Purification and properties of guinea-pig submandibular-gland kallikrein

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Guinea-pig submandibular kallikrein has been purified from the glands to electrophoretic homogeneity by conventional procedures. The enzyme is active as a kininogenase, releasing kallidin at a rate of 462 $\mu\text{g}/\text{min}$ per mg of protein from bovine kininogen, and proved potently hypotensive in the guinea pig and in the dog, properties which indicate its tissue kallikrein nature. The specific activity determined on the substrate *N*- α -benzoyl-L-arginine ethyl ester (11.1 $\mu\text{mol}/\text{min}$ per mg of protein) is much lower than that measured with *N*-acetyl-L-phenylalanyl-L-arginine ethyl ester (483 $\mu\text{mol}/\text{min}$ per mg of protein). The latter value is of an order of magnitude comparable with the specific activities of other tissue kallikreins determined with this sensitive kallikrein substrate. The enzyme is a glycoprotein consisting of 237 amino acid residues and containing three to four glucosamine molecules. Its amino acid composition is not identical with that reported for guinea-pig coagulating-gland kallikrein, but is remarkably similar to that of the porcine tissue kallikreins. Apparent M_r values are 29 000 (sodium dodecyl sulphate/polyacrylamide-gel electrophoresis) or 34 000 (gel filtration). The amino acid sequence of the first 31 *N*-terminal residues was determined and was found to be closely homologous with that of other tissue kallikreins.

Tissue or glandular kallikreins (EC 3.4.21.35) are serine proteinases with specific enzymic actions. From endogenous protein substrates (kininogens), tissue kallikreins release the decapeptide kinin (kallidin), which possesses high biological activity. The biochemical properties of highly purified tissue kallikreins from a number of species, in particular the porcine enzymes, have been characterized in considerable detail (see Fiedler, 1979; Fiedler *et al.*, 1981).

Of the tissue kallikreins in the guinea pig, experiments have been performed on the coagulating-gland (Moriwaki *et al.*, 1974b) and submandibular-gland enzymes. Immunological studies of Bhoola *et al.* (1979) failed to demonstrate a complete identity between these two enzymes. In contrast, human, porcine and rat tissue kallikreins

Abbreviations used: Bz-Arg-OEt, *N*- α -benzoyl-L-arginine ethyl ester; Ac-Phe-Arg-OEt, *N*-acetyl-L-phenylalanyl-L-arginine ethyl ester.

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from different organs show complete immunological identity (see Fiedler, 1979). The question therefore arose concerning the chemical nature and properties of submandibular- and coagulating-gland kininogenases. In the present study we report the isolation and properties of guinea-pig submandibular-gland kallikrein.

Experimental

Materials

Hydroxyapatite was Bio-Gel HTP obtained from Bio-Rad Laboratories. Sephadex G-75 (superfine grade), G-100 and G-200, DEAE-Sephadex and DEAE-Sepharose were purchased from Pharmacia, DEAE-cellulose from Whatman, Bz-Arg-OEt hydrochloride from E. Merck, bradykinin from Sandoz, kallidin (lysylbradykinin) from Sigma, and AnalaR-grade reagents from BDH. The synthesis of Ac-Phe-Arg-OEt acetate is described by Fiedler *et al.* (1978) and the isolation of porcine submandibular-gland kallikrein by Lemon *et al.* (1979). Neur-

aminidase-treated porcine pancreatic β -kallikrein B was generously given by Bayer AG (Kutzbach & Schmidt-Kastner, 1972). Partially purified bovine kininogen was prepared from heated bovine plasma by precipitation with $(\text{NH}_4)_2\text{SO}_4$ at half saturation, dialysis and freeze-drying (Moriwaki *et al.*, 1974a). Guinea-pig kininogen was enriched from plasma in a similar way. Human kininogen was partially purified from heat-treated plasma (human blood was kindly provided by the National Blood Transfusion Service, Bristol BS10 5ND, U.K.) by $(\text{NH}_4)_2\text{SO}_4$ precipitation and gel filtration on Sephadex G-200 (Hamberg *et al.*, 1975).

