T-kininogenase activity of the rat submandibular gland is predominantly due to the kallikrein-like serine protease antigen γ

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T-kininogen, the major kininogen in rat plasma, releases Ile-Ser-bradykinin (T-kinin) when incubated with trypsin, but is not a substrate for tissue kallikrein. Enzymes able to release T-kinins from T-kininogen have been found in the rat submandibular gland, but precise identification of these enzymes and their possible relationship to kallikrein-like enzymes has not been established. We studied T-kininogenase activity in fractionated submandibular gland homogenate. The main T-kininogen catalytic enzyme was purified and characterized, and found to be identical to antigen γ , a kallikrein-like enzyme which we have previously characterized. Of other identified kallikrein-like enzymes only tonin showed weak T-kininogenase activity, which was about 0.25 % of that of antigen γ . No other T-kininogen catalytic enzymes were observed. Antigen γ released a kinin which was identified as T-kinin by reverse-phase h.p.l.c. The T-kininogenase activity of antigen γ had a K_m of $29 \pm 4 \mu\text{M}$ and a k_{cat}/K_m of $140 \text{ M}^{-1} \cdot \text{s}^{-1}$, and was comparable with its high and low molecular mass-kininogenase activity (7.4 and $10 \mu\text{g}$ of kinin/h per mg respectively). In contrast, tissue kallikrein released 0.2 and $42\,200 \mu\text{g}$ of kinin/h per mg respectively. Thus antigen γ is a weak kininogenase. The isoelectric point of antigen γ , but not its molecular mass, differed from that of other kallikrein-like enzymes. Isoelectrofocusing in flat-bed gels combined with immunostaining was therefore a convenient method for identification. The kallikrein-like nature of antigen γ was demonstrated by its immunological similarity to tissue kallikrein and tonin and by 91 % and 87 % amino acid sequence similarity with tonin and kallikrein respectively (67 amino acids sequenced). Complete identity was also not observed with other sequenced kallikrein genes, mRNAs or proteins.

INTRODUCTION

T-kininogen is the main kininogen present in rat plasma (Okamoto & Greenbaum, 1983a). When incubated with trypsin, T-kininogen releases Ile-Ser-bradykinin (T-kinin) (Okamoto & Greenbaum, 1983b), which has the same pharmacological properties as bradykinin (Okamoto & Greenbaum, 1983c). T-kininogenase activity has been demonstrated in the rat submandibular gland (SMG) (Barlas *et al.*, 1987; Kato *et al.*, 1987; Gutman *et al.*, 1988; Xiong *et al.*, 1990). However, in these studies the characteristics and the identity of the isolated T-kininogenases were conflicting. The T-kininogenase isolated by Barlas *et al.* (1987) was not classified as a serine protease, since it was not inhibited by phenylmethanesulphonyl fluoride (PMSF). The enzyme reported by Gutman *et al.* (1988), called endopeptidase K, was, like other kallikrein-like enzymes, inhibited by PMSF, but did not react with an antiserum against tissue kallikrein and was thus not classified as a member of the kallikrein family. However, the amino acid sequences of the T-kininogenases isolated by Xiong *et al.* (1990) and Kato *et al.* (1987) indicated that these enzymes are members of the kallikrein family, although the two sequences were not the same. In addition, Gutman *et al.* (1988) found minor T-kininogenase activity in another yet uncharacterized enzyme fraction. Therefore the identity and the biochemical behaviour of rat SMG T-kininogen catalytic enzymes remain unclear, and it is not known whether SMG proteins with T-kininogenase activity belong to the kallikrein family.

The SMG of the rat is a rich source of the kininogenase tissue kallikrein (Brandtzæg *et al.*, 1976; Ashley & MacDonald, 1985)

as well as other closely related kallikrein-like enzymes, including tonin (Boucher *et al.*, 1974), esterase B (Khullar *et al.*, 1986), antigen γ (Berg *et al.*, 1987) and kallikrein-like protein S3 (KLP-S3; Berg *et al.*, 1992). Of these, esterase B and antigen γ have been reported to have weak kininogenase activity using dog high and low molecular mass HMM/LMM-kininogen as substrate (Khullar *et al.*, 1986; Berg *et al.*, 1987). It is therefore not unreasonable to assume that one of the previously identified or uncharacterized SMG kallikrein-like proteins is responsible for the T-kininogenase activity of this organ.

The purpose of the present investigation was therefore to clarify the identity of rat SMG T-kininogenases, with particular emphasis on their relationship to kallikrein-like proteins. We found that most of the T-kininogenase activity in the rat SMG was due to the kallikrein-like enzyme antigen γ (Berg *et al.*, 1987). After partial amino acid analysis, antigen γ was compared with the reported sequences of other kallikrein-like enzymes. In addition, the enzymic behaviour of antigen γ as a T-kininogenolytic enzyme was studied.

