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The Isolation and Properties of Pig Submandibular Kallikrein

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The kallikrein from pig submandibular glands was highly purified, with an overall yield of 31%. Affinity chromatography on bovine basic pancreatic trypsin inhibitor linked to Sepharose 4B was an especially effective step in the purification procedure, giving a purification factor of 80. The enzyme is a single-chain molecule, occurring, as does pig urinary kallikrein, as a major B-form of apparent mol.wt. 39600 and minor amounts of an A-form of apparent mol.wt. 35900; the two forms can be separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The amino acid composition of pig submandibular kallikrein is very similar to, but not quite identical with, that of the two-chain β-kallikrein isolated from pig pancreatic autolysates. Submandibular kallikrein contains notably more glucosamine and hexoses than does pancreatic β-kallikrein. Submandibular kallikrein, and also urinary kallikrein, exhibit an unusual biphasic hydrolysis of substrate esters that is not shared by pancreatic β-kallikrein. For the submandibular enzyme, the K_m for the initial reaction phase of the hydrolysis of α-*N*-benzoyl-L-arginine ethyl ester is 0.15 ± 0.01 mM (mean ± S.E.M.), but rises to 0.69 ± 0.04 mM (mean ± S.E.M.) in the stationary reaction phase; the $V_{max.}$ does not differ significantly between the two phases. The esterolytic activities of submandibular and urinary kallikreins on a number of esters of different amino acids resemble each other much more closely than those of pancreatic β-kallikrein.

The function of the glandular kallikreins (EC 3.4.21.8), serine proteinases found in the salivary glands, pancreas and urine of many species (Frey *et al.*, 1950), has been controversial for many years. As a step towards establishing the function of these enzymes, comparative information within a single species is desirable, to determine the properties that are common to the enzymes from the different tissues.

In the pig, pancreatic kallikrein has been characterized in detail (Fritz *et al.*, 1967; Kutzbach & Schmidt-Kastner, 1972; Zuber & Sache, 1974; Fiedler *et al.*, 1975; Fiedler, 1976), but relatively few data are available for submandibular and urinary kallikrein. We have isolated submandibular kallikrein in a highly purified form, and carried out chemical and enzymic studies to compare it with pancreatic and urinary kallikrein.

Experimental

Materials

Submandibular glands were obtained from freshly slaughtered pigs and stored at −20°C.

Abbreviation: SDS, sodium dodecyl sulphate.

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Urinary kallikrein was prepared as described by Tschesche *et al.* (1976*a*), with the affinity chromatography step included, and pancreatic kallikrein B (mainly two-chain β-kallikrein) was prepared by the method of Fiedler (1976).

α-*N*-Benzoyl-L-arginine ethyl ester (Bz-Arg-OEt) was obtained from Merck, Darmstadt, Germany, α-*N*-benzoyl-L-arginine methyl ester (Bz-Arg-OMe), α-*N*-toluene-*p*-sulphonyl-L-arginine methyl ester (Tos-Arg-OMe) and α-*N*-acetyl-L-tyrosine ethyl ester (Ac-Tyr-OEt) were from Serva, Heidelberg, Germany, and α-*N*-benzoyl-L-lysine methyl ester (Bz-Lys-OMe) and α-*N*-benzoyl-L-methionine methyl ester (Bz-Met-OMe) were from Cyclo Chemical, Los Angeles, CA, U.S.A., as hydrochlorides for the derivatives of basic amino acids. Ac-Phe-Arg-OMe acetate was synthesized from Ac-Phe and Arg-OMe,-2HCl by the carbodi-imide/*N*-hydroxysuccinimide procedure (Wünsch & Drees, 1966; Weygand *et al.*, 1966).

Basic bovine pancreatic trypsin inhibitor (Trasylol) was a gift of Bayer A.G., Wuppertal, Germany.

Methods

Kallikrein assay. All the assays described in this paper are based on the hydrolysis of Bz-Arg-OEt. During the initial steps of the purification, kallikrein

was assayed at 37°C by using a pH-stat method, as no other assay facilities were available. Subsequently, all the kallikrein activities were measured at 25°C, either by a pH-stat method or by an indirect spectrophotometric method in which the release of ethanol from Bz-Arg-OEt is linked to the reduction of NAD^+.

(*a*) pH-stat method (37°C). Assays were carried out with a Radiometer (Copenhagen, Denmark) Autotitrator (consisting of a PHM 63 meter, TTT 60 Titrator, ABU 13 Autoburette and a Titrigraph). Enzyme sample (20–500μl) was made up to 10.0ml with 15mM-sodium tetraborate, pH7.5, containing 0.25M-KCl and 2mM-EDTA. The reaction was started by adding 0.2ml of 2.06M-Bz-Arg-OEt, to give a final substrate concentration of 40.5mM, and the pH was maintained at 7.5 by titrating in 50mM-NaOH.

(*b*) pH-stat method (25°C). Assays were carried out with a Radiometer Autotitrator (PHM 26 meter, Titrator 11, Titrigraph SBR 2c and ABU 12 with a 0.25ml burette) under a stream of moist N_2 in a volume of 20ml at 25.0°C and pH8.0. The substrate was 10mM-Bz-Arg-OEt in 0.1M-NaCl containing 0.1mM-EDTA, and the titrating solution was 0.1M-NaOH (Fiedler, 1976). Reactions were started by the addition of kallikrein.

