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Characterization of a new kallikrein-like enzyme (KLP-S3) of the rat submandibular gland

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The submandibular gland of the rat contains several enzymes belonging to the kallikrein family. These include tissue kallikrein, antigen gamma (T-kininogenase), esterase B and tonin. In the present study, a new member of this family, which we have named KLP-S3, was identified and purified from the submandibular gland. KLP-S3 was classified as a kallikrein-like enzyme on the basis of its immunological similarity to other kallikrein-like enzymes and its showing 70% and 73% identity in partial amino acid sequence with tissue kallikrein and tonin respectively. Furthermore, the 44 sequenced amino acid residues showed complete correspondence to the mRNA S3 of the kallikrein gene family, which was the rationale for the name kallikrein-like protein (KLP) S3. KLP-S3 consisted of three isoenzymes with pI 6.75, 6.90 and 6.95, which significantly differed from those of other kallikrein-like enzymes. In conjunction with its immunological relationship to kallikrein, this parameter (pI) was considered robust enough to identify the enzyme during purification, since a specific physiological substrate for KLP-S3 has yet to be identified. In SDS/PAGE the three isoenzymes ran as one band with a molecular mass of 25800 Da, which after reduction with 2-mercaptoethanol was split into two chains with molecular masses of 16500 and 13300 Da. In common with other kallikrein-like enzymes, KLP-S3 was inhibited by phenylmethanesulphonyl fluoride, and was thus classified as a serine protease. It was also inhibited by soya-bean trypsin inhibitor but not by aprotinin. It showed weak reactivity against the chromogenic substrates S2288, S2266, S2366 and S2302 (D-Ile-Pro-Arg 4-nitroanilide, D-Val-Leu-Arg 4-nitroanilide, Glu-Pro-Arg 4-nitroanilide and D-Pro-Phe-Arg 4-nitroanilide respectively) and did not cleave rat T-kininogen or dog high-molecular-mass/low-molecular-mass kininogen. Its specific angiotensin II-generating activity (angiotensin I as substrate) was 0.04% of that of rat tonin. KLP-S3 (1–100 nM) induced a statistically significant angiotensin-independent contraction of isolated rat aorta rings. The maximum contraction was 15% of the response to the α-adrenoceptor agonist phenylephrine (1 μM). The concentration of KLP-S3 in the rat submandibular gland was by single radial immunodiffusion estimated to be 47±3 μg/mg of protein.

INTRODUCTION

The submandibular gland (SMG) of the rat has been shown to contain enzymes belonging to a group of serine proteases closely resembling tissue kallikrein (EC 3.4.21.35) (Brandtzæg *et al.*, 1976). Expression of numerous genes encoding proteins of the kallikrein family has been demonstrated in this organ (Ashley & MacDonald, 1985*a*,*b*). The kallikrein-like enzymes in the rat SMG that have so far been identified and characterized are kallikrein (Brandtzæg *et al.*, 1976), tonin (Boucher *et al.*, 1974), esterase B (Khullar *et al.*, 1986), antigen gamma (Berg *et al.*, 1987) and recently the vasoconstrictor SEV (submandibular enzymic vasoconstrictor) (Yamaguchi *et al.*, 1991*a*). Antigen gamma has been shown to be the enzyme largely responsible for SMG T-kininogenase activity (Berg *et al.*, 1991*a*) first discovered by Barlas *et al.* (1987), and it has also been named endopeptidase K (Gutman *et al.*, 1988) and kallikrein k10 (Gutman *et al.*, 1991). Antigen gamma is probably also identical with proteinase B and T-kininogenase described by Kato *et al.* (1987) and Xiong *et al.* (1990) respectively, despite some variations in amino acid sequence. The purpose of the present paper was to purify and characterize a new SMG kallikrein-like protein, first identified by its immunological similarity to kallikrein and other kallikrein-like proteins. Since known SMG kallikrein-like enzymes all directly or indirectly release vasoactive peptides, we elected to study the action of KLP-S3 vasomotor activity.

