

The method of Wrigley (1968) was used with chemical polymerization and application of the sample on top of the gel, and the anode at the bottom. Gels of 5 × 60 mm or 2.7 × 80 mm were run at 25°C for 4 to 6 h with 200 V constant voltage. The gels were stained and fixed by the method of Malik & Berrie (1972).

Polyacrylamide disc gel electrophoresis, pressure dialysis and phosphate determination

These were carried out as reported by Nustad & Pierce (1974), and conductivity measurements as described by Nustad *et al.* (1975).

Preparation of fluorescent antibody conjugate

A conjugate of the IgG fraction of the sheep antiserum to rat urinary kallikrein B_3 (Nustad & Pierce, 1974) with 35 μg tetramethylrodamine isothiocyanate per mg protein was prepared, filtered through a column of Sephadex G-50, and purified by DEAE-cellulose chromatography (Brandtzaeg, 1973a). The fraction eluted with 0.04 M NaCl–0.01 M Na-phosphate (pH 7.6) was used as the immunofluorescence reagent throughout. Its absorbance ratio A_{280}/A_{515} was 2.1. A working dilution of 0.47 mg/ml was established by performance testing on serial sections of alcohol-fixed rat submandibular glands (see below); it was the lowest conjugate concentration giving duct cell fluorescence of maximum intensity. The fluorescence end point of the conjugate corresponded to 4–8 μg/ml. To abolish non-specific background staining, the conjugate was adsorbed with acetone-extracted mouse liver powder before use, and the sections were preincubated with 25% bovine serum albumin (Brandtzaeg, 1973b).

Immunohistochemical procedures

Submandibular and sublingual glands from six adult female Sprague-Dawley rats were collected in ice-cold isotonic saline directly after excision, divided within 20 min into small pieces which were placed in cold 96% ethanol. After further tissue processing, paraffin embedding, and preparation of serial sections (6 μm) (Brandtzaeg, 1974), the sections were incubated with the conjugate for 30 min at room temperature, washed in 0.15 M NaCl–0.01 M Na-phosphate (pH 7.4) and mounted in Evanol (Brandtzaeg, 1973b). Serial control sections were incubated in parallel with aliquots of the conjugate that had been absorbed by addition of esterase B or C (0.17 mg of fraction $2B_1$ and 0.08 mg of 3C per ml working dilution).

Microscopy was carried out with a Leitz Ortholux equipped with an Osram HBO 200 W lamp and a Ploem-type vertical illuminator. Narrow-band excitation and filtration were selected for rhodamine fluorescence (Brandtzaeg, 1973b). The findings were recorded on 'Anscochrome' 500 daylight film.

Results

Purification of submandibular gland kallikreins: esterases C_1–C_4

Rat submandibular kallikreins were purified at 4°C except for step 3 (hydroxyapatite chromatography) which was performed at 20°C (Table 1 and Figure 1). Desoxycholate-solubilized kallikrein (Fraction 1) from homogenates of submandibular glands (see Methods section) was applied in 0.10 M NaCl–0.01 M Na-phosphate (pH 6.0) to a DEAE-Sephadex A-50 column equilibrated with the same buffer. Under these conditions 12% of the total esterase activity was not adsorbed (peak A_1 and A_2). Following application of a linear salt gradient (Figure 1a) 20% of the activity was eluted as a double peak (B_1 and B_2) at a conductivity of 20 mS (0.20 M NaCl–0.01 M Na-phosphate, pH 6.0); 68% of the activity was eluted as an asymmetrical peak (peak C) at 30 mS (0.31 M NaCl–0.01M Na-phosphate, pH 6.0). Peak C was identified as kallikrein by its ability to generate kinins and by immunochemical methods (see below). Peak C was concentrated by pressure dialysis, and equilibrated against 3 M NaCl–0.005 M Na-phosphate (pH 6.0) to give Fraction 2C. Fraction 2C (22 mg) was chromatographed on a hydroxyapatite column (Figure 1b). All of the Bz-Arg-OEt esterase activity was eluted as one peak (3C) with 0.018 M phosphate. Fraction 3C was concentrated by pressure dialysis, equilibrated against 0.01 M Na-phosphate (pH 7.4), and 4.2 mg was filtered through a Bio-Gel P-150 column. Protein and Bz-Arg-OEt esterase activity eluted in the same peak at 1.93 times the void volume (Figure 1c), suggesting a molecular weight of 36,000. A pool was collected, pressure dialysed, and equilibrated against 0.15 M NaCl–0.01 M Na-phosphate (pH 7.4) to give Fraction 4C. This step did not increase the specific activity which was constant throughout the peak (Figure 1c) indicating that hydroxyapatite chromatography produced a highly purified esterase preparation. One ml (4.2 mg) of Fraction 3C was placed in the middle of the stepwise gradient of and electrofocusing column which