(*c*) Spectrophotometric assay. The conditions used are a modification of the procedure described by Trautschold & Werle (1961). The incubation medium consisted of 0.1ml of 30mM-NAD^+, 50μl of a solution of yeast alcohol dehydrogenase (EC 1.1.1.1) [the suspension supplied by Boehringer (Mannheim, West Germany) at a stated activity of 9000μmol of ethanol converted/min per ml was diluted with an equal volume of water], 0.5ml of 6mM-Bz-Arg-OEt (final concentration, 1mM) and the enzyme sample (10–100μl), made up to a total volume of 3.0ml with 0.15M-semicarbazide hydrochloride buffer at pH8.4, containing 0.15M-sodium pyrophosphate and 44mM-glycine. The enzyme was added last, and the A_{366} measured every 60s in a single-beam Eppendorf automatic spectrophotometer at 25°C. The activity was calculated from a 10min period during which a constant rate was maintained. Spontaneous hydrolysis occurring under identical conditions, except for the absence of enzyme, was subtracted.

Units of activity. One unit of activity is defined as the amount of enzyme hydrolysing 1μmol of Bz-Arg-OEt/min. Unless otherwise indicated, values were obtained by the pH-stat method at 25°C.

Specific activities are expressed either per mg of protein (determined from amino acid analysis) or referred to the amount of material contained in 1ml of a solution that has an A_{280} of 1 along a 1cm light path.

Preparation of Sepharose-linked guanidinated Trasylol. Trasylol (100mg) was guanidinated with *O*-methylisourea sulphate (Serva), under the conditions described by Kummel (1967), for 4 days at 4°C. The guanidinated Trasylol was isolated on a Merckogel PGM 2000 column (Merck) (1.8cm×7.5cm), equilibrated and developed with water at a flow rate of 60ml/h. The guanidinated Trasylol fractions, detected by inhibition of trypsin as described by Fritz *et al.* (1966), were freeze-dried. The yield was 93mg. Freeze-dried CNBr-activated Sepharose 4B (2g; Pharmacia, Uppsala, Sweden) was swollen in 1mM-HCl at 4°C; 88mg of guanidinated Trasylol was added in 12ml of 0.1M-sodium tetraborate buffer (pH8.0) containing 0.5M-NaCl. The mixture was allowed to react for 2h at room temperature (20°C), with continuous shaking. The reaction supernatant was removed from the resin by centrifugation (5000*g* for 5min) followed by decantation, and the resin suspended in 12ml of 1M-ethanolamine (pH9) for a further 2h at room temperature. The resin was then extensively washed with the borate/NaCl buffer, followed by 0.1M-triethanolamine (pH7.8) containing 1M-NaCl. The resin was expanded to the required bed volume by mixing with Sephadex G-75.

Polyacrylamide gel electrophoresis. Cylindrical (5mm×65mm) polyacrylamide gels (10%, pH8.9) were prepared and run as described by Maurer (1971). SDS/polyacrylamide-gel electrophoreses were carried out as described by Weber & Osborn (1969), except that reduction was accomplished with 1% dithioerythritol instead of 2-mercaptoethanol. Molecular-weight marker proteins were turkey ovalbumin (43000), yeast alcohol dehydrogenase (41000), rabbit muscle lactate dehydrogenase (36000), pepsin (35000) and chymotrypsinogen A (25700).

Isoelectric focusing. This was carried out on 5% polyacrylamide plates containing 2.4% Ampholine (nominal range, pH3.5–9.5) (LKB, Stockholm, Sweden). The gels were focused on an LKB 2117 Multiphor bed at 3°C for 2.5h, the final voltage being 1200V. The pH gradient was determined by removing small segments of gel and measuring the pH of the Ampholines eluted into water. The focused gel was fixed with trichloroacetic acid solution (75g of trichloroacetic acid in 150ml of methanol+350ml of water), stained with Coomassie Brilliant Blue R_{250} (0.115%, w/v, in destaining solution) and destained with methanol/acetic acid/water (5:2:13, by vol.).

Neuraminidase treatment of kallikrein. α-Neuraminidase (EC 3.2.1.18) solution (0.5ml; from *Vibrio cholerae*; Serva; stated activity per ml: 500μg of *N*-acetylneuraminic acid released from human glycoprotein in 15min at 37°C) was added to 0.5ml of a solution of about 5mg of kallikrein in 50mM-sodium acetate buffer, pH6.0, containing 0.15M-NaCl and 10mM-$CaCl_2$. The mixture was incubated for 20h at 37°C. Kallikrein alone and α-neuraminidase alone were incubated under identical conditions as controls. The samples were then passed through a 1.1cm×56cm column of Ultrogel AcA 54 (LKB),

with 10mM-triethanolamine, pH7.5, containing 0.1M-NaCl, as the eluting buffer. The fractions containing material reacting in the resorcin test (Whitehouse & Zilliken, 1960) were pooled, and the sialic acid content of the pooled sample was then determined quantitatively, by using synthetic *N*-acetylneuraminic acid (Serva) as a standard. The kallikrein-containing fractions were pooled, desalted on the same column, but with 10mM-ammonium acetate, pH6.7, as the eluting buffer, and freeze-dried.