EXPERIMENTAL

Reagents

BSA fraction V, papain (twice crystallized; type III) and Tween 20 were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.; T-kinin and bradykinin were from Peninsula Laboratories, Belmont, CA, U.S.A.; trypsin (TPL3) was from Millipore, Freehold, NJ, U.S.A.; 1,10-phenanthroline and EDTA were from Fisher Scientific, Fair Lawn, NJ, U.S.A.; and

Abbreviations used: SMG, submandibular gland; PMSF, phenylmethanesulphonyl fluoride; KLP-S3, kallikrein-like protein S3; HMM/LMM-kininogen, high and low molecular mass kininogen; PAP, peroxidase-antiperoxidase; IEF, isoelectric focusing; PBS, phosphate-buffered saline.

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Fig. 1. Anion-exchange chromatography of SMG homogenate

SMGs from 36-month-old male Wistar rats were separated from the sublingual gland and homogenized in a Potter-Elvehjem homogenizer (20 strokes, 4 °C) in 0.05 M-ammonium acetate buffer (pH 5.8) (1:10, w/v). After centrifugation (13000 g, 20 min, 4 °C), the precipitate was washed in an equal volume of buffer and the two supernatants were combined. The supernatant (117 ml, 2.1 g of protein) was applied to a Whatman DE-52 ion-exchange column (460 ml, 2.5 cm × 94 cm) (0.05 M-ammonium acetate, pH 5.8; 0.05–0.5 M-ammonium acetate gradient; 10 ml fractions, 4 °C). The elution profile was monitored by absorbance at 280 nm (—). Kallikrein activity (---) was measured using the chromogenic substrate for tissue kallikrein, S2266 (Val-Leu-Arg-nitroanilide), as substrate, and tonin enzyme activity (●) was measured with angiotensin I as substrate. Fractions were pooled as indicated above the elution profile (fractions 1–12). The kininogenase activities of pooled fractions using rat T-kininogen or dog HMM/LMM-kininogen as substrate are shown in Table 1. For further details, see the Experimental section. - - - - -, Conductivity.

3,3'-diaminobenzidine tetrahydrochloride was from Ega-Chemi, Steinheim/Albuch, Germany. Whatman DE-52 anion-exchange material was obtained from Whatman Chemical Separation Ltd., Maidstone, Kent, U.K.; Sepharose 4B and MonoQ and MonoP prepacked columns were from Pharmacia Fine Chemicals (Uppsala, Sweden); and S2266 was from Kabi Diagnostica, Stockholm, Sweden. Pig anti-rabbit immunoglobulin G anti-serum and peroxidase-antiperoxidase (PAP) reagent were from Boehringer, Mannheim, Germany.

Preparation of dog HMM/LMM-kininogen and rat T-kininogen

Semi-purified dog HMM/LMM-kininogen was prepared as previously described (Carretero *et al.*, 1976). T-kininogen was purified from rat plasma using a modification of the method described by Johnson *et al.* (1987), as follows. Blood from rats uninephrectomized 70 h previously was collected from the abdominal aorta into sodium-citrate-containing tubes (13 mm final concentration). Plasma was obtained by centrifugation (1000 g, 15 min, 22 °C), and NaCl was added to a final concentration of 2 M. The precipitate was removed by centrifugation (2000 g, 15 min, 4 °C), and the supernatant was applied to a papain-Sepharose 4B affinity column (25 cm × 2.5 cm) equilibrated with 50 mM-sodium phosphate buffer (pH 7.5) containing 2 M-NaCl. Kininogens adhering to the papain column were eluted with 3 M-NaSCN (pH 8.3). After concentration and desalting using a Diaflo YM30 ultrafiltration membrane (Amicon Division, Grace & Co., Danvers, MA, U.S.A.), the kinin moiety in HMM- and LMM-kininogens was removed by incubation with rat tissue kallikrein (2 mg of enzyme/44 µg of protein, 6 h, 37 °C) and dialysis against 0.9% NaCl (2 × 4 litres, 10 h, 4 °C) and 0.1 M-phosphate buffer (pH 8.0; containing 0.02% Na₂S₂O₃) (1 × 4 litres, 5 h, 4 °C) in dialysis bags retaining molecules above 12–14 kDa. When 88 µg of substrate was incubated with 2 mg of trypsin (1.5 h, 37 °C), T-kinin, but not bradykinin, was released, as documented by reverse-phase h.p.l.c. as described below. The substrate released 92% of its theoretical kinin content.