Methods

Protein. Protein was determined by its A_{280} in cuvettes of 1 cm light-path. One A_{280} unit is that amount of protein showing an absorbance of 1 when contained in 1 ml of solution. For determination of the specific activities of the purified enzyme, the protein content was obtained from quantitative amino acid analysis.

Esterolytic activity of guinea-pig submandibular-gland kallikrein. This was determined, with Bz-Arg-OEt as substrate, by the method of Schwert & Takenaka (1955). Cuvettes of 1 cm light-path, thermostatically controlled at 25.0°C, contained 3.00 ml of 0.1 M-Tris/HCl buffer, pH 8.0, containing 0.1 mM-EDTA, 0.5 mM-Bz-Arg-OEt hydrochloride, and an amount of kallikrein, such that $\Delta A_{253}/10$ min did not exceed a value of 0.10 (Fiedler *et al.*, 1977b). The reaction was started with the enzyme solution, and the increase in absorbance was monitored at 253 nm for 10 min in a Zeiss PM6 spectrophotometer. Hydrolysis of 1 μmol of substrate results in an increase of absorbance of 0.3833 (Trautschold & Werle, 1961). One unit of kallikrein is defined as that amount of enzyme hydrolysing 1 μmol Bz-Arg-OEt/min under the above conditions. Ac-Phe-Arg-OEt hydrolysis was measured with 0.5 mM-substrate as described previously (Fiedler *et al.*, 1978). The coefficient of variation of this assay has been redetermined as 1.8%.

Kallidin release by guinea-pig submandibular-gland kallikrein. This was assayed by the method of Gau *et al.* (1982). A 10 mg portion of partially purified bovine kininogen [forming a total of 1.6 μg (1.2 nmol) of kallidin with porcine pancreatic kallikrein] was incubated (at 37°C in 1.0 ml of 0.5 M-phosphate buffer, pH 8.0, for 4.0 min) with 37 or 110 ng (based on amino acid analysis) of submandibular-gland kallikrein. Samples of 50 or 100 μl were analysed by high-pressure liquid chromatography as described by Gau *et al.* (1982). Kallidin was identified by virtue of its elution position relative to kinin standards, and the procedure was calibrated with kallidin samples quantified by weighing and

assumed to be pure kallidin tetra-acetate in the calculations.

Biological activity of the kinin released from kininogen by guinea-pig submandibular-gland kallikrein. This was demonstrated qualitatively by contraction of the guinea-pig ileum (at 35°C) and relaxation of the rat duodenum (at 31°C). The isolated organ preparations were suspended in 10 ml of Tyrode's solution gassed with CO_2/O_2 (1:19). Atropine (100 ng/litre) and mepyramine (100 ng/litre) were added before each test. Substrate [partially purified human kininogen (12.5 mg of protein) or guinea-pig kininogen (28 mg of protein)] and submandibular-gland kallikrein (11–44 ng of protein) were added directly to the organ bath.

Measurement of the hypotensive action of the enzyme on arterial blood pressure. Guinea pigs were anaesthetized by intraperitoneal injection of 1.75 g of urethane/kg and a dog with 45 mg of sodium pentobarbitone/kg. Blood pressure was recorded from the carotid artery with a pressure transducer linked through a George Washington oscillograph (model 400 MD/2) to a Bryans pen recorder. Injection of test substances was made through a venous cannula inserted into the external jugular vein of guinea pig and the femoral vein of the dog and washed into the circulation with a constant volume of 0.9% NaCl. The enzyme was match-assayed against bradykinin and the hypotensive effect expressed as bradykinin equivalents/mg of enzyme protein.

Apparent M_r of the enzyme. This was determined by gel filtration on a column (0.9 cm $\times$ 76 cm) of Sephadex G-75 (superfine grade) equilibrated with 0.1 M-Tris/HCl buffer, pH 8.0, containing 0.1 M-NaCl and calibrated with bovine pancreatic ribonuclease A, chymotrypsinogen A and chicken ovalbumin. A 2–4 mg sample of the kallikrein preparation (before the hydroxyapatite steps), dissolved in 0.5 ml of the above buffer, was applied to the column and was eluted with the same buffer at room temperature at a rate of 3 ml/h. Fractions (1 ml) were collected and assayed for esterolytic activity and for A_{280} .