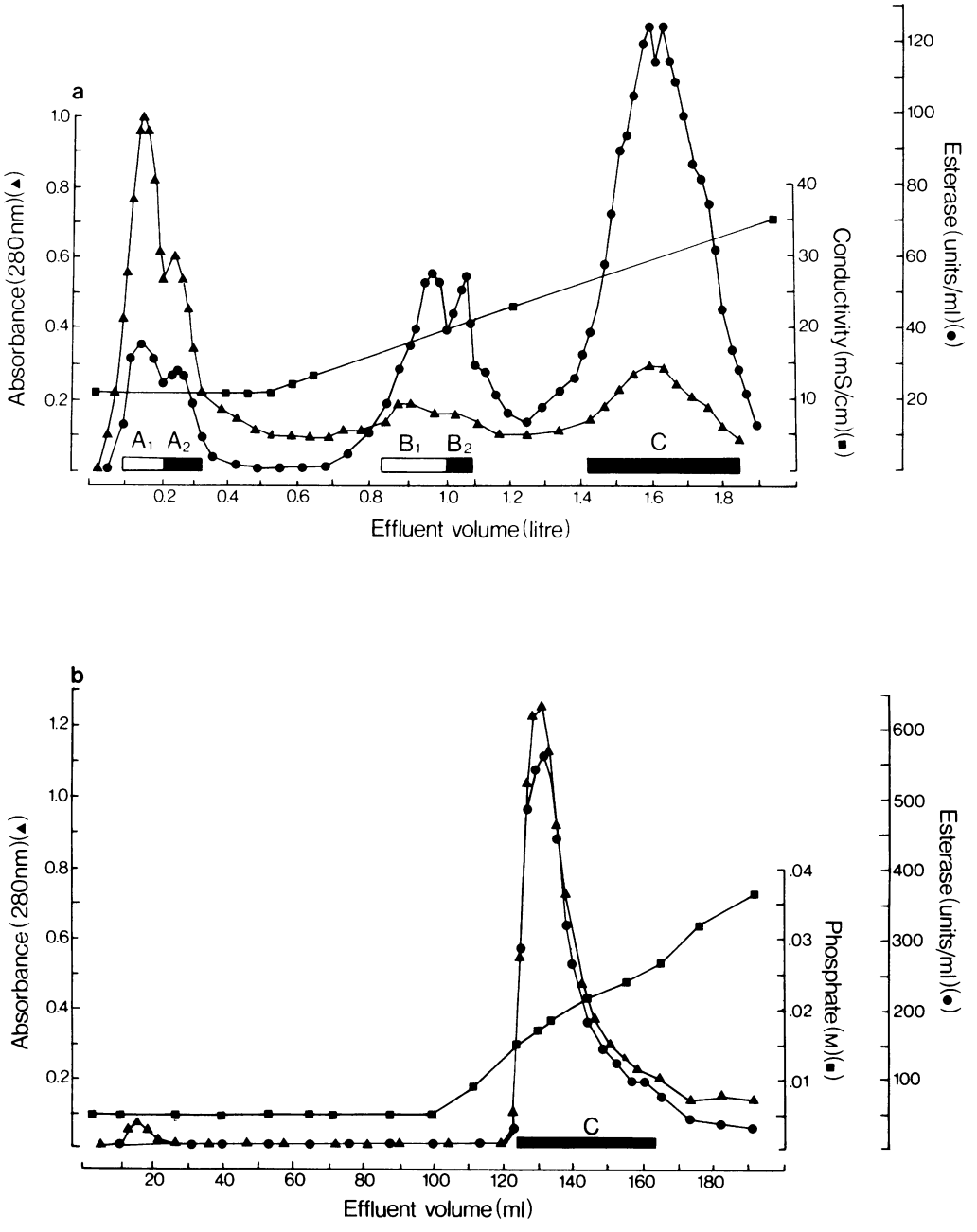
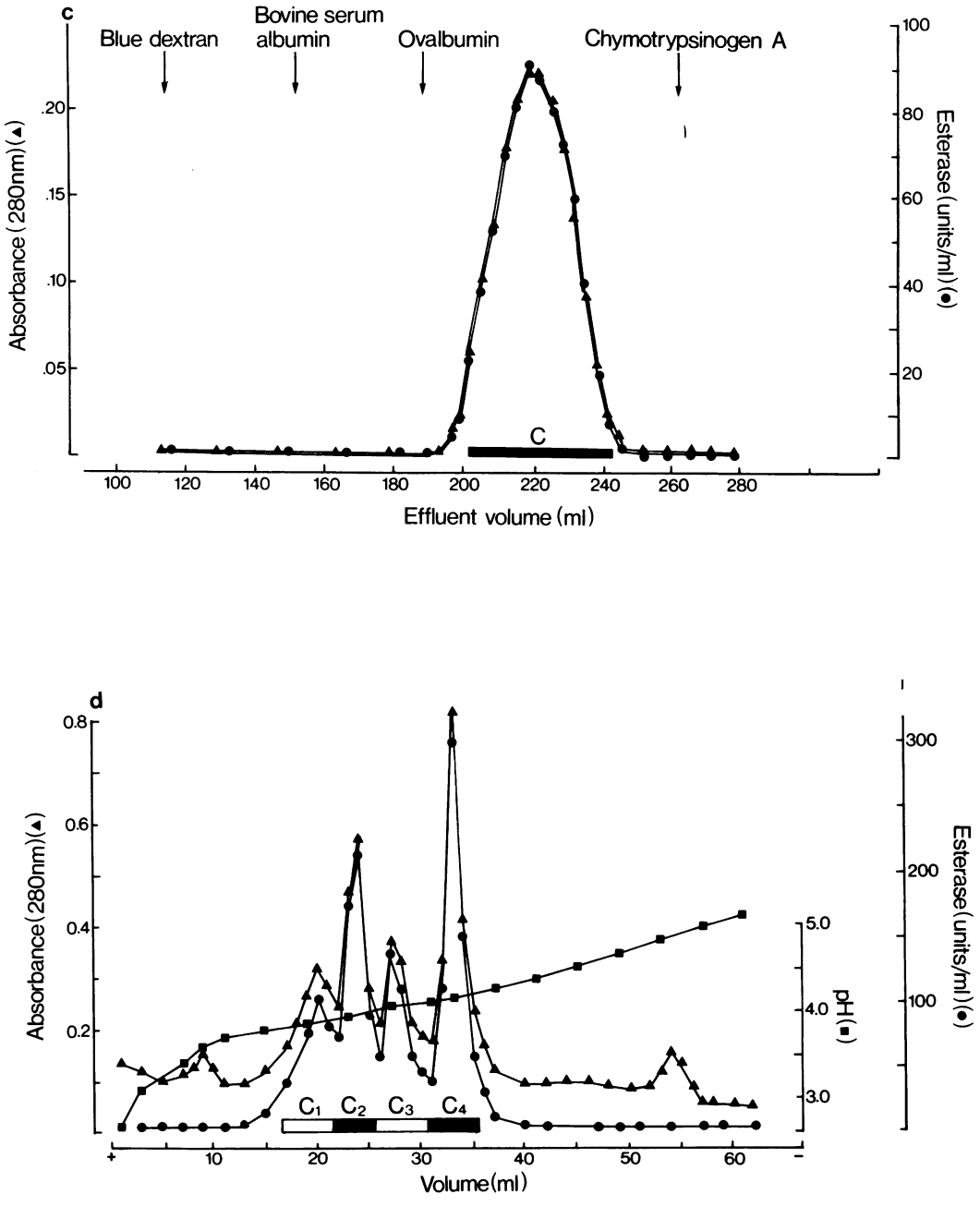