Purification of rat SMG enzymes

The SMG antigen γ and other kallikrein-like enzymes were purified as previously described (Berg *et al.*, 1987; Johansen *et al.*, 1987). In short, enzymes were initially separated on a DE-52 Whatman column (pH 5.8) (see Figs. 1 and 2). The antigen γ - and kallikrein-containing fractions (Fig. 1, pooled fractions 10 and 12 respectively) were further purified on a MonoQ column

eluted with a 0.1–1.0 M-ammonium acetate buffer, pH 4.8, (flow rate 1 ml/min) using the Pharmacia f.p.l.c. system (Johansen *et al.*, 1987). Antigen γ was subsequently purified by affinity chromatography on an aprotinin-Sepharose 4B CNBr column. The affinity column was prepared as described by the manufacturer, equilibrated with 0.02 M-NaPO₄ buffer (pH 7.0)/0.2 M-NaCl and eluted with 0.1 M-sodium acetate buffer (pH 3.5). Fractions of 1 ml were collected directly into 1 ml of 0.5 M-Tris/HCl buffer (pH 8.5). Tonin and esterase B from the Whatman column void fraction (Fig. 1, pooled fraction 1) were separated by a second run on the same column, this time equilibrated with 0.02 M-Tris/HCl buffer, pH 8.0 (0–0.4 M-NaCl gradient), and purified to homogeneity on a MonoP chromatofocusing column on the f.p.l.c. system (pH gradient 7.4–4.0) (Johansen *et al.*, 1987). The purification procedure was followed by isoelectric focusing (IEF) as described below.

Enzyme assays

S2266 amidolytic activity. The chromogenic substrate S2266 (H-D-Val-Leu-Arg-4-nitroanilide) was used. The enzyme was dissolved in 900 µl of assay buffer (0.2 M-Tris/HCl, pH 9.0) and incubated for 5 min at 37 °C with 100 µl of substrate (2 mM). The reaction was stopped with 100 µl of 50% acetic acid and absorbance was measured at 405 nm (Amundsen *et al.*, 1979). One unit is equal to the amount of enzyme that hydrolysed 1 µmol of substrate/min.

Tonin enzyme activity. Tonin enzyme activity was determined by allowing angiotensin I (200 µl, 0.5 g/l; pH 6.8) to react with 100 µl of enzyme solution for 10 min at 37 °C as described by Boucher *et al.* (1974). His-Leu was used as standard. One unit of tonin enzyme activity is equivalent to the amount of protein that hydrolysed 1 nmol of angiotensin I/min.

T-kininogenase and HMM/LMM-kininogenase activity. Unless otherwise indicated, 44 µg (10 µl) of rat T-kininogen or 3.33 mg (0.5 ml) of dog HMM/LMM-kininogen was incubated with about 0.5–1 µg (10–50 µl) of enzyme for 1 h at 37 °C. Incubations were done in 0.1 M-sodium phosphate buffer (pH 8.5) containing 1.485 mg of 1,10-phenanthroline/ml and 27.9 mg of EDTA/ml in a total incubation volume of 700 µl. The reaction was stopped by heat (100 °C, 10 min), followed by addition of 2.8 ml of 100% ethanol. After centrifugation (2200 g, 10 min, 4 °C), the supernatant was evaporated to dryness under N₂ (60 °C), and frozen (–20 °C) until assayed for kinin by radioimmunoassay as described by Carretero *et al.* (1976). For T-kininogenase activity,

the assay was run in the presence of Tween 20 (5% final concentration). To calculate enzyme kinetic constants, 90 nM-antigen γ was incubated with 3.7–147.1 μ M-T-kininogen. A computer program for non-linear regression analysis described by Duggleby (1984) was employed. The pH-dependency of antigen γ activity was studied by replacing the sodium phosphate buffer with 0.1 M-Tris (pH 7.0–11.0) with or without Tween 20.

Protein. The concentration of protein was measured using the method of Lowry *et al.* (1951) with BSA as standard.

Immunological characterization of antigen γ

IEF and immunoblotting. Protein bands from IEF gels were blotted on to nitrocellulose membrane (0.2 μ m). The membrane, moistened with transfer buffer (0.025 M-Tris/0.192 M-glycine/20% methanol, pH 8.3), was placed on to the gel, wrapped in layers of filter paper stabilized with a perforated polyacrylamide frame, and incubated for 30 min, with shaking, in a buffer-containing chamber (37 °C). The nitrocellulose membrane was subsequently immersed (22 °C) successively in: (1) 6% BSA in phosphate-buffered saline (PBS; 0.01 M- $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, pH 7.4, 0.14 M-NaCl) (60 min), (2) rabbit primary antiserum (1:5000, v/v), (3) pig anti-rabbit immunoglobulin G antiserum (1:1000, v/v), and (4) PAP reagent (1:1000, v/v). Polyclonal rabbit antibodies against rat SMG kallikrein, tonin or antigen γ (Ørstavik *et al.*, 1982; Berg *et al.*, 1987) were used as primary antibodies. Antisera were diluted in PBS containing 0.1% BSA, and incubations lasted 45 min with the protein side of the membrane downwards. Between incubations the membrane was washed in PBS containing 0.1% Tween 20 for 3 $\times$ 15 min. Staining was developed using 3,3'-diaminobenzidine tetrahydrochloride as described by Taylor (1974).