Preparation of reduced carboxymethylated kallikrein (*Crestfield et al.*, 1963; *White*, 1967). First 6M-guanidinium hydrochloride was prepared by dissolving 2g of guanidinium hydrochloride in 2ml of 2M-Tris/HCl, pH8.6, containing 0.02% EDTA. Then 16mg of the enzyme was dissolved under N_2 in 1.6ml of this solution; 40μl of 2-mercaptoethanol was added, and the solution kept at room temperature for 18h. Next 65.9mg of freshly recrystallized iodoacetic acid, dissolved in 0.42ml of 1.4M-NaOH, was added, and 15min later 20μl of 2-mercaptoethanol. After a further 30min, 2ml of acetic acid and 0.5ml of water were added, and the solution was applied to a column (1.5cm×146cm) of Sephadex G-75, equilibrated with 50% (v/v) acetic acid. Elution at 17ml/h was carried out with the same solvent and 2.5ml fractions were collected. The fractions containing reduced carboxymethylated enzyme (detected by A_{280}) were combined, and the material was recovered under reduced pressure in a rotary-film evaporator. The fractions (including the salt peak) after the protein peak were also combined and evaporated.

Amino acid analysis. Samples containing about 0.15mg of material were hydrolysed under vacuum at 110°C in 1.5ml of concentrated reagent-grade HCl (Merck), diluted with 1.5ml of water, to which 20μl of phenol (liquefied with 20% water) had been added. The periods of the hydrolyses are indicated in the Results section.

The analyses were carried out on a Beckman Unichrom amino acid analyser equipped with microcuvette, multi-sample injector and Infatronics integrator CRS-110A. The procedure was either the two-column system (Spackman *et al.*, 1958), or a single-column, two-buffer system described by the manufacturers of the instrument. For some of the analyses a Durrum D-500 amino acid analyser has been used.

Results

Isolation of submandibular kallikrein (*Table* 1)

The first steps of the following isolation procedure were based on the procedure outlined by Werle & Trautschold (1963). An affinity step was included, since exploratory studies (Fritz *et al.*, 1969; Fritz & Förg-Brey, 1972) had shown the benefits of such a procedure for the isolation of glandular kallikreins.

Extraction. Frozen gland tissue (8.45kg) was minced in a heavy-duty stainless-steel mill, and the semi-frozen pulp homogenized in 0.1M-acetic acid (5 litres/kg of tissue) in a Waring-type blender (1min at maximum speed) at 5°C. The homogenate was centrifuged at 19000*g* at 20°C in a continuous centrifuge and the supernatant neutralized to pH7 with 5M-NaOH.

Acetone precipitation. Acetone precooled to 5°C was added with vigorous stirring to give a concentration of 35% (v/v), and left overnight at 5°C. The precipitate was removed by centrifugation (19000*g* at 20°C in a continuous centrifuge) and the concentration of acetone in the supernatant raised to 65% (v/v). Stirring was continued for 2h at 5°C, and the precipitate collected by centrifugation in the same way. The precipitate was dissolved by stirring overnight at 5°C in 14 litres of water that had been adjusted to pH7.5 with ammonia solution.

DEAE-cellulose adsorption. DEAE-cellulose (Whatman DE 23; 300g dry wt. equilibrated with 0.1M-ammonium acetate, pH6.7) was stirred into the solution and after 1h the supernatant removed by filtration through a glass sinter under reduced pressure. The DEAE-cellulose was washed with 2×5 litres of 0.1M-ammonium acetate, pH6.7, and then the kallikrein eluted with 5 litres of ammonium acetate (40g/l), pH6.7, containing NaCl (50g/l). No further kallikrein was eluted by raising the concentration of

Table 1. *Isolation of kallikrein from pig submandibular glands*
The starting material was 8.45kg of frozen glands.

Purification step	Total activity (unit) pH-stat (37°C)	Total activity (unit) Spectrophotometric assay	Specific activity from the spectrophotometric assay (unit/A_{280})	Yield (%)
Neutralized supernatant	187500			(100)
65%-acetone precipitate	134000			71
DEAE-cellulose eluate	115000	34000	1.2	61
Affinity-chromatography eluate		20900	95.7	38
DEAE-Sephadex eluate		17000	97.3	31

the elution buffer to ammonium acetate (50g/l), pH6.7, containing NaCl (50g/l). The eluate was dialysed twice against 10vol. of water (pH adjusted to 7 with ammonia solution) and freeze-dried. Freeze-dried material (28.3g) was obtained from 8.45kg of gland tissue.