EXPERIMENTAL

Reagents

Whatman DE-52 anion-exchange DEAE-cellulose was obtained from Whatman Chemical Separations (Maidstone, Kent, U.K.). The chromogenic substrates S2266 (D-Val-Leu-Arg 4-nitroanilide dihydrochloride), S2302 (D-Pro-Phe-Arg 4-nitroanilide dihydrochloride), S2366 (Glu-Pro-Arg 4-nitroanilide dihydrochloride) and S2288 (D-Ile-Pro-Arg 4-nitroanilide dihydrochloride) were purchased from Kabi Diagnostica (Stockholm, Sweden), Aquacide was from Calbiochem (La Jolla, CA, U.S.A.) and 3,3′-diaminobenzidine tetrahydrochloride was from Ega-Chemi (Steinheim/Albuch, Germany). BSA, soya-bean trypsin inhibitor, angiotensin I, angiotensin II, His-Leu and phenylephrine were from Sigma Chemical Co. (St. Louis, MO, U.S.A.), phenylmethanesulphonyl fluoride was from Serva (Heidelberg, Germany), aprotinin (Trasylol) was from Bayer (Leverkusen, Germany) and trypsin (1:250) was from Difco Laboratories (Detroit, MI, U.S.A.). Angiotensin II antagonist DuPont753 was from Du Pont, Wilmington, DE, U.S.A. Pig anti-(rabbit immunoglobulin G) serum and peroxidase–anti-peroxidase reagent were from Boehringer (Mannheim, Germany).

F.p.l.c. system

The f.p.l.c. system consisted of an LCC-500 chromatography controller, two P-500 pumps, a V-7 valve injector, a UV-1

Abbreviations used: KLP, kallikrein-like protein; SMG, submaxillary gland; IEF, isoelectric focusing.

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monitor with an HR 10 cell, a pH monitor, a Frac-100 collector and an REC-482 recorder. The prepacked column MonoP HR 5/20 was used for chromatofocusing, MonoS HR 5/5 and MonoQ HR 5/5 and HR 10/10 for cation and anion-exchange chromatography respectively, and Superose 12 HR 10/30 for gel filtration. The f.p.l.c. system, the prepacked columns and Polybuffer 74 and 96 were from Pharmacia Fine Chemicals (Uppsala, Sweden).

Preparation of tissue homogenates

SMG from 4-month-old male Wistar rats were extirpated during Nembutal anaesthesia (70 mg/kg, intraperitoneally). The glands were cut into pieces and homogenized with a Potter–Elvehjem homogenizer (20 strokes, at 4 °C) in 0.05 M-ammonium acetate buffer, pH 5.8 (1:5, w/v). The homogenate was centrifuged (20000 *g* for 15 min at 4 °C) and the supernatant was collected. The precipitate was rehomogenized in an equal volume of buffer and the supernatants were combined. The supernatants were filtered (0.8 μm pore size) and dialysed against the same buffer (48 h at 4 °C), ionic strength and pH were adjusted with 1 M-ammonium acetate buffer, pH 5.8, to equal that of the 0.05 M-ammonium acetate buffer, and used for enzyme purification.

SMG homogenates that were used for enzyme quantification were prepared from individual glands as above in phosphate-buffered saline (0.14 M-NaCl in 0.01 M-sodium phosphate buffer, pH 7.4) (1:8, v/v) without filtration.

Enzyme assays

Amidolytic activity. The chromogenic substrates S2266, S2288, S2366 and S2302, mostly used for tissue kallikrein, tissue plasminogen activator, Factor XI and plasma kallikrein respectively, were used to test amidolytic activity. Enzyme solution (100 μl) and 800 μl assay buffer (0.2 M-Tris/HCl buffer, pH 9.0, 8.4, 8.0 and 8.0 for the four substrates respectively) were incubated for 5–120 min at 37 °C with 100 μl of substrate (2, 10, 2 and 4 mM for the four substrates respectively). The amount of enzyme and incubation time were chosen so as to be well within the limit of substrate exhaustion. The reaction was stopped with 100 μl of 50 % (v/v) acetic acid, and absorption was measured at 405 nm (Amundsen *et al.*, 1979). One unit was equal to the amount of enzyme that hydrolysed 1 μmol of substrate/min.