Immuno-electrophoresis and double immunodiffusion. Protein preparations were tested by immuno-electrophoresis (Weeke, 1973) and by double immunodiffusion (Ouchterlony, 1958) using rabbit antisera against rat SMG kallikrein, tonin (Ørstavik *et al.*, 1982) and antigen γ (Berg *et al.*, 1987).

Biochemical characterization of antigen γ

Isoelectric point IEF. Proteins were run in flat-bed isoelectrofocusing gels (PhastGel; IEF 3–9) in the Pharmacia Fast System as described in the manufacturer's manual. Isoelectric points were determined using the Pharmacia IEF calibration kit (pH 3–10) as standard. The gels were stained with silver as described in the manufacturer's manual or used for immunoblotting as described above.

Molecular mass by SDS/PAGE. Purified antigen γ was treated with SDS (25 mg/ml, 2 min, 100 °C) with or without addition of β -mercaptoethanol (5%), run on SDS/PAGE using PhastGel (gradient 8–25) in the Pharmacia Fast System, and stained with silver as above. The Pharmacia electrophoresis calibration kit (low molecular mass proteins) was used as standard.

Amino acid composition and partial sequence. Antigen γ , tonin and kallikrein were run on SDS/PAGE using Excel gel SDS (gradient 8–18) as described by the manufacturer (Pharmacia LKB Biotechnology). Protein bands were electrophoretically transferred to an Immobilon transfer membrane (Millipore, Bedford, MA, U.S.A.) in a Pharmacia Multiphor II Novablot System as described by the manufacturer. After staining with Coomassie Blue, protein bands were cut out and used for amino acid analysis. N-Terminal analyses were performed using an automatic 477A Protein/Peptide Sequencer with an on-line 120A phenylhydantoin amino acid analyser from Applied Biosystems (Foster City, CA, U.S.A.) (Cornwell *et al.*, 1988). The amino acid composition of antigen γ was determined in a Biotronik LC

5000 amino acid analyser after hydrolysis with 6 M-HCl (Sletten *et al.*, 1987).

Hydrolysis of T-kininogen by antigen γ and trypsin revealed by SDS/PAGE

Degradation of T-kininogen was studied by SDS/PAGE run as described above after incubation of 44 μ g (10 μ l) of substrate with 0.07 or 0.7 μ g (10 μ l) of antigen γ for 0–10 h, or with 1 or 10 μ g of trypsin for 1 h (37 °C).

Reverse-phase h.p.l.c. of T-kinin released from T-kininogen

Kinins, generated from T-kininogen (44 μ g) by incubation with antigen γ (0.7 μ g, 37 °C, 6 h), were separated by reverse-phase h.p.l.c. on a 5 μ m Ultrasphere-ODS column (Mindrou *et al.*, 1987). The column was equilibrated with 19% acetonitrile in 0.04 M-triethylamine formate, pH 3.8, and eluted by isocratic elution (17 min) followed by a linear gradient of 19–50% acetonitrile in triethylamine formate (20 min) and isocratic elution with 50% acetonitrile in triethylamine formate (3 min). The flow rate was 1 ml/min, and the fraction volume was 0.5 ml. Kinins were detected by radioimmunoassay as described above. T-kinin was identified by co-elution with added radiolabelled T-kinin.

RESULTS

Identification of the main SMG T-kininogenase as antigen γ , a member of the kallikrein family

Antigen γ and tissue kallikrein were initially separated by DE-52 Whatman anion-exchange chromatography (Fig. 1). When pooled column fractions were tested, T-kininogenase specific activity peaked in the antigen- γ -containing fraction (3.3 μ g of kinin/h per mg of protein), whereas the highest HMM/LMM-kininogenase activity was detected in the tissue kallikrein fraction (Table 1). Antigen γ was purified to homogeneity by MonoQ and aprotinin affinity chromatography as demonstrated by IEF (Fig. 2), giving a specific T-kininogenase activity of 7.4 μ g of kinin/h per mg (Table 1). The HMM/LMM-kininogenase activity of antigen γ was 9.5 μ g of kinin/h per mg, compared with 42 200 μ g of kinin/h per mg for the Whatman column kallikrein fraction.