Affinity chromatography. Batches of 3–12g of the freeze-dried kallikrein were dissolved in 250ml of affinity-wash buffer (0.1M-triethanolamine, pH7.8, containing 1M-NaCl) and mixed with 145ml (bed volume) of Sepharose-linked Trasylol. After decantation of the supernatant, the resin was washed with 250ml of affinity-wash buffer and packed into a 3.5cm-diameter column, cooled to 12°C with a water jacket. The resin was washed at a flow rate of 30–40ml/h until the eluate no longer contained detectable amounts of material absorbing at 260nm (400ml was normally sufficient). The wash buffer was replaced by eluting buffer (0.1M-triethanolamine, pH6.45, containing 0.5M-NaCl and 0.5M-benzamidine hydrochloride). All the kallikrein was eluted in the first 300ml. Kallikrein can be directly assayed in the eluate, in spite of the benzamidine, because dilution of the inhibitor in the assay cuvette decreases inhibition almost to zero. The combined eluate from three affinity separations was concentrated to 50ml by ultrafiltration (Amicon UM2 filter); the remaining benzamidine was removed by filtration through a column (2.2cm×60cm) of Sephadex G-25, with 10mM-ammonium acetate, pH6.7, as the buffer, and the kallikrein-containing fractions were combined and freeze-dried.

DEAE-Sephadex chromatography. A final purification was carried out on DEAE-Sephadex by a procedure that was very successful with pancreatic kallikrein (Kutzbach & Schmidt-Kastner, 1972). The kallikrein (10mg/ml of 0.1M-ammonium acetate, pH6.7) was loaded on a column (1.8cm×55cm) of DEAE-Sephadex A-50 (equilibrated with 0.1M-ammonium acetate, pH6.7, and cooled to 12°C with a water jacket). The kallikrein was eluted by means of a convex ammonium acetate gradient formed by passing 0.8M-ammonium acetate (pH6.7) into a constant-volume mixing chamber containing 150ml of 0.1M-ammonium acetate (pH6.7). A peak of material absorbing strongly at 253nm was eluted in fractions 27–35, and may be due to nucleic acids not removed by affinity chromatography. Protein was eluted in a single peak in which absorption at 280nm corresponded well to kallikrein activity. The kallikrein was desalted through Sephadex G-25 (2.2cm×60cm) with 10mM-ammonium acetate, pH6.7, as the buffer, and freeze-dried.

Specific activity and molecular weight

The purified submandibular kallikrein had a specific activity of 166 units/A_{280} or 312 units/mg of protein. This was not increased by repeating the chromatography step on DEAE-Sephadex. It was, however, necessary to have 0.1mM-EDTA or 0.1mM-thioglycollic acid present in the assay, otherwise the activities were about 10% lower; this effect resembles that observed with pig pancreatic kallikrein (Fiedler & Werle, 1968), and is most probably due to the presence of inhibitory amounts of heavy metals in the assay medium. Under the same conditions, urinary kallikrein had a specific activity of 156 units/A_{280}, which could be raised to 168 units/A_{280} by repeating the DEAE-Sephadex-chromatography step. These values may be compared with a specific activity of 141 ± 5 units/A_{280} obtained for pig pancreatic β-kallikrein (Fiedler *et al.*, 1975).

The homogeneity of the submandibular kallikrein was checked by rechromatography on DEAE-Sephadex A-25. The kallikrein was eluted as a single rather broad peak with a slight tail. SDS/polyacrylamide-gel electrophoresis of submandibular kallikrein (and also of urinary kallikrein) resulted in two bands, although these are much less clearly resolved for submandibular kallikrein than for urinary kallikrein (Fig. 1). By analogy to Habermann's (1962) nomenclature for electrophoretically discernible forms of pancreatic kallikrein, the slower-running dominant band is called the B-form, and the faster-running minor band is called the A-form. However, SDS/polyacrylamide-gel electrophoresis of the fractions eluted from DEAE-Sephadex showed that,

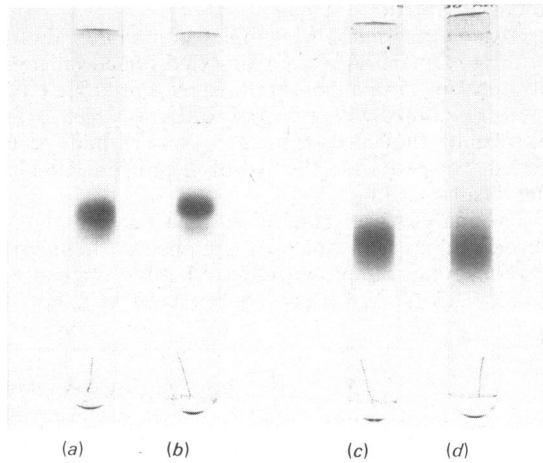

Fig. 1. *SDS/polyacrylamide-gel and disc-gel electrophoresis of pig submandibular and urinary kallikreins*

SDS/polyacrylamide-gel electrophoresis was run for 17h at 15V and 2.3mA/gel. (*a*) Reduced submandibular kallikrein; (*b*) reduced urinary kallikrein. Disc-gel electrophoresis was run for 100min at 100V and 1.2mA/gel. (*c*) Submandibular kallikrein; (*d*) urinary kallikrein. To all gels 20μg samples were applied. The anode was at the bottom.

whereas early fractions of the peak contained only a B-form, about one-third of the kallikrein in fractions eluted towards the tail of the peak was present as the A-form. Nevertheless, since different fractions across the peak had similar specific activities it appears that both forms are enzymically active and have similar specific activity. Urinary kallikrein behaved in the same way as regards the position and shape of the peak eluted from DEAE-Sephadex, and the existence of A- and B-forms with similar specific activity. In the absence of SDS, electrophoresis of either kallikrein on polyacrylamide gel yielded a single broad diffuse band (Fig. 1). In ultracentrifugation studies the preparations of both submandibular and urinary kallikrein behaved as homogeneous proteins (Fritz *et al.*, 1977).