Polyacrylamide-gel electrophoresis. This was run in cylindrical gels (5 mm $\times$ 65 mm) in a Shandon apparatus. Disc electrophoresis was performed at pH 8.9 in the 7.5% (w/v)-acrylamide no. 1 gel system of Maurer (1971), with or without spacer gel. Protein samples were applied in 50 μl of 20% (w/v) sucrose solution in 25-fold-diluted electrode buffer and run for 2 h at 90 V (1.3 mA/gel). Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed as described by Weber & Osborn (1969), except that protein was reduced with 1% dithioerythritol for 2 min at 100°C and run at 14 V (2 mA per gel) for 19 h. For determination of the M_r of guinea-pig submandibular-gland kalli-

krein, chicken ovalbumin, pepsin, bovine chymotrypsinogen A and bovine trypsin were used as markers. Gels were stained with Coomassie Brilliant Blue R-250.

Amino acid analysis. This was performed on 24, 48 and 72 h hydrolysates of 0.4 nmol of kallikrein in 0.3 ml of HCl [concentrated 37% (w/v) HCl (Merck 'pro analysi') diluted with an equal volume of water] containing 0.08% phenol (Sanger & Thompson, 1963; Africa & Carpenter, 1970) and hydrolysed at 110°C in evacuated sealed ampoules. After being evaporated to dryness in a vacuum desiccator over CaCl_2 and KOH, portions were analysed on a Durrum D-500 analyser with a single-column three-buffer system in the 2 nmol range, calibrated with an equal number of amino acid standards (batch 05247.13; Pierce Eurochemie, Rotterdam, The Netherlands). In order to achieve separation of glucosamine from phenylalanine (and galactosamine), the second buffer was adjusted as described by Fiedler & Hirschauer (1981). Cysteine was determined after oxidation with performic acid (Hirs, 1967).

N-Terminal amino acid sequence. This was determined by the Edman degradation method (Edman & Henschen, 1975) in the sequenator version on a 4.3 nmol sample of the native enzyme. A second sequenator run was performed after reduction and carboxymethylation (Lottspeich *et al.*, 1979) of the enzyme. In cycle 13, where proline was suspected from homology with other serine proteinases, the cleavage time was prolonged (Brandt *et al.*, 1976). The phenylthiohydantions were identified by a newly developed isocratic high-pressure-liquid-chromatographic system (Lottspeich, 1980).

Purification of guinea-pig submandibular kallikrein (Table 1). Extraction. All steps were performed at 4°C, unless stated otherwise. Frozen submandibular-gland tissue (130 g) was thawed and homogenized at full speed for 30 s. The homogenate was centrifuged (5 min, 3000 g) and the supernatant decanted and neutralized with 2 M-NaOH.

Adsorption on DEAE-cellulose. The supernatant was mixed with DEAE-cellulose (10 g dry wt.; Whatman DE-11 medium, equilibrated with 0.1 M-ammonium acetate, pH 6.7) and stirred intermittently for 1 h. The mixture was centrifuged (5 min, 3000 g) and the DEAE-cellulose precipitate mixed with 250 ml of 0.1 M-ammonium acetate, pH 6.7, stirred for 1 h and centrifuged (5 min, 3000 g) again. Esterolytic activity in the DEAE-cellulose precipitate was eluted by stirring with 3×100 ml of 0.8 M-ammonium acetate, pH 6.7, the supernatant on each occasion being collected by centrifugation (5 min, 3000 g). The three eluate fractions were combined, dialysed against 2×5 litres of deionized water (pH adjusted to between 7 and 8 with 1 M- NH_3) and freeze-dried.

Gel filtration. The freeze-dried material was dissolved in 30 ml of 0.1 M-ammonium acetate, loaded on a Sephadex G-100 column (80 cm $\times$ 1.6 cm) and eluted with 0.1 M-ammonium acetate, pH 6.7, with a flow rate of 30 ml/h and fraction size of 4 ml (Fig. 1). Fractions with Bz-Arg-OEt-hydrolysing activity were combined, desalted and freeze-dried.