Minor T-kininogenase (2.6% of total eluted activity) and HMM/LMM-kininogenase (0.5% of total eluted activity) activities were also found in the DE-52 Whatman column void fraction (Fig. 1, fraction 1). On further purification of this fraction most of the T-kininogenase activity was found to be due to tonin (16 ng of kinin/h per mg), whereas the HMM/LMM-kininogenase activity was due to tonin as well as esterase B, both of which after purification expressed catalytic activity (11 and 31 μ g of kinin/h per mg respectively) (Table 1). No other submandibular gland kininogenases were observed.

Immunological characterization of the T-kininogenase antigen γ

The T-kininogenase antigen γ was recognized by antiserum against tissue kallikrein, weakly by flat-bed IEF with immunoblotting (Fig. 2) but, as previously documented (Berg *et al.*, 1987), clearly in double immunodiffusion (not shown) and immuno-electrophoresis (Fig. 3) studies, in a pattern indicating partial immunological identity with tissue kallikrein. Antigen γ was poorly recognized by antiserum against tonin (Figs 2 and 3). This pattern of immunoreactivity was observed with several different antisera against both kallikrein and tonin, indicating that antigen γ is a member of the rat kallikrein family. The rabbit antiserum previously raised against antigen γ (Berg *et al.*, 1987) reacted with the presently isolated T-kininogenase enzyme and showed little reactivity towards other enzymes of the kallikrein family (Figs. 2 and 3).

Table 1. T-kininogenase and HMM/LMM-kininogenase activity of DE-52 Whatman column fractions and purified kallikrein-like enzymes

Pooling of the fractions is indicated above the elution profile of the anion-exchange column in Fig. 1. KLP-S3 is another newly identified kallikrein-like enzyme with vasoconstrictor ability (identical with the S3 mRNA) purified from pooled fraction 1 (Berg *et al.*, 1992).

	T-kininogenase		HMM/LMM-kininogenase	
	(μ g of kinin/h per mg)	(% of total activity eluted)	(μ g of kinin/h per mg)	(% of total activity eluted)
Pooled fraction:				
1	0.070	2.6	434	0.5
2	0	0	173	0.1
3	0.027	0.1	76	0.01
4	0	0	89	0.01
5	0	0	72	0.01
6	0	0	113	0.05
7	0	0	45	0.01
8	0.065	0.6	33	0.01
9	0.495	6.5	89	0.03
10	3.295	53.4	209	0.1
11	1.698	28.9	25049	12.5
12 (kallikrein)	0.158	7.9	42200	86.8
Purified enzymes:				
Antigen γ	7.424		10	
Tonin	0.016		11	
Esterase B	0		31	
KLP-S3	0		0

Fig. 2. IEF and immunoblotting of antigen γ

After IEF the samples were stained with silver (a) or blotted on to nitrocellulose membrane and stained with antiserum against SMG kallikrein (b), tonin (c) or antigen γ (d). The kallikrein, antigen γ and tonin isoenzymes are indicated with horizontal bars on the left. The isoelectric points were 3.50, 3.60, 3.70 and 3.85 for kallikrein, 3.95, 4.05 and 4.25 for antigen γ , and 6.05 and 6.15 for tonin. Lane H, SMG homogenate; lane 1, antigen γ fraction from the DE-52 Whatman column (fraction 10 in Fig. 1); lane 2, antigen γ fraction from the MonoQ column containing several antigen γ isoenzymes; lane 3, pure antigen γ after aprotinin affinity chromatography to select the major antigen γ band; lane 4, kallikrein fraction from the DE-52 Whatman column (fraction 12 in Fig. 1); lane 5, pure kallikrein after MonoQ chromatography; lane 6, purified tonin.

Biochemical characterization of antigen γ

Isoelectric point. IEF after MonoQ fractionation indicated that antigen γ consisted of several isoenzymes (Fig. 2, lane 2)

Fig. 3. Immuno-electrophoresis of SMG homogenate and antigen γ

SMG homogenate (Hom), tissue kallikrein (RSK) and antigen γ (γ) were immuno-electrophoresed with polyclonal rabbit antisera against rat SMG kallikrein (a-RSK), tonin (a-tonin), and antigen γ (a- γ). Antigen γ reacted with the anti-kallikrein serum in a pattern of partial immunological identity with kallikrein, thus demonstrating a close immunological relationship between the two enzymes. We therefore classified antigen γ as a member of the kallikrein family. Antigen γ was, however, not recognized by antiserum against tonin. This pattern of immunoreactivity was observed with several different antisera. Antigen γ therefore seemed to be immunologically more closely related to kallikrein than to tonin, even though a closer similarity in amino acid sequence was observed between antigen γ and tonin than between tissue kallikrein and antigen γ . However, only 55 amino acids were sequenced, and the sequence identity pattern may be different for the whole polypeptide chain. It is also possible, since antibody recognition is dependent on recognition of similar antigenic epitopes, that those epitopes that are shared with tissue kallikrein may be more exposed or more antigenic than those that are shared with tonin. The particular antiserum against antigen γ used in the present study did not react with kallikrein. This was a rare immune response, since all other antisera we have produced against antigen γ reacted very strongly with tissue kallikrein, often without distinguishing between the two enzymes (results not shown).