Figure 1 Purification of rat submandibular gland kallikreins, esterases C_1–C_4. (a) DEAE-Sephadex A-50 chromatography. Column: 1.5 x 74 cm. Sample: 476 mg of Fraction 1 (Table 1) in 70 ml. Eluents: 300 ml equilibration buffer (0.10 M NaCl–0.01 M Na-phosphate, pH 6.0), and a 2000 ml linear gradient to 0.50 M NaCl–0.01 M Na-phosphate, pH 6.0. Flow rate: 19 ml/hour. Fraction volume: 6.5 ml. Temperature: 4°C. (b) Hydroxyapatite chromatography. Column: 0.70 x 29 cm. Sample: 22 mg of Fraction 2C (Table 1) in 6.0 ml. Eluents: 50 ml equilibration buffer (3 M NaCl–0.005 M Na-phosphate, pH 6.0), and a 200 ml linear gradient to 3 M NaCl–0.05 M Na-phosphate, pH 6.0. Flow rate: 2.6 ml/hour. Fraction volume: 2.6 ml. Temperature: 20°C. Horizontal bars indicate the pools collected.



(c) Bio-Gel P-150 gel filtration. Column: 1.5 x 91 cm. Sample: 4.2 mg of Fraction 3C (Table 1) in 1.3 ml equilibration buffer (1 M NaCl–0.01 M Na-phosphate, pH 7.4). Flow rate: 13 ml/hour. Fraction volume: 3.3 ml. Temperature: 4°C. (d) Electrofocusing. Column: 110 ml containing 0.8% Ampholine pH 3–5. Sample: 4.2 mg of Fraction 3C (Table 1) in 1.0 ml 0.01 M Na-phosphate, pH 7.4. Voltage: 200 V/3 h, 400 V/17 h, and 800 V/4 hour. Elution flow rate: 120 ml/hour. Fraction volume: 1.0 ml. Temperature: 4°C. Horizontal bars indicate the pools collected.

contained 0.8% (v/v) Ampholine pH3–5. After focusing four esterase peaks were obtained with pI values: 3.87, 3.96, 4.07 and 4.16 (Figure 1d). These were collected, dialysed against 3 M NaCl–0.01 M Na-phosphate (pH 6.0), concentrated by pressure dialysis, and equilibrated against 0.01 M Na-phosphate (pH 7.4) to give Fractions $5C_1$–$5C_4$ (Table 1). Their specific activities were 5–19% lower than that of Fraction 3C. Thus the kallikreins were slightly unstable in the electrofocusing conditions used.

Purification of esterases B

The esterases B_1 and B_2 (Figure 1a) were purified by exactly the same procedures as the kallikreins (Figure

Table 1 Purification of rat submandibular gland kallikreins, esterases C_1–C_4

Step	Treatment	Protein (mg)	Specific activity (EU*/mg)	Purification	Recovery (%)
1	Submandibular gland homogenate, centrifuged extracted and dialysed to give fraction 1	476	144		
	Esterase C activity†		97	(1)	(100)
2	DEAE-Sephadex A-50 chromatography of fraction 1 to give fraction 2C	55	633	6.5	74
3	Hydroxyapatite chromatography of 22 mg of fraction 2C to give fraction 3C‡	26	738	7.6	42
4	Bio-Gel P-150 gel filtration of 4.2 mg of fraction 3C to give fraction 4C	2.8	725		
5	Electrofocusing of 4.2 mg of fraction 3C to give fractions:				
	$5C_1$ pI 3.87	0.39	603		
	$5C_2$ pI 3.96	0.50	700		
	$5C_3$ pI 4.07	0.48	610		
	$5C_4$ pI 4.16	0.77	636		

* EU (esterase unit) = 1.0 μmol of Bz-Arg-OEt/min (pH 8.5, 37°C).
† Calculated on the assumption that 68% of the total esterase activity is due to esterase C, as found by DEAE-Sephadex A-50 chromatography.
‡ Protein and recovery have been calculated assuming that all of fraction 2C was fractionated.

Table 2 Purification of rat submandibular gland esterases B

Step	Treatment		Protein (mg)	Specific activity EU*/mg	Purification	Recovery (%)
1	Fraction 1 (see step 1, Table 1)					
	Esterase B activity†			31	(1)	(100)
2	DEAE-Sephadex A-50 chromatography of fraction 1 to give fractions	$2B_1$	24	288	9	44
		$2B_2$	12	342	11	29
3	Hydroxyapatite chromatography of fraction $2B_1$ to give fraction	$3B_1$	6.2	448	14	19
	fraction $2B_2$ to give fraction	$3B_2$	2.2	830	27	12
4	Bio-Gel P-150 gel filtration of fraction $3B_1$ to give fraction	$4B_1$	2.2	950	31	14
	fraction $3B_2$ to give fraction	$4B_2$	0.7	427‡	14	2

* EU (esterase unit) see Table 1.
† Calculated on the assumption that 21% of the total esterase activity is due to esterase B.
‡ The specific activity before concentration was 823 EU/mg.