Angiotensin I catalytic 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). The concentration of purified enzyme was 0.5, 1.0, 0.6, 0.9, and 0.003 g/l for KLP-S3, tissue kallikrein, antigen gamma, esterase B and tonin respectively. His-Leu was used as standard. One unit of tonin enzyme activity was equivalent to the amount of protein that hydrolysed 1 nmol of angiotensin I/min.

Protein determination

Protein was determined by the method of Lowry *et al.* (1951). BSA was used as standard.

Activation of proenzymes

KLP-S3 (20 μl, 0.25 g/l) was incubated with trypsin (10 μl, 1 g/l, at 22 °C for 20 min). Trypsin activity was subsequently inhibited by aprotinin (30 μl, 1000 kallikrein-inactivator units/ml, at 22 °C for 30 min), and activity towards S2288 was tested as above. Equivalent volumes of buffer, trypsin and aprotinin were used as blank; aprotinin blocked 99.9 % of S2288 activity induced by trypsin alone.

Sensitivity to enzyme inhibitors

Phenylmethanesulphonyl fluoride dissolved in 10 μl of propanol (0–250000 μM) was incubated with 50 μl of enzyme for 24 h at 22 °C. Soya-bean trypsin inhibitor (50 μl; 0–880 μM) was incubated with 50 μl of enzyme for 30 min at 22 °C. Aprotinin (50 μl; 0–172 μM) was incubated with 50 μl of enzyme for 30 min at 22 °C. Enzyme concentrations are given in Table 3. Enzyme activity was subsequently tested as above with S2288 as substrate. Equivalent incubations of buffer (phosphate-buffered saline) and the highest concentration of inhibitor were used as blank. Purified tonin, esterase B, kallikrein, and antigen gamma were obtained as previously described (Berg *et al.*, 1987, 1991*b*; Johansen *et al.*, 1987).

Isoelectric focusing (IEF) and SDS/PAGE with immunoblotting

IEF and SDS/PAGE were run in the Pharmacia Phast System as described in the manufacturer's manual. For IEF we used PhastGel (IEF 3–9) with Pharmacia Isoelectric focusing calibration kit (pH 3–10) as standard. For SDS/PAGE PhastGel Gradient 8–25 and Pharmacia electrophoresis calibration kit (low-molecular-mass proteins) were used, and the proteins were pretreated with SDS (25 g/l) with or without 2-mercaptoethanol (5 %, v/v, for 2 min at 100 °C). The gels were stained with the silver-staining technique as described in the manufacturer's manual or used for immunoblotting as previously described (Berg *et al.*, 1991*a*). Rabbit antisera prepared as described below against rat SMG kallikrein, tonin, antigen gamma and KLP-S3 (1:2500–5000, v/v) were used as primary antisera, followed by pig anti-(rabbit immunoglobulin G) serum and peroxidase–antiperoxidase reagent. Staining was developed with 3,3′-diaminobenzidine tetrahydrochloride.

Determination of amino acid composition and partial amino acid sequence

KLP-S3 was run in SDS/PAGE in ExcelTM Gel (SDS gradient 8–18) and transferred electrophoretically to Immobilon [poly-(vinylidene difluoride)] transfer membrane (Millipore, Bedford, MA, U.S.A.) in a Pharmacia Multiphor II Novablot System as described by the manufacturer (Pharmacia LKB Biotechnology, Uppsala, Sweden). After staining with Coomassie Blue, the two protein bands were cut out and used for amino acid analyses. *N*-Terminal analyses were performed by using an automatic 477A Protein/Peptide Sequencer with an on-line 120A amino acid phenylthiohydantoin analyser from Applied Biosystems (Foster City, CA, U.S.A.) (Cornwell *et al.*, 1988). Amino acid composition was determined in a Biotronik LC 5000 amino acid analyser after hydrolysis with 6 M-HCl (Sletten *et al.*, 1987).