with pI values of 3.95, 4.05 and 4.25. The major antigen γ band (pI 4.05) was selected by aprotinin affinity chromatography. IEF also revealed that the various kallikrein-like enzymes showed clear differences in pI. IEF was therefore a convenient method for identification of antigen γ as well as the other kallikrein-like enzymes (Fig. 2). After the sensitive silver staining technique, this method also offered easy detection of contaminants.

Molecular mass. Antigen γ was found to have a molecular mass of 28 400 Da by SDS/PAGE (Fig. 4). After treatment with mercaptoethanol, two bands were observed of molecular masses of 19 000 and 10 000 Da.

Amino acid composition and sequence. The amino acid composition of antigen γ (whole molecule) is shown in Table 2. Some variations compared with the T-kininogenases endopeptidase K (Gutman *et al.*, 1988) and proteinase B (Kato *et al.*, 1987) were observed (Table 2). A partial amino acid sequence of the two chains is given in Fig. 5. A large degree of sequence similarity with other kallikrein-like enzymes was observed, confirming the kallikrein-like nature of antigen γ , with 87 and 91 % identity in the 67 sequenced amino acids when compared with kallikrein and tonin respectively. Complete identity with any of the other sequenced rat kallikrein-like enzymes, including the T-kininogen catalytic enzyme isolated by Xiong *et al.* (1990), was not observed (Fig. 5). When comparing antigen γ with the T-kininogen catalytic enzyme proteinase B (Kato *et al.*, 1987) complete identity was observed for the light chain, whereas the sequence of the heavy chain was different. The obtained sequences (first 20 amino acids) for SMG kallikrein and tonin agreed with those previously reported (Ashley & MacDonald, 1985; Lazure *et al.*, 1987).

Fig. 4. SDS/PAGE of antigen γ

The molecular mass of antigen γ with (lane 2) and without (lane 3) reduction with β -mercaptoethanol was determined by SDS/PAGE in the Pharmacia Fast System as described in the Experimental section. Lane 1 contains low molecular mass standards.

Table 2. Amino acid composition of antigen γ

The composition of endopeptidase K is from Gutman *et al.* (1988), and that of proteinase B is from Kato *et al.* (1987). n.d., not determined.

Amino acid	Composition (residues/molecule)		
	Antigen γ	Endo-peptidase K	Proteinase B
Asparagine/aspartic acid	35	25	31
Threonine	14	11	11
Serine	18	14	14
Glutamine/glutamic acid	30	26	22
Proline	19	18	15
Glycine	21	20	24
Alanine	12	11	10
Valine	15	15	13
Methionine	5	4	4
Isoleucine	11	11	10
Leucine	24	22	21
Tyrosine	12	10	11
Phenylalanine	7	6	5
Histidine	6	5	5
Lysine	17	14	16
Cysteine	8	5	10
Arginine	5	4	4
Tryptophan	n.d.	n.d.	7
Total number of residues	259	221	231

The ability of antigen γ to cleave T-kininogen and release T-kinin

Hydrolysis of T-kininogen by antigen γ revealed by SDS/PAGE. When hydrolysis of T-kininogen by antigen γ was studied by SDS/PAGE (Fig. 6), the intensity of the 63 kDa T-kininogen was decreased, with the simultaneous appearance of fragments of 46.5, 42.5, 18.2 and 17.1 kDa (substrate/enzyme molar ratio = 30; 1 h incubation). On longer incubation the 17.1 kDa band remained, whereas the 18.2 kDa band disappeared, possibly further hydrolysed to the 17.1 kDa fragment by removal of a fragment comparable in size to kinin. On prolonging the incubation time and/or increasing the concentration of antigen γ ,

Fig. 5. Partial amino acid sequence of antigen γ fragments

The amino acid sequences of the two fragments of antigen γ obtained by SDS/PAGE after mercaptoethanol treatment are compared with those of other kallikrein-like enzymes and genes [the proteins kallikrein (Ashley & MacDonald, 1985), tonin (Lazure *et al.*, 1987), the T-kininogen catalytic enzyme isolated by Xiong *et al.* (1990) and named T-kininogenase, proteinases A and B (Kato *et al.*, 1987), the kallikrein family mRNAs P1 (Brady *et al.*, 1989), S1 and S3 (Ashley & MacDonald, 1985), and the kallikrein family genes RSKG-3, RSKG-7 (Chen *et al.*, 1988) and RSKG-50 (Shai *et al.*, 1989)]. Of the proteins, antigen γ , T-kininogenase, and proteinase B have all been isolated as the main T-kininogen catalytic enzyme of the rat SMG. (a) Light chain, (b) heavy chain. Identical amino acids are framed, whereas those different from antigen γ within the frame are boxed. X, unidentified amino acid. Open spaces are used to align identical sequences in the different proteins. The sequences start at the N-terminal end to the left.