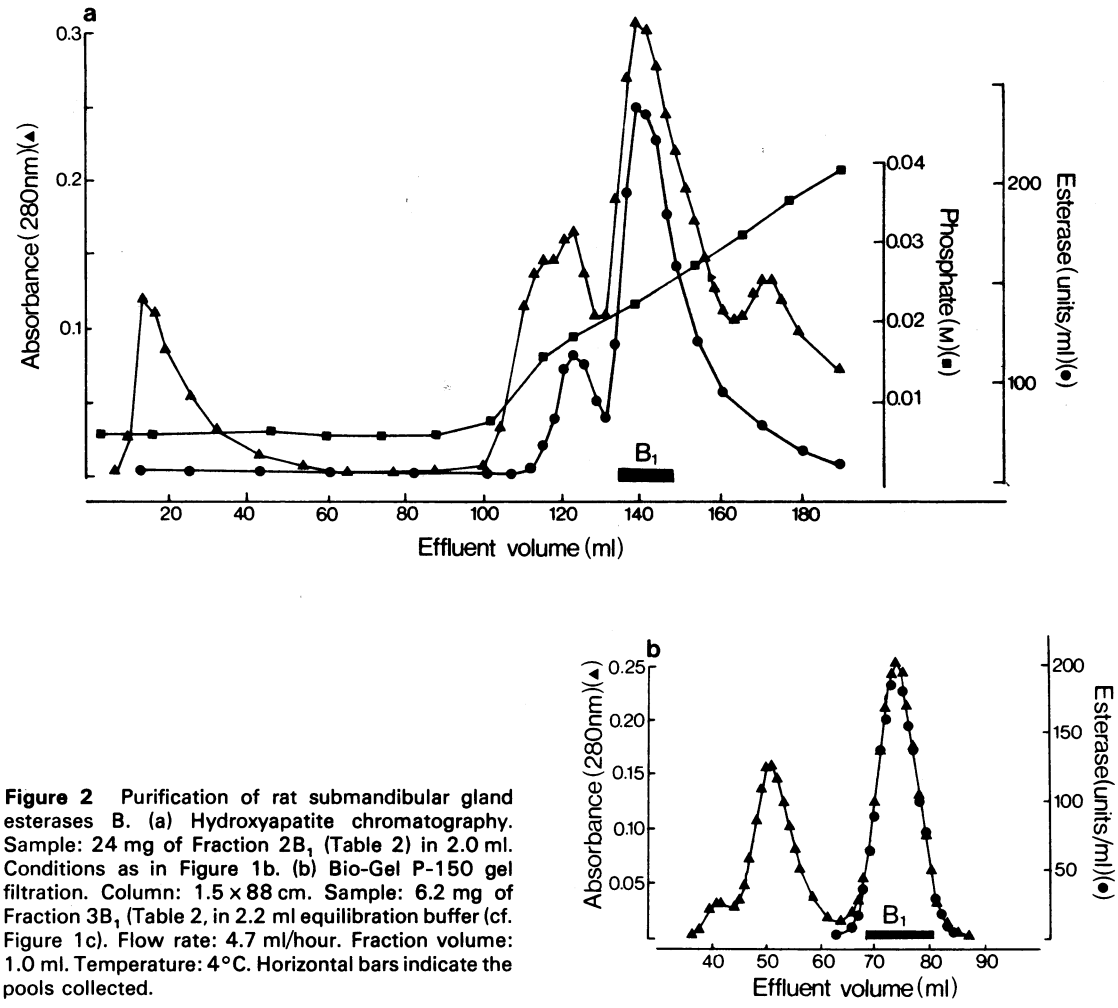


Figure 2 Purification of rat submandibular gland esterases B. (a) Hydroxyapatite chromatography. Sample: 24 mg of Fraction $2B_1$ (Table 2) in 2.0 ml. Conditions as in Figure 1b. (b) Bio-Gel P-150 gel filtration. Column: 1.5 x 88 cm. Sample: 6.2 mg of Fraction $3B_1$ (Table 2, in 2.2 ml equilibration buffer (cf. Figure 1c). Flow rate: 4.7 ml/hour. Fraction volume: 1.0 ml. Temperature: 4°C. Horizontal bars indicate the pools collected.

2 and Table 2). Eighty per cent of esterase B_1 was eluted from the hydroxyapatite column at 0.022 M phosphate (Figure 2a), whereas 90% of B_2 was eluted at 0.028 M (data not shown). Esterases B_1 and B_2 were eluted from the Bio-Gel P-150 column at 1.81 and 1.84 times the void volume, respectively (Figure 2b). Only the protein peak corresponding to the esterase activity was found when B_2 was gel filtered (data not shown). The esterases B were unstable after gel filtration (Table 2) and electrofocusing in gradients of pH 3–10 and 4–6 caused more than 75% loss of enzyme activity. Fraction $4B_1$ focused as one major peak with a pI value of 5.30, and several small peaks at pH 4.84, 5.12, 5.55 and 5.70 (data not shown). Fraction $4B_2$ gave one major peak at pH 5.10 with a shoulder at 4.8 (data not shown).

Purification of esterases A

The esterase activity not adsorbed by DEAE-Sephadex A-50 was collected as two fractions, A_1 and A_2 (Figure 1a), concentrated by pressure dialysis, and equilibrated against 0.15 M NaCl–0.01 M Na-phosphate (pH 7.4). These fractions contained 39 and 9 mg protein and 95 and 214 EU/mg of esterase activity, respectively.