First chromatography on hydroxyapatite. The freeze-dried enzyme was dissolved in 5 ml of 0.01 M-sodium phosphate buffer, pH 7.0, and dialysed overnight against 3 litres of the same buffer. The enzyme solution was applied at a rate of 8 ml/h

Table 1. *Purification of guinea-pig submandibular-gland kallikrein*

Owing to the low A_{280} of column eluates and some turbidity, the values for protein and specific activities for the hydroxyapatite steps are rather imprecise.

Purification step	Volume (ml)	Protein (A_{280} units)	Activity towards Bz-Arg-OEt (units)	Specific activity (units/ A_{280} unit)	Yield (%)
1. Neutralized supernatant from acidified homogenate	330	476	119	0.25	100
2. Combined DEAE-cellulose eluates	280	100	113	1.13	95
3. Sephadex G-100 gel filtration	29	70.5	98	1.39	87
4. First hydroxyapatite chromatography					
Fraction I	40	5.4	48	8.9	71
Fraction II	75	5.9	37	6.3	
Fraction III	100	20.8	8	0.4	
5. Hydroxyapatite rechromatography of:					
Fraction I (Fraction Ir)	86	3.4	24	7.1	37
Fraction II (Fraction IIR)	100	2.9	20	6.1	
Fraction III (Fraction IIIrA)	50	4.4	1.8	0.4	
(Fraction IIIrB)	80	7.8	0.48	0.06	
6. DEAE-Sepharose chromatography (after dialysis)	6.7	2.04	16.7	8.17	14

Fig. 1. Gel filtration on Sephadex G-100
For conditions see the text. Fractions were pooled as indicated by the horizontal bar.

Fig. 2. First chromatography on hydroxyapatite
Three fractions (I-III) were pooled as indicated by the horizontal bars.

to a column (2.5 cm $\times$ 8 cm was the minimal retaining column) of hydroxyapatite (Bio-Gel HTP) equilibrated with the phosphate buffer and run at room temperature. The column was washed with 35 ml of buffer and eluted with a linear gradient (total volume 400 ml) rising from 0.01 M- to 0.1 M-sodium phosphate buffer, pH 7.0, at a rate of 20 ml/h. Fractions (5 ml) were collected. The eluted material was pooled into three fractions (I-III) as indicated in Fig. 2, dialysed in the cold against 10 litres of water and freeze-dried.

Second chromatography on hydroxyapatite. Each of the fractions I-III was dialysed and rechromatographed separately on a fresh column of hydroxyapatite exactly as described above. Enzymic activity from fraction I as well as fraction II of Fig. 2 was now eluted in the same position (Fig. 3), similar to that of fraction II in the preceding step. Presumably peak I of Fig. 2 represents enzyme bound only very weakly due to overloading of the column. Material pooled as indicated in Fig. 3 was dialysed overnight against 10 litres of water. The losses of enzymic activity between the last two steps (Table 1) aroused the suspicion that the enzyme might not be stable on freeze-drying. The rechromatographed fractions Ir and Iir were therefore stored frozen.

Chromatography on DEAE-Sephacrose. In addition to equal elution positions in the preceding step, fractions Ir and Iir showed comparable specific activities (Table 1) and the same behaviour on disc and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The two fractions were therefore combined and subjected to chromatography on DEAE-Sephacrose, in order mainly to concentrate the enzyme solution and remove hydroxyapatite fines. A 230 ml portion of the solution was applied at

Fig. 3. Rechromatography of fractions I-III on hydroxyapatite
Fractions were pooled as indicated by the horizontal bars.

a rate of 5 ml/h to a column (0.82 cm $\times$ 15 cm) of DEAE-Sephacrose equilibrated with 0.05 M-ammonium acetate solution, pH 6.7. Elution was performed at the same rate with 200 ml of a linear gradient rising from 0.1 M- to 0.8 M-ammonium acetate, pH 6.7. Fractions (2 ml) were collected,

pooled as indicated in Fig. 4 and dialysed overnight against 6 litres of water. The enzyme solution was distributed among a number of polypropylene vials and stored frozen at -20°C .

Fig. 4. Chromatography on DEAE-Sepharose. Fractions were pooled as indicated by the horizontal bar.