Preparation of antisera

Rabbit antisera against rat SMG kallikrein, tonin and antigen gamma were prepared as previously described (Ørstavik *et al.*, 1982; Berg *et al.*, 1987). Antiserum against SMG KLP-S3 was produced in rabbits by multiple intracutaneous injections on the back with 30 μg of KLP-S3 in complete Freund's adjuvant in phosphate-buffered saline (3:1, v/v; 0.6 ml). The rabbits were boosted after 3, 4 and 5 months. At 2 weeks after the last immunization, antiserum with a satisfactory precipitin titre was collected for a month.

Double immunodiffusion and immunoelectrophoresis

Purified protein preparations were tested in double immunodiffusion (Ouchterlony, 1958) with the antisera against rat SMG

kallikrein, tonin, antigen gamma and KLP-S3. Immunoelectrophoresis was performed as described by Weeke (1973) on SMG homogenate and KLP-S3 preparations against the same antisera.

Quantification of KLP-S3 in tissue homogenate

Individual SMG homogenates from ten male rats (250–280 g body wt.) were prepared as described above. Their KLP-S3 concentration was determined by single radial immunodiffusion as described by Mancini *et al.* (1965). The concentration of anti-KLP-S3 serum was 20 μl/ml of 1.5 % agarose, and purified SMG KLP-S3 was used as standard (0.02–0.17 g/l). For control purposes, the main SMG cross-reacting enzymes were tested for assay interference, i.e. tonin (5.1 g/l), tissue kallikrein (1.0 g/l), antigen gamma (1.6 g/l) and esterase B (1.6 g/l). A 5 μl portion of purified enzyme or homogenate in one of two different dilutions was added per well.

Effect of KLP-S3 on isolated rat aortic rings

Thoracic aortic rings (3 mm length) were obtained from male Wistar rats (250–350 g body wt.) that had been killed by a sharp blow to the head. The endothelium was removed by gentle rubbing of the intimal surface with a roughened 0.5 mm-diam. stainless-steel rod (Furchgott & Zawadzki, 1981). Aortic rings were set up for isometric recording at 37 °C in a modified Krebs–Henseleit buffer [composition (mM): Na^+ 143, K^+ 5.9, Ca^{2+} 0.25, Mg^{2+} 1.2, Cl^- 128, $H_2PO_4^-$ 2.2, HCO_3^- 24.9, SO_4^{2-} 1.2, glucose 10], gassed with O_2/CO_2 (19:1). The aortic rings were stretched with an initial pre-load of 3 g and the preparations were washed four times over the following hour. To establish tissue viability, the α-adrenoceptor agonist phenylephrine was added (1 μM), and the contraction was allowed to develop. Half of the preparations were then washed five times over the next hour (relaxed preparations, $n = 4$) while the remainder were maintained in a contracted state (contracted preparations, $n = 4$). KLP-S3 (0.01–100 nM) was then added in a cumulative manner, and any contractile or relaxant activity was measured isometrically. The effect of an angiotensin II antagonist on the KLP-S3-induced vasoconstriction was tested in a separate set of experiments with a different batch of KLP-S3: 16 aortic rings, obtained from four rats, were prepared as above for relaxed preparations, and divided into four groups of four rings each. The groups were treated with angiotensin II (0.1–100 nM) alone or in the presence of the angiotensin II antagonist DuPont753 (0.4 μM), or with KLP-S3 (10 nM) with or without the presence of angiotensin antagonist (0.4 μM). In similar experiments with relaxed tissue, tonin was added at concentrations of 0.05 and 0.5 μM with subsequent addition of phenylephrine (1 μM) to test tissue viability. Vehicle controls (Krebs) were conducted with up to 5 % of bath volume.

Statistical methods

All results are expressed as mean values ± S.E.M. Evaluation of statistical significance was done with the Student's t test. The exclusion limit for statistical significance was set at $P = 0.05$.