Polyacrylamide gel electrophoresis

Kallikreins (esterases C). Electrophoresis of different fractions (Table 1) in 16% polyacrylamide gels (Figure 3a) left kallikreins $5C_1$, $5C_2$ and $5C_3$ probably confined to single protein bands. However,

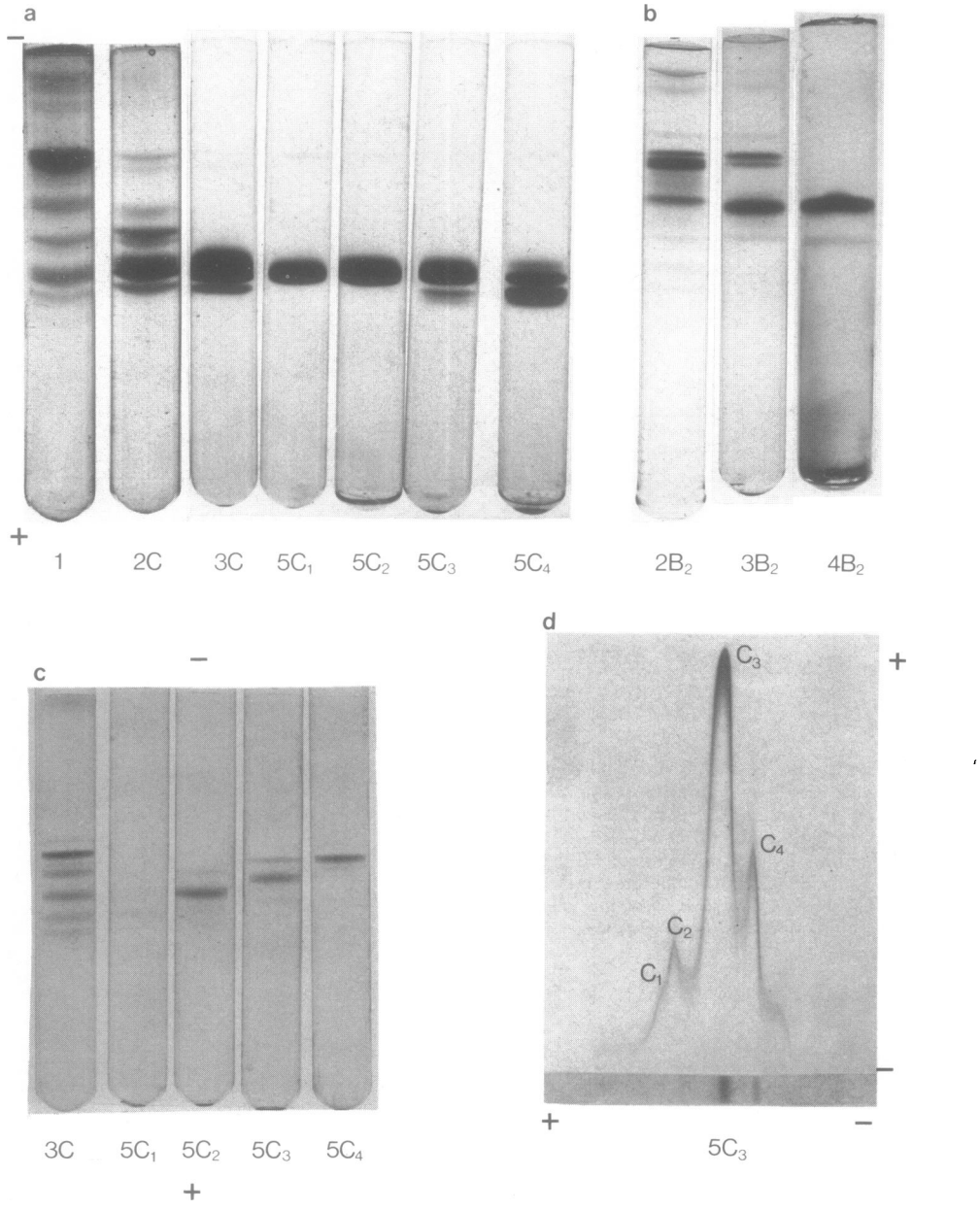


Figure 3 Electrophoresis (a and b) and electrofocusing (c and d) in polyacrylamide gels. (a) Fractions from the purification of rat submandibular gland kallikreins (21–39 μg/gel) identified by their numbers in Table 1. (b) Fractions (see Table 2) from the purification of rat submandibular gland esterases B_2 (50 μg/gel). (c) Fractions from the purification of rat submandibular gland kallikreins (10–40 μg/gel) identified by their numbers in Table 1. Gel size 5 × 60 mm. (d) Fraction $5C_3$ (Table 1) 4 μg electrofocused in a polyacrylamide gel (2.7 × 70 mm) and transferred to crossed immunoelectrophoresis (see Methods section).

each fraction was contaminated by neighbouring fractions in the electrofocusing gradient (Figure 1c). The bands corresponding to C_1, C_2, C_3 and the slowest component of C_4 had similar mobilities, and constituted the wide protein band seen in all fractions prior to separation of C_1–C_4. Kallikrein C_4 appeared as two major bands and a slower minor one due to contaminating C_3. The fastest component of C_4 also

appeared in Fractions 1, 2C, 3C and $5C_3$. Unstained gels of $5C_1$–$5C_4$ were sectioned, homogenized in 0.15 M NaCl–0.01 M Na-phosphate (pH 7.4). Assays of esterase activity showed a 60–70% recovery. It was located in the protein bands of $5C_1$–$5C_4$.

Esterases B. Electrophoresis in 16% polyacrylamide gels of esterases B_2 are shown in Figure 3b. Bz-Arg-OEt esterase activity was detected only in the fastest heavy protein band. Esterases B_1 were found in a protein band with the same electrophoretic mobility as esterases B_2 (data not shown).