Results

Electrophoretic properties and apparent M_r

On disc electrophoresis with or without spacer gel (which did not influence significantly the appearance of the band), guinea-pig submandibular-gland kallikrein showed only a single component, even when $25\text{ }\mu\text{g}$ were applied to the gel (result not shown). It migrated distinctly faster and as a somewhat sharper band than porcine submandibular-gland kallikrein. Only a single, though relatively broad protein band (apparent M_r , 29 000) was also seen on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis after reduction (result not shown). The apparent M_r determined by gel filtration was 34 000.

Amino acid analysis of guinea-pig submandibular-gland kallikrein. Results are shown in Table 2. The values are based on a content of three arginine residues per molecule. The amino acid composition and the total number of amino acid residues are similar to those of guinea-pig coagulating-gland kallikrein, but more especially to those of porcine

Table 2. Comparison of the amino acid composition of guinea-pig submandibular-gland kallikrein, guinea-pig coagulating-gland kallikrein and porcine pancreatic β -kallikrein B

The results are given as residues per molecule ($\pm$ s.e.m.) obtained from duplicate analyses of 24, 48 and 72 h hydrolysates. The values for threonine and serine were obtained by linear extrapolation to zero-hydrolysis time. Isoleucine was taken from 72 h and glucosamine from 24 h hydrolysates. Cysteine has been determined after performic acid oxidation and tryptophan spectrophotometrically as described in the text.

	Composition (residues/molecule)		
	Guinea-pig submandibular-gland kallikrein	Guinea-pig coagulating-gland kallikrein	Porcine pancreatic β -kallikrein
	Most probable integer	(Moriwaki <i>et al.</i> , 1974b)	(Fiedler <i>et al.</i> , 1977a)
Asp	27.7 $\pm$ 0.3	32	28
Thr	12.1 $\pm$ 0.4	13	15
Ser	14.8 $\pm$ 0.5	12–13	14
Glu	22.1 $\pm$ 0.1	25	23
Pro	16.4 $\pm$ 0.2	19*	16
Gly	21.0 $\pm$ 0.1	20–21	22
Ala	13.27 $\pm$ 0.03	15	13
Cys	9.8 $\pm$ 0.1	8–9	10
Val	17.0 $\pm$ 0.2	20	10
Met	5.55 $\pm$ 0.04	7	4
Ile	11.4	9	12
Leu	22.9 $\pm$ 0.1	24–25	20
Tyr	3.96 $\pm$ 0.02	6	7
Phe	5.84 $\pm$ 0.04	4	10
His	15.0 $\pm$ 0.1	10	8
Lys	8.6 $\pm$ 0.1	4	10
Arg	2.90 $\pm$ 0.06	7	3
Trp	5.18	7	7
Total	237	242–246	232
Glucosamine	(1.7)	13*	

* Recalculated from the data of Moriwaki *et al.* (1974b). The 31 proline residues given there are probably in error and resulted from reading 7.75 instead of 4.75 for the molar ratio of this amino acid.

pancreatic β -kallikrein B (Table 2); the latter is the only tissue kallikrein for which the amino acid composition is backed by an amino acid sequence (Fiedler *et al.*, 1981). An M_r value of 25 900 results for the protein part of the enzyme.

About 1.7 residues of glucosamine per molecule, but no galactosamine, were found in 24 h hydrolysates. From previous experience with porcine pancreatic kallikrein it is assumed that about half of the glucosamine content is destroyed under the conditions of hydrolysis used. So a probable glucosamine content of three to four residues per molecule is inferred for guinea-pig submandibular-gland kallikrein, indicating the glycoprotein nature of this tissue kallikrein also.