Fig. 6. T-kininogen breakdown by antigen γ and trypsin studied by SDS/PAGE

T-kininogen was incubated at 37 °C alone, with antigen γ (substrate/enzyme molar ratios of 30 and 3) or with trypsin (substrate/enzyme molar ratios of 175 and 17.5). The incubation time is indicated below the columns. The incubates were subsequently run on SDS/PAGE as described in the Experimental section. The protein band representing antigen γ is marked with an arrowhead.

the larger fragments disappeared, with the appearance of additional fragments of lower molecular mass. When the hydrolysis of T-kininogen by trypsin was studied using a substrate/enzyme molar ratio of 17.5, the degradation products resembled those observed after extensive action of antigen γ (molar ratio of 3; 3 h incubation). However, the kininogen band did not disappear to

Fig. 7. Identification of the peptide released by antigen γ as T-kinin

Reverse-phase h.p.l.c. separation of kinin immunoreactivity released by antigen γ (1 μ g) from rat plasma T-kininogen (44 μ g) during 6 h of incubation at 37 °C. Kinin immunoreactivity was detected by radioimmunoassay, and the T-kinin peak was identified by co-elution with added radiolabelled T-kinin. The chromatographic conditions are described in the Experimental section. BK, bradykinin.

Fig. 8. pH-dependence of antigen γ T-kininogenase activity

Antigen γ (0.6 μ g; pooled fraction 10 from the ion-exchange column, Fig. 1) and 44 μ g of rat T-kininogen were used in a total incubation volume of 700 μ l. The assay was run in 0.1 M-Tris buffer (pH 7.0–11.0) with (○) or without (●) Tween 20 (5% final concentration). The high-pH optimum was not due to alkaline hydrolysis of the substrate and is in agreement with previous studies using S2266 (Val-Leu-Arg-nitroanilide) as substrate (Berg *et al.*, 1987).

the same extent. With a 10 times higher concentration of trypsin an almost complete degradation of all protein bands was observed.

Antigen γ releases T-kinin from T-kininogen. The peptide released from T-kininogen by antigen γ was, as for trypsin (results not shown), found by h.p.l.c. to have the same elution time as T-kinin standard (Fig. 7). In addition another uncharacterized kinin-immunoreactive peptide was observed.

Antigen γ T-kininogenase enzyme kinetics. For antigen γ , using T-kininogen as a substrate, we found a K_m of 29 ± 4 μ M, a V_{max} of 0.37 ± 0.03 nm/s (means $\pm$ S.E.M.), and a k_{cat}/K_m of 140 $M^{-1} \cdot s^{-1}$. The activity was observed to be pH-dependent (Fig. 8). Activity at pH 11.0 was slightly higher in the presence of Tween 20, whereas no effect of Tween 20 was observed in the pH range 7.0–9.5 (Fig. 8).

DISCUSSION

On the basis of its chromatographic and enzymic behaviour, pI and immunological characteristics, we found that the main T-

kininogen catalytic enzyme of the rat SMG was identical to our previously characterized SMG antigen γ , a kallikrein-like enzyme (Berg *et al.*, 1987). Other SMG enzyme fractions contained only negligible amounts of T-kininogenase activity, i.e. less than 3% of the total, which was found to be due to the weak T-kininogenase activity of tonin. By studying the effect of antigen γ on T-kininogen by SDS/PAGE it could be confirmed that antigen γ has the ability to cleave T-kininogen. The initial cleavage appeared to involve two peptide bonds, since two pairs of fragments were obtained, and the difference in mass between the two was comparable with the molecular mass of T-kinin. The release of T-kinin was confirmed by reverse-phase h.p.l.c. H.p.l.c. also revealed the presence of another kinin-immunoreactive polypeptide which could represent a fragment after cleavage at only one site. T-kininogen was subsequently further degraded into smaller polypeptide chains which may have been due to the further action of antigen γ . It was therefore concluded that antigen γ is the major T-kininogen catalytic enzyme of the rat SMG. However, on comparing its rate of kinin release with that caused by SMG kallikrein reacting with HMM/LMM-kininogen, or its k_{cat}/K_m value (1.4×10^2 $M^{-1} \cdot s^{-1}$) with that of human urinary kallikrein with HMM-kininogen as substrate (8.6×10^4 $M^{-1} \cdot s^{-1}$) (Maier *et al.*, 1983), it was concluded that antigen γ is a weak kininogenase.