RESULTS

Identification of SMG KLP-S3

In silver-stained IEF gels SMG homogenate was found to contain three bands at pI 6.75, 6.90 and 6.95, each of which showed a strong reaction with polyclonal anti-kallikrein as well as anti-tonin serum after blotting. These three protein bands were defined as KLP-$S3_1$, KLP-$S3_2$ and KLP-$S3_3$ respectively (Fig. 1).

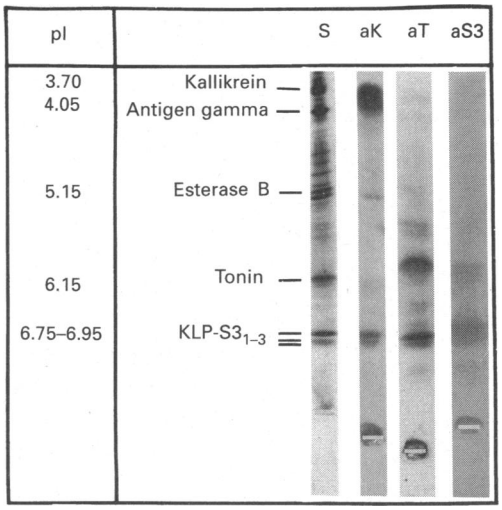

Fig. 1. Identification of SMG KLP-S3 by IEF and immunoblotting

SMG homogenate was run in flat-bed isoelectrofocusing gels and stained with silver (S) or antiserum after blotting on to nitrocellulose membrane. Initial identification of KLP-$S3_{1-3}$ was obtained by the detection of three protein bands at pI 6.75, 6.90 and 6.95 that reacted with the anti-kallikrein serum (aK) or anti-tonin serum (aT). Polyclonal antisera with reactivity against common antigenic epitopes were needed to demonstrate this immunological relationship to members of the kallikrein family. When antiserum was raised against KLP-S3, these bands were shown to react strongly also with the anti-KLP-S3 serum (aS3). White lines indicate application points.

Purification of KLP-S3 from the rat SMG

When SMG homogenate was applied to a Whatman DE-52 DEAE-cellulose anion-exchange column (pH 5.8), KLP-$S3_{1-3}$ was found in the void volume (Pooled Fr. 1 in Fig. 2). This fraction was further purified on an f.p.l.c. MonoS cation-exchange column (Fig. 3), where KLP-$S3_{1-3}$ was separated from tonin and esterase B. Contaminating proteins with pI < 6.2 were removed by subsequent chromatofocusing on a MonoP (pH 7.4–4.0) column, where most of the KLP-$S3_{1-3}$ was eluted in the void volume, but some also adhered to the column (chromatogram not shown). This void volume showed one contaminating band in silver-stained IEF gels as well as minor tonin enzyme activity, and reacted in double immunodiffusion against anti-tonin serum with a weak line of identity with tonin in addition to the main KLP-$S3_{1-3}$ precipitin band. A pure preparation of KLP-$S3_{1-3}$ was obtained by a second run on the MonoS column (Fig. 4): in the eluted KLP-S3 peak the first fraction (Fr. 1 in Fig. 4) contained one minor, unidentified, contaminant revealed by IEF, whereas no contaminants were observed in the remaining four fractions that were pooled (Fr. 2 in Fig. 4) (Fig. 5). This pure preparation of KLP-$S3_{1-3}$ showed an unambiguous pattern of partial immunological identity with tonin when tested against anti-KLP-S3 serum (Fig. 8), and had a specific angiotensin I catalytic activity of 3.2 nmol/min per mg of protein, i.e. 0.04 % of that of purified tonin. KLP-$S3_{1-3}$ during purification was identified by IEF (Fig. 5) since a specific assay for KLP-S3 has not yet been described. The purification procedure is outlined quantitatively in Table 1.