Optical properties and tryptophan content

$A_{280,1\text{cm}}^{1\%}$ (on a protein basis) for guinea-pig submandibular-gland kallikrein was determined as 13.6 and A_{280}/A_{260} as 1.85. With the above M_r for the protein part, the molar absorption coefficient (ϵ_{280}) is $35\,200\text{ M}^{-1}\cdot\text{cm}^{-1}$. By using the molar absorption coefficients given by Edelhoch (1967) for tryosine, cystine and tryptophan, a tryptophan content of 5.18 residues per molecule is calculated. The validity of this procedure, i.e. working in the absence of guanidinium chloride in contrast with Edelhoch (1967), is illustrated by the data of Whitaker & Granum (1980) for serine proteinases and by the case of porcine pancreatic β -kallikrein. For the latter enzyme, 7.21 residues of tryptophan per molecule are calculated from the molar absorption coefficient (Fiedler *et al.*, 1975) by the above procedure, whereas seven residues were found by other methods (Fiedler *et al.*, 1975).

N-Terminal amino acid sequence

When 4.3 nmol of guinea-pig submandibular-gland kallikrein were subjected to Edman degradation, 4 nmol valine phenylthiohydantoin derivative were recovered in the first cycle. This finding

confirms the M_r of the enzyme derived above. The sequence of the first 31 residues is given in Table 3. The N-terminal sequence of guinea-pig submandibular-gland kallikrein proved closely homologous to that of other tissue kallikreins, only seven of the 31 residues having no equivalent in one or more of the tissue kallikreins investigated so far, and consists of the same number of amino acid residues up to the definitely homologous position 31. The results of the sequenator degradation demonstrate the purity of the enzyme preparation and the absence of an intrachain split.

Esterolytic activity

The rate of Ac-Phe-Arg-OEt hydrolysis by guinea-pig submandibular-gland kallikrein was much higher than that obtained with Bz-Arg-OEt (Table 4). For comparison, hydrolytic rates of this substrate by porcine submandibular-gland kallikrein, a single-chain tissue α -kallikrein (Fiedler & Gebhard, 1980; Lemon *et al.*, 1979) and by porcine pancreatic β -kallikrein B carrying a split in its polypeptide chain were also determined (Table 4).

Kininogenase and hypotensive activity

The kinin released by guinea-pig submandibular-gland kallikrein from partially purified bovine kininogen was identified to be kallidin (Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg, lysylbradykinin) by high-pressure liquid chromatography. The rate of kallidin formation from $1.2\text{ }\mu\text{M}$ -bovine kininogen was $462 \pm 6.7\text{ mg (0.32 }\mu\text{mol)/min per mg of protein (n=4) or }0.14\text{ s}^{-1}$. No release of bradykinin was detected. On the basis of the sensitivity of detection, the upper limit for a possible release of bradykinin was at most 8% of that of kallidin.

The enzyme when incubated with partially purified guinea-pig and human kininogen contracted the guinea-pig ileum and relaxed the rat duodenum, pharmacological properties which characterize kinins. Guinea-pig submandibular-gland kallikrein

Table 4. Specific activities ($\mu\text{mol/min per mg of protein}$) of several tissue kallikreins as determined with Bz-Arg-OEt or Ac-Phe-Arg-OEt as substrates

Substrate	Guinea-pig submandibular kallikrein	Porcine submandibular α -kallikrein	Porcine pancreatic β -kallikrein	Human urinary kallikrein
Bz-Arg-OEt*	11.1	140†	230‡	24§
Ac-Phe-Arg-OEt	483	920	2090	1100

* Measured at 253 nm as described in the present paper, except in the case of human urinary kallikrein, where the alcohol dehydrogenase-linked assay had been used.

† Calculated from data in Lemon *et al.* (1979).

‡ Calculated from data in Lemon *et al.* (1979) and Fiedler *et al.* (1975).

§ Calculated from data in Fiedler *et al.* (1978) and Geiger *et al.* (1980).

|| Geiger *et al.* (1980).

Fig. 5. Hypotensive effects of guinea-pig submandibular-gland kallikrein on guinea-pig blood pressure. The chart speed was 3 cm/min, with paper moving from right to left. Each horizontal division (time) is equivalent to 20s and each vertical division (blood pressure) is equivalent to 10mmHg. Bradykinin (BK) or submandibular kallikrein (GPSK) was injected intravenously at the arrow.

possessed marked hypotensive activity, consistently lowering the blood pressure with a time course similar to that of bradykinin in seven guinea pigs (300 μ g of bradykinin equivalents/mg of protein; Fig. 5) and in one dog (1130 μ g of bradykinin equivalents/mg of protein).