The molecular weights of the A- and B-forms of submandibular kallikrein were estimated to be respectively 35900 ± 700 and 39600 ± 800 from SDS/polyacrylamide-gel-electrophoresis studies of the reduced enzyme. The molecular weights of the A- and B-forms of urinary kallikrein were 36100 ± 600 and 39600 ± 700 respectively. Since the molecular weights of reduced submandibular and urinary kallikrein have nearly the same values as those obtained for the native enzymes (38000 ± 1000 and 40000 ± 1500 respectively) in ultracentrifugation studies (Fritz *et al.*, 1977), it appears that these two kallikreins are single-chain molecules. Nevertheless, submandibular and urinary (Tschesche *et al.*, 1976*a*) kallikreins are glycoproteins, so that it is likely that the molecular weights obtained by SDS/polyacrylamide-gel electrophoresis are too high; similarly, the values obtained from ultracentrifugation studies (Fritz *et al.*, 1977) are also probably too high, for reasons discussed in that paper.

Isoelectric focusing and the action of neuraminidase

Submandibular kallikrein is resolved into eight bands when focused on an Ampholine gradient in polyacrylamide-gel plates. The isoelectric points were estimated to be 3.15, 3.4, 3.65, 3.85, 3.95, 4.1, 4.2 and 4.4, which is in good agreement with the values previously reported for a sucrose-density-gradient system (Fiedler *et al.*, 1970*b*). After treatment with neuraminidase for 20h the pattern changed, with the material of higher pI values becoming relatively more heavily represented. Incubation for up to 86h did not result in any further change in the pattern of focused bands; under the same conditions, only a single band was observed on isoelectric focusing of pancreatic β-kallikrein B treated for 20h with neuraminidase, confirming previous observations on that enzyme (Fritz *et al.*, 1967; Fiedler *et al.*, 1970*a*, 1975). After incubation for 20h at 37°C with neuraminidase (see under 'Methods'), 2.0mol of free neuraminic acid/mol of kallikrein was recovered, but the behaviour of the isoelectric-focusing pattern on treatment of the kallikrein with neuraminidase suggests that part of the neuraminic acid content of the enzyme may be resistant to the action of neuraminidase. The specific activity (determined by the spectrophotometric assay) of neuraminidase-treated kallikrein (87.5 units/A_{280}) was not appreciably different from the specific activity of kallikrein incubated without neuraminidase (88.5 units/A_{280}).

Amino acid composition

A number of amino acid analyses have been carried out on submandibular kallikrein, under a variety of different conditions. The results of the analyses are recorded in Table 2, together with what is concluded to be the most probable composition. In view of the uncertainty of the molecular-weight values obtained by physical methods, absolute values for amino acid residues per molecule were calculated by using the mol.wt. 26500 (Fiedler *et al.*, 1977) of the protein component of pancreatic β-kallikrein. The validity of such a procedure is supported by the nearly identical composition that results.

In the single-column systems, glucosamine is eluted in the region between methionine and leucine, precluding determination of the amino acids in this region. Threonine and serine were corrected for destruction during hydrolysis by multiplying the results from the 24h hydrolyses by factors of 1.04 and 1.09 respectively. These factors have been derived from numerous analyses with pig pancreatic kallikrein.

The molar absorption coefficient of submandibular kallikrein at 280nm has been determined as $48.8 \times 10^3 \text{M}^{-1} \cdot \text{cm}^{-1}$ (Fritz *et al.*, 1977). This is practically equal to the molar absorbance of pancreatic kallikrein, i.e. $(50.6 \pm 1.3) \times 10^3 \text{M}^{-1} \cdot \text{cm}^{-1}$ (Fiedler *et al.*, 1975). The A_{280}/A_{260} ratio obtained for submandibular kallikrein was 1.76 ± 0.02, which is also very similar to the value of 1.74 found for pancreatic kallikrein (Kutzbach & Schmidt-Kastner, 1972). In view of this, and because the tyrosine and cysteine contents of the two enzymes are equal (Table 2), it may be inferred that the tryptophan content is also equal. The total number of residues in the submandibular kallikrein molecule exceeds that of pancreatic kallikrein probably by three residues: a threonine, a histidine and a lysine. The molecular weight of the protein part of pig submandibular kallikrein is thus 25970.

The reduced carboxymethylated submandibular kallikrein, which is eluted as a single sharp peak from Sephadex G-75 (see under 'Methods'), has a composition identical with that of native kallikrein. No additional amino acids were detected by amino acid analysis of HCl hydrolysates of the fractions (including the salt peak) eluted after the kallikrein peak.

Table 2. *Amino acid analyses of pig submandibular kallikrein*
For full details see the text. Values are means ± S.E.M.