The three KLP-S3 proteins did not separate on any of the above columns or by MonoQ anion-exchange chromatography (start buffer 0.02 M-Tris, pH 8.4; elution buffer 0.02 M-Tris, pH 8.4, containing 0.5 M-NaCl) on a DE-52 Whatman DEAE-cellulose column run at pH 8.0 (0.02 M Tris, pH 8.0; 0.04–0.4 M-NaCl gradient), or by gel filtration on a Superose 12 f.p.l.c.

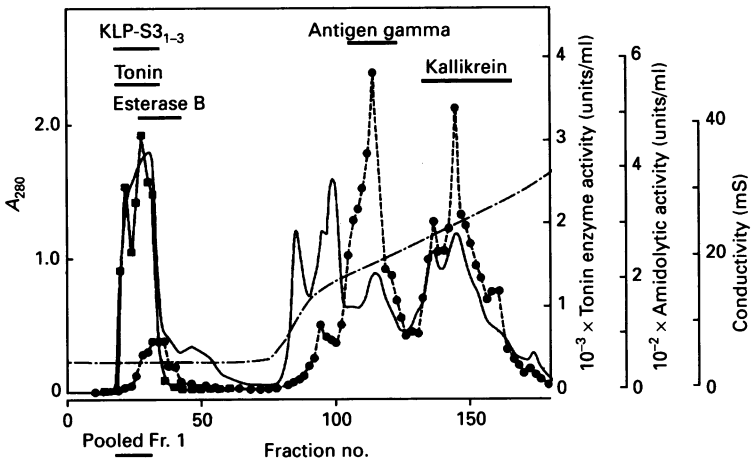

Fig. 2. Whatman DE-52 DEAE-cellulose anion-exchange chromatography (pH 5.8) of SMG homogenate

SMG homogenate was prepared as described in the Experimental section, and 0.8 g of protein (120 ml) was applied to a 450 ml Whatman DE-52 DEAE-cellulose anion-exchange column. Starting material and the column were equilibrated with 0.05 M-ammonium acetate buffer, pH 5.8, and the column was eluted with a 0.05–0.5 M-ammonium acetate buffer, pH 5.8, gradient (700 ml and 700 ml; flow rate 38 ml/min). The elution profile was monitored by conductivity (-—-), A_{280} (——), tonin enzyme activity (angiotensin I as substrate) (■—■) and amidolytic activity (S-2288 as substrate) (●--●). Fractions (10 ml) were collected, and proteins were identified in IEF stained with silver or after blotting with anti-kallikrein and anti-tonin sera. The KLP-S3-containing tubes were pooled as indicated on the chromatogram (Pooled Fr. 1). For further details see the Experimental section.

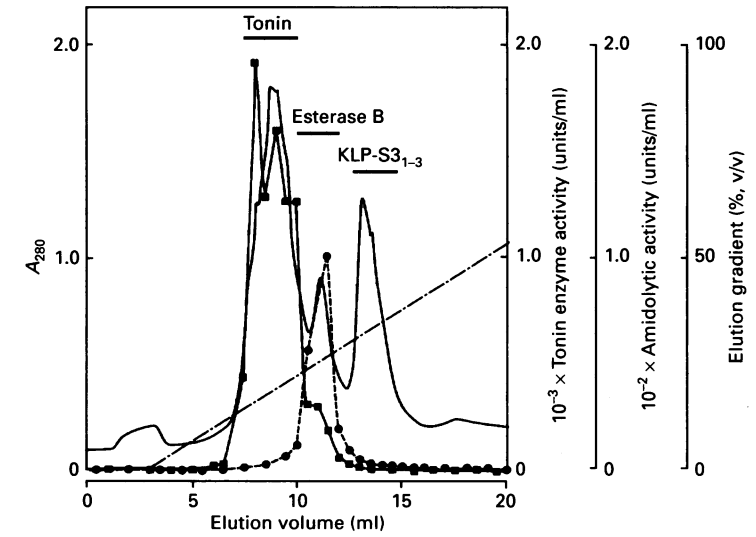

Fig. 3. MonoS cation-exchange chromatography of the Whatman DE-52 DEAE-cellulose column (pH 5.8) SMG KLP-S3 fraction