We previously classified antigen γ as a member of the rat kallikrein family, since it is a serine protease and is immunologically partially identical to tissue kallikrein, a characteristic which in fact was the initial criterion for isolation of this enzyme (Berg *et al.*, 1987). This immunological cross-reactivity pattern was confirmed for the presently isolated T-kininogenase/antigen γ with antisera against both kallikrein and tonin. Partial amino acid sequencing further confirmed the kallikrein-like nature of antigen γ , showing 91% and 87% sequence identity with tonin and kallikrein respectively for the 67 amino acids sequenced. This indicates that, although the enzymes of the kallikrein family are closely related, minor variations in amino acid sequence alter their behaviour and catalytic activity against biological substrates. In the variable region included in the sequenced segments of the light chain only one amino acid differed between tonin and antigen γ , whereas more variation was observed between antigen γ and kallikrein. On the other hand, in the amino acids of the heavy chain, antigen γ resembled kallikrein more than tonin. Due to the clear differences in substrate specificity of the enzymes it is likely that further differences will be observed in the as yet unsequenced regions of the polypeptide chains. The sequence for antigen γ did not match that of any of the sequenced members of the kallikrein gene family, thus indicating that antigen γ did not correspond to any of the kallikrein-like genes or mRNAs identified so far.

Antigen γ seemed to be identical to the T-kininogenase (endopeptidase K) isolated by Gutman *et al.* (1988), based on similarities in pI, molecular mass, and chromatographic and enzymic behaviour, although minor differences were observed in amino acid composition. Gutman *et al.* (1988) did not observe an immunological relationship between endopeptidase K and tissue kallikrein, but this can be explained by the fact that their antiserum against kallikrein was more monospecific than the one we used in the present study. It is our experience that most antisera against members of the kallikrein family will have cross-reactivity to other family members; however, more monospecific polyclonal antisera are occasionally obtained, as documented for the particular antiserum against antigen γ used in the present paper. When comparing the amino acid sequence of antigen γ with that of the T-kininogen catalytic enzyme isolated by Xiong *et al.* (1990), differences were observed in the first variable region of the light chain. However, it is possible that this may be due to

the difference in rat strains used in the two studies, i.e. Wistar rats (present study) and Sprague-Dawley rats (Xiong *et al.*, 1990) respectively, since we have observed in preliminary studies that the dominant SMG antigen γ isoenzyme may vary from one strain to another. However, on comparison with the T-kininogenase isolated by Kato *et al.* (1987), also from Sprague-Dawley rats, complete sequence identity with antigen γ was observed in the light chain including the first variable region, although three amino acids were found to differ in the conserved areas of the heavy chain *N*-terminal end. The T-kininogen catalytic enzyme isolated by Barlas *et al.* (1987) was not sequenced and thus its amino acid composition could not be compared with that of the others. However, this enzyme differed from all SMG enzymes with T-kininogen catalytic activity detected in the present study (i.e., antigen γ , tissue kallikrein and tonin) in that it was not inhibited by the serine protease inhibitor PMSF (Barlas *et al.*, 1987; Berg *et al.*, 1987, 1992). The discrepancies between these studies were surprising and are not easily explained, since all studies presumably isolated the main T-kininogen catalytic enzyme of the rat SMG.

Although differences in pI may be caused by variations in post-translational events or by purification procedures, the present study shows that the different identified kallikrein-like enzymes with different substrate specificities were clearly separated by their differences in pI. IEF in flat-bed gels was an efficient method with which to separate the various enzymes of the kallikrein family and was, therefore, when combined with immunoblotting, an excellent method for identifying these enzymes, which are very similar in molecular mass. Micro-heterogeneities in charge, as observed for tissue kallikrein and antigen γ , may be due to minor differences in amino acid sequence or may be a result of differences in post-translational handling. Contaminants were also easily detected, by the sensitive silver staining technique.

In conclusion, the present work demonstrates that the main T-kininogen catalytic enzyme of the rat SMG is identical to the previously isolated kallikrein-like serine protease antigen γ . Both immunologically and by its amino acid sequence it was clear that antigen γ belongs to the kallikrein family. However, complete identity with other previously sequenced kallikrein-like genes or mRNAs was not observed, indicating that the gene or mRNA for antigen γ has not yet been identified. Hydrolysis of T-kininogen by antigen γ and release of T-kinin was confirmed by SDS/PAGE and h.p.l.c. respectively. Although antigen γ was the only major T-kininogen catalytic enzyme in the rat SMG, the rate at which it released T-kinin from T-kininogen was much lower than that at which tissue kallikrein released bradykinin from HMM/LMM-kininogen.

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