	Amino acid (residues/molecule)								
	I	II	III	IV	V	VI	VII		
Hydrolysis time (h) ...	24, 48, 72	24	164	24	24	24, 48, 72	24	Most	Pig
Analytical system ...	Single column	Two column	Two column	Two column	Single column	Durrum	Durrum	probable integral	pancreatic β-kallikrein B†
No. of samples ...	3	3	3	3*	3	3	4	value	
Asp	28.02	28.5 ± 0.5	28.4 ± 0.5	28.4 ± 0.5	27.3 ± 0.2	28.0 ± 0.2	27.4 ± 0.4	28	28
Thr	14.62‡	15.5 ± 0.1		15.8 ± 0.2	15.7 ± 0.2	15.9‡	15.7 ± 0.2	16	15
Ser	13.54‡	14.0 ± 0.1		14.5 ± 0.4	14.3 ± 0.2	14.4‡	13.3 ± 0.1	14	14
Glu	22.69	23.1 ± 0.3	23.1 ± 0.3	22.9 ± 0.3	23.2 ± 0.3	22.9 ± 0.2	23.6 ± 0.3	23	23
Pro	15.33	15.2 ± 0.2		15.0 ± 0.3	16.2 ± 0.5	19.0 ± 0.3	15.9 ± 1.7	16	16
Gly	22.49	21.8 ± 0.4	22.2 ± 0.4	22.3 ± 0.5	21.6 ± 0.1	22.7 ± 0.1	21.5 ± 0.2	22	22
Ala	13.26	12.8 ± 0.1	12.9 ± 0.2	13.1 ± 0.2	12.9 ± 0.1	13.1 ± 0.1	13.2 ± 0.1	13	13
½CyS	9.4§			9.8 ± 0.3				10	10
Val	9.76	9.8 ± 0.1	9.8 ± 0.1	9.8 ± 0.1	9.7 ± 0.0	9.2 ± 0.2	9.6 ± 0.1	10	10
Met	4.0§	3.4 ± 0.1		3.4 ± 0.2				4	4
Ile		10.4 ± 0.1	11.2 ± 0.1	10.4 ± 0.1				11	12
Leu		21.4 ± 0.2	21.3 ± 0.1	21.6 ± 0.2				21	20
Tyr	6.80	6.9 ± 0.1		6.9 ± 0.1		6.3 ± 0.3	7.3 ± 0.1	7	7
Phe	10.09	9.9 ± 0.1		9.8 ± 0.1		9.7 ± 0.2	9.9 ± 0.7	10	10
Lys	10.45	11.4 ± 0.1		11.5 ± 0.1	12.6 ± 0.1	11.8 ± 0.1	11.0 ± 0.1	11	10
His	7.59	8.6 ± 0.1		8.7 ± 0.1	8.8 ± 0.1	8.8 ± 0.1	8.9 ± 0.1	9	8
Arg	2.68	3.0 ± 0.1		2.9 ± 0.1	2.8 ± 0.0		3.2 ± 0.1	3	3
Trp‖								7	7
Total								235	232

* Reduced carboxymethylated sample.
† Values taken from Fiedler *et al.* (1977).
‡ Extrapolated to zero hydrolysis time.
§ Performic acid-oxidized sample.
‖ Estimated spectrophotometrically as described in the text.

This is additional evidence that submandibular kallikrein is a single-chain molecule, and that it does not contain a small peptide fragment that escapes detection during SDS/polyacrylamide-gel electrophoresis.

A single sample of submandibular kallikrein was hydrolysed for 3.5h at 100°C in 6M-HCl (containing phenol, as described under 'Methods') and run on the long column of the Unichrom amino acid analyser. This yielded a preliminary value of 14 glucosamine and 0.6 galactosamine residues/molecule. An analysis of submandibular kallikrein by the orcinol procedure (François *et al.*, 1962), with mannose as a standard, gave a value of about 25 hexose residues/molecule. These values for the carbohydrate content, from which a molecular weight of about 34000 may be calculated for the enzyme, are regarded as preliminary.

Esterolytic activity

The hydrolysis of Bz-Arg-OEt by porcine submandibular and urinary kallikrein follows a remarkable biphasic time course. The initial rate of hydrolysis falls until a slower maintained rate is achieved. The effect is more pronounced, and the time taken to achieve the maintained rate is longer, when low concentrations of Bz-Arg-OEt are used. With 0.2mM-Bz-Arg-OEt, at pH8.0 and 25°C, about 5min elapses before the stationary phase is reached, but at concentrations of 1mM and above the initial rapid phase is barely detectable. The effect is not due to substrate depletion, because of the constancy of the rate in the stationary phase. Furthermore, when the reaction commenced with a Bz-Arg-OEt concentration of 0.2mM, only about 10% of the substrate had been hydrolysed when the stationary phase was reached. Nor is the effect due to product inhibition: with 0.2mM-Bz-Arg-OEt as substrate, the presence of 0.2mM-Bz-Arg or 0.2 or 2mM-Bz-ArgNH$_2$ had no influence on the course of the hydrolysis; this was true whether the reaction was started by the addition of enzyme, or whether the enzyme was first incubated for 8min with the Bz-Arg or Bz-ArgNH$_2$ and the reaction then begun by addition of the Bz-Arg-OEt. After complete hydrolysis of the substrate, further additions of Bz-Arg-OEt again provoked the biphasic reaction. The effect was independent of the presence of 0.1mM-thioglycollic acid or EDTA. The phenomenon is not an artifact of the pH-stat assay, since it was also observed when the reaction was followed spectrophotometrically at 253nm in 0.1M-Tris/HCl (containing 0.1mM-EDTA) at pH8.0 and 25°C, at a Bz-Arg-OEt concentration of 0.2 or 0.5mM.