Electrofocusing in polyacrylamide gel

The composition of kallikrein fractions 3C and $5C_1$–$5C_4$ are shown in Figure 3c. In addition to neighbouring kallikreins, fractions $5C_3$ and $5C_4$ contained 3 faint bands on either side of C_3 and C_4. These minor bands might represent kallikreins with different pI, since they reacted with the antiserum in crossed immunoelectrophoresis (Figure 3d).

Molecular weight determinations

Dodecyl sulphate polyacrylamide electrophoresis showed that each of the four kallikreins ($5C_1$–$5C_4$) gave a single band with identical mobility corresponding to a molecular weight of 34,000. A faster band of kallikrein C_4 (Figure 3a), visible with higher protein loads, may represent a kallikrein with a pI of 4.16, but with a smaller molecular weight.

Similar analysis of Fraction $4B_1$ showed a major protein band (calculated mol. wt. 37,500) followed by a minor impurity (data not shown).

Antigenic relationship between the kallikreins and other esterases

The antiserum to rat urinary kallikrein produced a reaction of identity between four urinary kallikreins, but did not react with a non-kallikrein esterase found in rat urine (Nustad & Pierce, 1974). This antiserum also reacted with the submandibular esterases A, B and C. The immunoelectrophoretic patterns of rat submandibular extract (RSE; Fraction 1 in Table 1) and of esterases A, B and C, are shown in Figure 4a, b and c. The antigen in the major precipitin arc had a faster mobility than serum albumin, and corresponded to the four kallikreins C_1–C_4 (Figure 4a). The esterases B gave a continuous double arc merging with the cathodal part of the kallikrein line in a reaction of 'partial identity' (Figure 4a, b and c). Esterases A gave a continuous double arc and this was spurred over by the B-line (Figure 4c). When the antiserum was absorbed with esterases B only the C-line was retained (Figure 4a). Our interpretation of these results is summarized in Figure 4d.

In double diffusion tests with antiserum to rat urinary kallikrein (RUK) a reaction of identity was produced between that antigen (RUK) and submandibular gland extract (RSE) as well as between the four forms of esterase C (Figure 4e). The latter formed distinct spurs over the esterases A and B, thus confirming that A and B were antigenically deficient compared with kallikrein (Figure 4f). The two forms of esterase A, and the two forms of B, appeared antigenically similar, wherease the relationship between A and B could not be clearly established because of mutual contamination (Figure 4f). However, they clearly did not share all antigenic determinants.

Biological activity of submandibular gland esterases

The purified kallikreins $5C_1$–$5C_4$ released kinin equivalent to 42, 55, 36 and 42 μg of bradykinin $min^{-1} \cdot mg^{-1}$ when incubated with rat kininogen. By contrast, the purified fractions of esterase B (Fractions $3B_1$ and $3B_2$, Table 2) and esterase A showed no detectable kinin releasing activity (far below 0.1 μg bradykinin $mg^{-1}\ min^{-1}$).

Each of the four kallikreins (1 μg of Fractions $5C_1$–$5C_4$) alone caused maximal contractions of the rat isolated uterus, comparable to effects of 0.2 μg of bradykinin. Esterases A and B had no effect in amounts 32 and 80 times those of esterases C. Intravenous injections of 0.1–0.3 EU of esterases C produced falls in the blood pressure of rats, 20–60 EU of esterases A caused small falls, whereas 20–40 EU of esterases B had no effect.

Caseinolytic activity of submandibular gland esterases

The caseinolytic activities expressed as caseinolytic units per Bz-Arg-OEt esterase unit (CU/EU) were: $5.2 \cdot 10^{-4}$ (esterases A); $2.6 \cdot 10^{-4}$ (esterases B); $1.6 \cdot 10^{-5}$ (Fraction 3C, Table 1); and $3.2 \cdot 10^{-1}$ (trypsin). The specific caseinolytic activities (CU/mg) were: 0.05 (esterases A); 0.08 (esterases B); 0.01 (3C); and 11.3 (trypsin).

Cellular localization of submandibular gland kallikreins

Very intense cytoplasmic fluorescence was localized in cells of granular tubules (Figure 5a). The cells were morphologically identified by examining neighbouring sections stained with haematoxylin and eosin, and subsequently by histochemistry on formalin-fixed tissue (Örstavik, Brandtzaeg, Nustad & Halvorsen, 1975). The intensity of fluorescence decreased where the granular tubules merged with the striated ducts which showed only faint cytoplasmic staining except for a bright luminal rim (Figure 5a). Similar rims in striated ducts were the only fluorescent feature of