Discussion

In the dog, guinea-pig submandibular-gland kallikrein was a potent vasodepressor. The hypotensive potency is very similar to the value of 1000 μ g of bradykinin equivalents/mg of protein calculated for porcine pancreatic kallikrein from the data of Moriwaki *et al.* (1974a), a specific activity of 282 units/mg of protein (Fiedler *et al.*, 1975) and a relationship of 1 unit = 6.37 kallikrein units (Kutzbach & Schmidt-Kastner, 1972). Still more important, guinea-pig submandibular-gland kallikrein proved to be potently hypotensive in the guinea pig itself. These are properties expected for a tissue kallikrein. Further convincing evidence for the tissue-kallikrein nature of the enzyme is the fact that kinin released from bovine kininogen is kallidin. The value for the rate of kallidin release compares favourably with that determined for porcine pancreatic kallikrein (Moriwaki *et al.*, 1974a; Dittmann & Wimmer, 1979; Gau *et al.*, 1982).

The specific activity of guinea-pig submandibular-gland kallikrein obtained with Bz-Arg-OEt as

substrate was significantly lower than that of the porcine tissue kallikreins (Table 4). However, activity determined with Ac-Phe-Arg-OEt was much closer to that of other tissue α -kallikreins. Ac-Phe-Arg-OEt resembles more closely structural features of the natural kallikrein substrate, kininogen. It is not only a much more sensitive substrate for tissue kallikreins, but evidently is hydrolysed by different enzymes of this group at a much more uniform rate.

The present work adds another *N*-terminal sequence to the growing list of known whole and partial sequences of tissue kallikreins and closely related enzymes (Table 3). All these enzymes with a specificity for basic amino acids are relatives of trypsin, as is evident from their sequences, especially from the presence of Cys-7 (in tissue-kallikrein numbering; Cys-22 in chymotrypsinogen numbering), which is characteristic within the family of the pancreatic serine proteinases for the trypsins. They are distinguished by one additional amino acid residue of somewhat uncertain position in the region from residues 20 to 25 (see the legend to Table 3), and also by a very high degree of homology when compared to each other (Fiedler, 1982; Fiedler & Fritz, 1981). Another closely related enzyme, rat tonin (Lazure *et al.*, 1981a,b; Fiedler & Fritz, 1981), differs by two deletions in positions 21 and 22 (which may also be differently aligned in this variable region).

Unclear are the relationships between submandibular-gland kallikrein and the coagulating-gland kallikrein of the guinea pig isolated by Moriwaki *et al.* (1974b). This enzyme appears also to be a tissue kallikrein as judged by the release of kallidin from crude dog kininogen (Kizuki *et al.*, 1980) and by its potent vasodilator activity in the dog (Moriwaki *et al.*, 1974b), even though no studies have been reported on its effect on guinea-pig blood pressure. The three to four residues of glucosamine, determined presently for the submandibular enzyme, are in contrast with the 13 amino-sugar residues recalculated for coagulating-gland kallikrein and could be taken to indicate a higher total carbohydrate content in the latter enzyme. This would allow one to rationalize the differences in apparent M_r , 40 000 (Moriwaki *et al.*, 1974b) for coagulating-gland kallikrein and 34 000 for submandibular-gland kallikrein obtained by gel filtration, in spite of a similar number of amino acid residues. In the case of porcine tissue kallikreins, enzymes from different organs have been shown to contain the same protein moiety, but various amounts of carbohydrate (Fiedler *et al.*, 1981). However, the reported amino acid analysis of guinea-pig coagulating-gland kallikrein (Table 2), although generally similar to that of the submandibular enzyme, shows distinct quantitative differences in several of the amino acids. As yet it is not possible to determine whether the differences in amino acid composition are due to different degrees of purity of the two preparations or to non-identity in the protein segments of the two molecules. At any rate, the amino acid compositions reported argue against the possibility that several amino acids could have been lost from one of the enzymes, of otherwise similar structure. If the two enzymes should prove to have protein moieties of differing composition, then the guinea-pig tissue kallikreins would display an important property and would appear to be different in that respect from the porcine and human tissue kallikreins.

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