The biphasic hydrolysis reaction was not confined to Bz-Arg-OEt. The arginine esters Bz-Arg-OMe and Ac-Phe-OMe behaved similarly, but to different extents. With Bz-Lys-OMe, the stationary phase was achieved very slowly with both submandibular and urinary kallikreins. None of the other substrates listed in Table 3 gave any indication of a biphasic response over the 15min period in which the reaction was followed. The phenomenon was never observed with β-pancreatic kallikrein B, irrespective of the substrate used.

The esterolytic profiles of submandibular and urinary kallikrein resemble each other remarkably closely over a wide range of different substrates (Table 3). In comparison with these two enzymes, the profile of pancreatic β-kallikrein, although generally similar, differs notably in certain details.

Kinetic constants for the hydrolysis of Bz-Arg-OEt by submandibular and urinary kallikreins have been determined for the initial and the stationary reaction phases (Table 4). The hydrolysis was followed on the Autotitrator as described under 'pH-stat method (25°C)', except that 5mM-NaOH was used as titrating agent and the substrate concentration was as given in Table 4. Initial rates were determined from tangents drawn to the Autotitrator curves. Kinetic constants were calculated by the method of Wilkinson (1961) after inspection of the data in Lineweaver–Burk and Eadie diagrams. In each case Michaelis–Menten-type kinetics were exhibited. The resulting $V_{max.}$ values are quite similar, but K_m is much lower in the initial reaction phase. The latter observation reconciles the value of 0.114mM obtained for submandibular kallikrein (Lemon *et al.*, 1976) and of 0.125mM for urinary kallikrein (Tschesche *et al.*, 1976*a*) with values of 0.60mM and 0.44mM respectively obtained previously (Trautschold & Werle, 1961). In contrast with the similarity of the data for submandibular and urinary kallikrein, those for pancreatic β-kallikrein are distinctly different.

The different values of the Michaelis constants for pancreatic kallikrein on the one hand and for submandibular and urinary kallikreins on the other have a bearing on the activities of the enzymes as deter-

Table 3. *Relative rates of ester hydrolysis catalysed by the three pig glandular kallikreins as measured in the stationary reaction phase on the Autotitrator*

Values are related to rate = 100 for each kallikrein at 10mM-Bz-Arg-OEt. Conditions: 1mM-substrate, 0.1 = M-NaCl, 0.1mM-EDTA, pH8.0, at 25°C.

Substrate	Kallikrein		
	β-Pancreatic B	Submandibular	Urinary
Ac-Phe-Arg-OMe	578	257	266
Bz-Arg-OMe	112	87.3	95.5
Bz-Arg-OEt	95.5	69.8	73.6
Tos-Arg-OMe	2.73	2.57	2.99
Bz-Lys-OMe	4.23	0.86	1.19
Ac-Tyr-OEt	0.25	0.21	0.19
Bz-Met-OMe	0.18	0.12	0.12

mined with the various assay systems in use for glandular kallikreins (Table 5). The activity of pancreatic β-kallikrein depends much less on the substrate concentration than does the activity of the other two enzymes. Nevertheless the variations observed with submandibular and urinary kallikrein are similar under a wide variety of assay conditions. The especially low values obtained with the NAD^+-linked assay indicate some inhibitory influence of a component of this assay system.

We do not wish to discuss in greater depth in the present paper the cause of the unique kinetic behaviour of submandibular and urinary kallikrein. The phenomenon has been described here to emphasize the very close relationship between the two enzymes.

Discussion

We have described a procedure for the isolation of highly purified pig submandibular kallikrein. The incorporation of an affinity-chromatography step is particularly advantageous, since the kallikrein solution eluted from DEAE-cellulose was extremely viscous, and unsuitable for processing on a conventional column. The use of the Sepharose-linked Trasylol allows a batchwise washing step to be inserted before the resin and adsorbed kallikrein are loaded on a column for more extensive washing. The affinity chromatography yields kallikrein with little contamination; the major impurity, which may be nucleic acid, is removed by a final chromatography step through DEAE-Sephadex.

Sufficient data have now been accumulated to warrant a detailed appraisal of the relationship between the three pig glandular kallikreins (submandibular, pancreatic and urinary).

Submandibular kallikrein probably has an identical composition to the pancreatic enzyme, apart from the presence of three extra residues (one each of threonine, histidine and lysine). This is reflected in the amino acid sequence: the sequence so far available for both submandibular and pancreatic kallikreins

Table 4. *Kinetic constants for the hydrolysis of Bz-Arg-OEt by the three pig glandular kallikreins*
Measurements were made in an Autotitrator, in 0.1 M-NaCl containing 0.1 mM-EDTA at pH 8.0 and 25.0°C. For submandibular and urinary kallikreins, data were obtained at Bz-Arg-OEt concentrations of 0.05–0.7 mM (initial rates) or 0.05–10 mM (stationary rates). For pancreatic kallikrein, with which only a stationary phase is observed, a range of 0.01–1 mM-Bz-Arg-OEt was used (Fiedler *et al.*, 1973). Values are means ± S.E.M for at least three titrations.