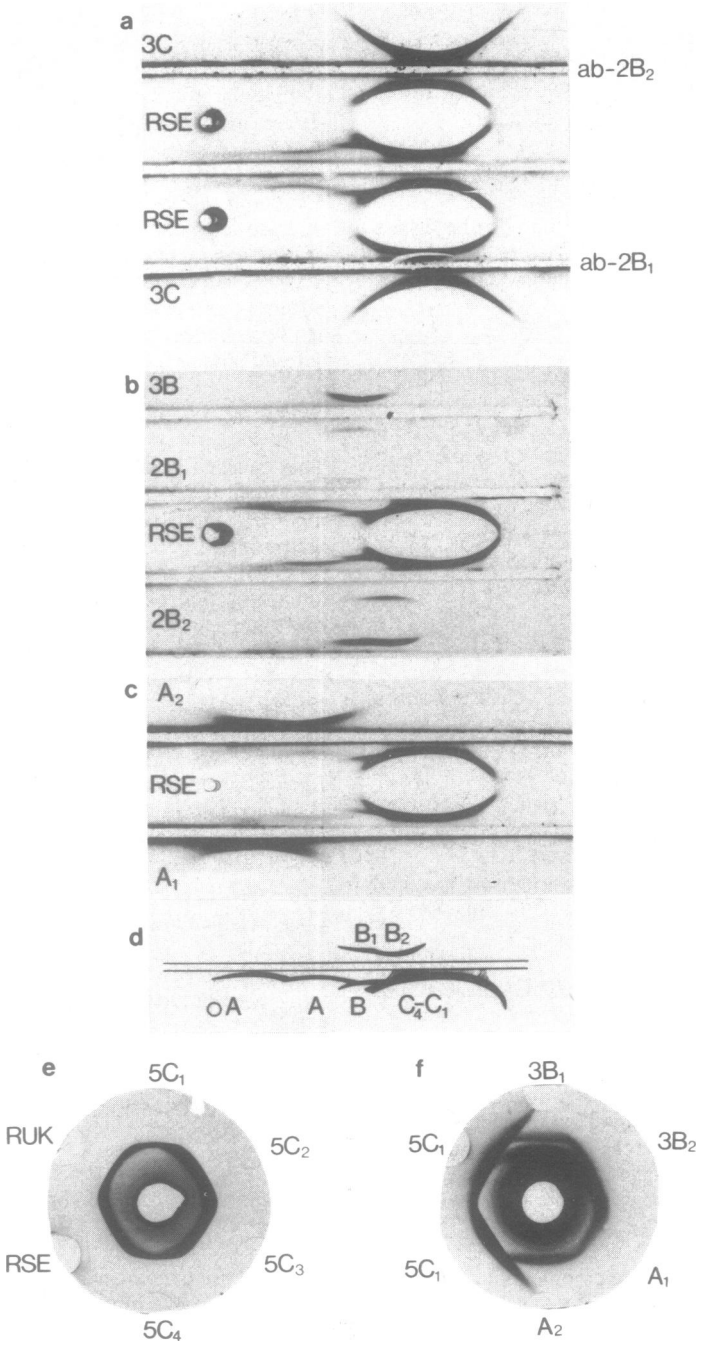


Figure 4 Immunoelectrophoresis and double immunodiffusion. The fractions are identified by their numbers (Tables 1 and 2). Rat submandibular extract (RSE) is a 30% (w/v) extract of the gland in 0.1 M NaCl–0.15 mM NaN_3–0.01M Na-phosphate, pH 7.4 (Fraction 1, Table 1, before addition of desoxycholate and dialysis at pH 6.0). Rat urinary kallikrein (RUK) B_3, Fraction 4C, Table 1, 1.0 mg/ml from Nustad & Pierce, 1974). The esterase A, B and C fractions (2–5 µl) were analysed at 0.5–1 mg/ml. Undiluted antiserum to rat urinary kallikrein (RUK) was used in all experiments except in Figure 4a where the middle trough contained antiserum diluted (1:2) with buffered saline, and the upper and lower troughs contained antiserum absorbed with 0.50 mg/ml of Fractions $2B_1$ and $2B_2$ (ab–$2B_1$ and ab–$2B_2$). (e) and (f) Double diffusion tests with antiserum to RUK, see text.