	Initial reaction phase			Stationary reaction phase		
	K_m (mM)	$k_{cat.}$ (s^{-1})	$V_{max.}$ (μmol/min per A_{280} unit)	K_m (mM)	$k_{cat.}$ (s^{-1})	$V_{max.}$ (μmol/min per A_{280} unit)
Submandibular kallikrein	0.15 ± 0.01	138 ± 6	169 ± 7	0.69 ± 0.04	154 ± 6	189 ± 7
Urinary kallikrein	0.19 ± 0.02		172 ± 6	0.61 ± 0.03		173 ± 3
Pancreatic β-kallikrein				0.104 ± 0.008	123 ± 8	

Table 5. *Relative rates of Bz-Arg-OEt hydrolysis by the three glandular kallikreins in different test systems*
Values are related to rate = 100 at 10 mM-Bz-Arg-OEt in the Autotitrator assay. The values for submandibular and urinary kallikreins refer to the stationary phase of the reaction. All assays were run at 25.0°C.

				Relative measured activity		
Assay	Reaction medium	Bz-Arg-OEt (mM)	pH	Submandibular kallikrein	Urinary kallikrein	Pancreatic β-kallikrein B
Autotitrator	0.1 M-NaCl/0.1 mM-EDTA	10	8.0	100	100	100
		0.5	8.0	54	48	87
		0.5	8.7	62	57	100
Photometric* (direct)	0.05 M-Triethanolamine	0.5	8.0	48	45	78
	0.1 M-Tris/0.1 mM-EDTA	0.5	8.0	45	41	82
Photometric*† (NAD^+-linked)		0.5	8.7	33	32	73

* Trautschold & Werle (1961).
† Fritz *et al.* (1967).

(that of the 28 residues of the *N*-terminal region) is identical for the two enzymes (Fritz *et al.*, 1977). However, pig pancreatic kallikrein consists of two polypeptide chains (Fiedler *et al.*, 1977; Tschesche *et al.*, 1976*b*), whereas both submandibular and urinary kallikreins were found to be single chain molecules. Hence the speculation arises that the three additional residues in submandibular kallikrein represent a tripeptide fragment originally present between the two chains of pancreatic kallikrein. The fact that the pancreatic kallikrein was obtained from tissue autolysates in which a great deal of proteolytic activity would be expected to be present suggests that the cleavage of such a fragment during the isolation procedure is not unlikely. Additional indirect support for this proposal may be derived from the *C*-terminal residue of the A-chain of pancreatic β-kallikrein (i.e. one of the sites of cleavage); in about half of the chains this residue is serine, but in the other half the serine has evidently been cleaved off (Tschesche *et al.*, 1976*b*).

It is tempting to consider whether a single-chain precursor to the two-chain pancreatic kallikrein can be isolated that would have properties much closer to those of submandibular kallikrein. So far, evidence for such a pancreatic kallikrein is scant. Tentative support may be gleaned from the report of pancreatic kallikrein B′, obtained by spontaneous activation of pancreatic prekallikrein (Fiedler *et al.*, 1970*a*). This kallikrein resembled submandibular kallikrein (cf. Table 5) in giving a much lower activity in an NAD^+-linked spectrophotometric assay at a low Bz-Arg-OEt concentration (0.5mM) than in an Autoanalyser assay at a high Bz-Arg-OEt concentration (4mM). Probably pancreatic kallikrein B′ is an undegraded single-chain form of pancreatic kallikrein.

The amino acid composition of urinary kallikrein (Tschesche *et al.*, 1976*a*; Fritz *et al.*, 1977) is not yet sufficiently precise for it to be seen whether or not it is identical with that of submandibular kallikrein. Certainly, though, in view of the very similar physical and chemical characteristics and the indistinguishable enzymic behaviour of submandibular and urinary kallikrein, it would not be surprising if the amino acid sequences were identical. Such minor differences as are observed (for example, in electrophoretic behaviour) are likely to be due to differences between the carbohydrate moieties of the two enzymes.

Because of the very close similarity of the three pig gland kallikreins it is strange that pancreatic kallikrein should be synthesized as an inactive precursor (prekallikrein), whereas there is no evidence for such a precursor in the submandibular gland or the urine. The likelihood of genetic alteration resulting in the synthesis of an active sequence in one tissue and a precursor sequence in another is remote (Neurath & Walsh, 1976). The explanation is not known.

The origin of pig urinary kallikrein may also be considered in the light of our results, since it has often been proposed that urinary kallikrein is not synthesized in the kidney, but merely represents submandibular or pancreatic kallikrein filtered from the blood. The filtration theory seems unlikely since there are minor but distinct differences between all three enzymes. This is important physiologically: if active kallikrein in the urine were derived from a distant gland it would imply the presence of gland kallikrein in the blood in a potentially active form, a situation that has been hotly debated for many years.

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