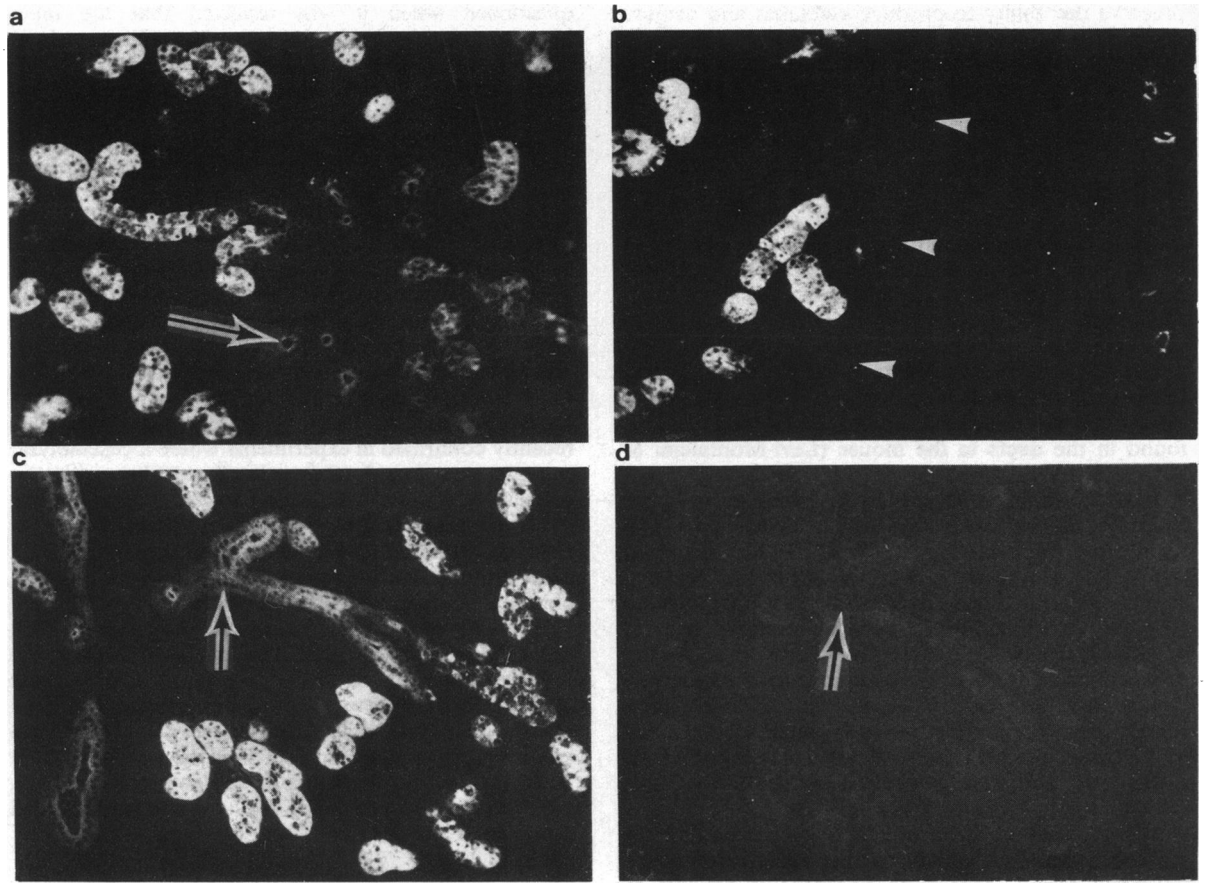


Figure 5 Immunohistochemical demonstration of kallikrein in alcohol-fixed sections of rat submandibular and sublingual glands. (a) Intense cytoplasmic immunofluorescence of submandibular granular tubules. Faint fluorescence of striated ducts, with a brigher luminal rim (arrow). (b) The border between submandibular (left) and sublingual (right) glands is indicated by arrow heads. Note four striated ducts with apical cytoplasmic fluorescence and absence of granular tubules in the sublingual gland. (c) and (d). Comparable fields in neighbouring submandibular sections incubated with antibody conjugate which had been absorbed with esterase B (c) or with esterase C (d). Esterase C abolished the staining reaction; the extremely faint fluorescence of ducts was revealed by overexposure. The arrow marks an identical site in the two pictures. Original magnification: 45×.

sublingual glands (Figure 5b). Faint cytoplasmic staining was seen in some larger ducts, but not in acini and stroma (Figure 5c).

Fluorescent staining was abolished by conjugate absorption with esterases C (Figure 5d), but not with esterases B (Figure 5c). This clearly demonstrated that the fluorescence specifically reflected the presence of kallikrein, and that the binding of the conjugate was specific (Brandtzaeg, 1973b). Specificity was substantiated by the extremely low staining end-point of the conjugate (4–8 μg/ml).

Discussion

The bulk of the high kallikrein content of the rat submandibular gland has been found in the granular tubules. A small amount was detected in the luminal border of striated duct cells. The latter is the main localization of kallikrein in the sublingual gland, which is devoid of granular tubules (Snell & Garrett, 1958). The granular tubules are differentiated from striated duct cells (Junqueira, 1967). Our results suggest that during their postnatal differentiation, granular tubules

preserve the ability to produce kallikrein and acquire the ability to store it. The finding of kallikrein in tubules differs from the reported acinar localization in rat and mouse submandibular glands (Beraldo *et al.*, 1972; Bhoola *et al.*, 1973). Amongst enzymes found in granular tubules, is a trypsin-like enzyme which acts on ϵ-amino caproic acid naphthol ester (Lagunoff, Benditt & Watts, 1962). This substrate is probably not cleaved by kallikreins. In mouse granular tubules several antigenically similar Bz-Arg-OEt esterases have been demonstrated by indirect immunofluorescence (Ekfors & Hopsu-Havu, 1971; Ekfors, Malmiharju & Hopsu-Havu, 1972). They were characterized as plasminogen activators and permeability increasing enzymes, but not as kallikreins (Ekfors, Suominen & Hopsu-Havu, 1972). The nerve growth factor, another Bz-Arg-OEt esterase, is also found in the ducts in the mouse (Levi-Montalcini & Angeletti, 1961).

It is interesting that kallikrein, which is probably one of the regulators of local blood flow and membrane permeability (Hilton, 1970; Gautvik, 1970), has a different localization from the digestive enzyme amylase (Kraus & Mestecky, 1971). The large amount of kallikrein found in saliva strongly suggests an extracellular function. The ducts modify the primary saliva formed by the acinar cells (Schneyer, Young & Schneyer, 1972), and extracellular kinin formation may ensure adequate functional hyperaemia, and possibly facilitate transport of water, electrolytes and macromolecules across the tubular cells.

Werle, Vogel & Lendrodt (1960) reported that the rat submandibular gland was the richest source of kallikrein found, containing hypotensive activity of 3000 biological units/g wet wt., compared to 1–2 units/g wet wt. in man. This conclusion was questioned when it was reported that the main hypotensive enzyme in the rat submandibular gland and its saliva was a trypsin-like enzyme (salivain) which, unlike kallikrein, exhibited a strong caseinolytic activity (Riekkinen, Ekfors & Hopsu, 1966; Riekkinen & Ekfors, 1966; Ekfors, Malmiharju, Riekkinen & Hopsu-Havu, 1967a). Four kallikrein-like enzymes purified from the same gland had a low caseinolytic activity, but represented a minor part of the hypotensive activity of the gland (Ekfors *et al.*, 1967a, b). Our results clearly show that the main hypotensive enzymes in the gland can be identified as kallikreins by their kininogenase activity, and oxytocic activities, low caseinolytic activity, and their immunochemical identity with rat urinary kallikrein. We must therefore assume that salivain is a mixture of kallikreins and a caseinolytic enzyme. This was recently confirmed in experiments where a caseinolytic enzyme was found to be eluted together with the esterases C from DEAE-Sephadex A-50 columns, but could be separated by preparative disc gel electrophoresis (Venneröd, Laake & Nustad, unpublished observations). In the present work, this separation was obtained by hydroxyapatite chromatography.

The esterases B shared antigenic determinants with the kallikreins, and so did components present in the less pure fractions A, probably representing esterases A. The latter enzymes appeared antigenically even more deficient than the esterases B. These cross reactions may indicate that the esterases A, B and C all originate from a common trypsin-chymotrypsin like enzyme. We have not yet identified esterases A and B.

Requests for reprints to K.N. at The Norwegian, Radium Hospital.

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