The KLP-S3$_{1-3}$ fraction from the Whatman DE-52 DEAE-cellulose column (Pooled Fr. 1 in Fig. 2) was equilibrated with starting buffer (0.05 M-sodium acetate buffer, pH 5.0) and applied to a MonoS HR 5/5 column using the f.p.l.c. system. The column was eluted with 0.5 M-sodium acetate buffer, pH 5.0 (-—-). The elution profile was monitored by A_{280} (——), tonin enzyme activity (angiotensin I as substrate) (■—■) and amidolytic activity (S2288 as substrate) (●--●), measured as described in the Experimental section. KLP-S3$_{1-3}$, identified by IEF with silver staining, was eluted as one peak after tonin and esterase B as indicated on the chromatogram. The flow rate was 1 ml/min and the fraction volume was 1 ml. A 11 mg portion of protein (4 ml) was applied per run, and this was repeated nine times to obtain sufficient material for further purification.

column (in phosphate-buffered saline). Chromatofocusing on the MonoP column with a pH range 9.3–6.0, where KLP-S3$_{1-3}$ in total adhered to the column, also failed to effect separation of the KLP-S3 proteins. These observations indicated that the three bands observed in IEF represented isoenzymes of KLP-S3.

Determination of KLP-S3 molecular mass

In SDS/PAGE (Fig. 6), KLP-S3 migrated as one band giving a molecular mass of 25800 Da, which after treatment with 2-mercaptoethanol was split into two bands with molecular masses of 16500 and 13300 Da.

Amino acid composition and partial amino acid sequence

The amino acid composition of KLP-S3 in comparison with antigen gamma and kallikrein is given in Table 2. The partial amino acid sequence of the two KLP-S3 bands obtained in SDS/PAGE after reduction with 2-mercaptoethanol is shown in Fig. 7 in comparison with other kallikrein-like enzymes. KLP-S3

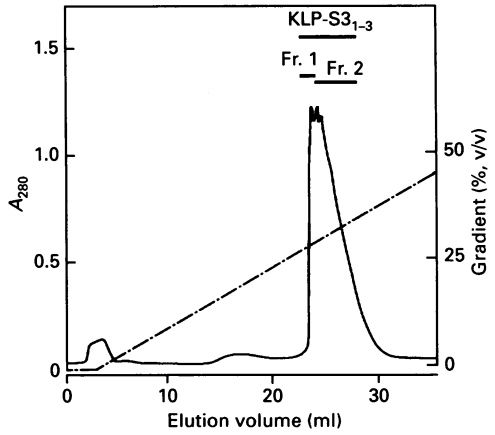

Fig. 4. Repeated cation-exchange chromatography of the KLP-S3 fraction obtained by chromatofocusing

The KLP-S3$_{1-3}$ fraction from the MonoS column (Fig. 3) was further purified on a MonoP chromatofocusing column (start buffer 0.05 M-imidazole, pH 7.4, eluted with Polybuffer 74, 1:10, v/v, pH 4.0). Most of KLP-S3$_{1-3}$ was eluted in the void fraction but some also adhered to this column (chromatogram not shown). The void was after dialysis against 0.05 M-sodium acetate buffer, pH 5.0, re-applied to the MonoS column under the same conditions as above (Fig. 3) (-·—·-). The elution profile was monitored by A_{280} (——) and by IEF stained with silver for the detection of contaminants. On the basis of the purity of the column fractions, KLP-S3$_{1-3}$ was pooled into two main fractions as indicated on the chromatogram (Fr. 1 and Fr. 2). Fr. 2 contained KLP-S3$_{1-3}$ only (Fig. 5, lane 5), whereas the presence of one minor, unidentified, contaminant was detected in Fr. 1 (results not shown). The start buffer was 0.05 M-sodium acetate buffer, pH 5.0, and the elution buffer was 0.50 M-sodium acetate buffer, pH 5.0. The flow rate was 1 ml/min and the fraction volume was 1 ml. Two samples, each of 4.7 mg of protein (2 ml), were applied to the column.

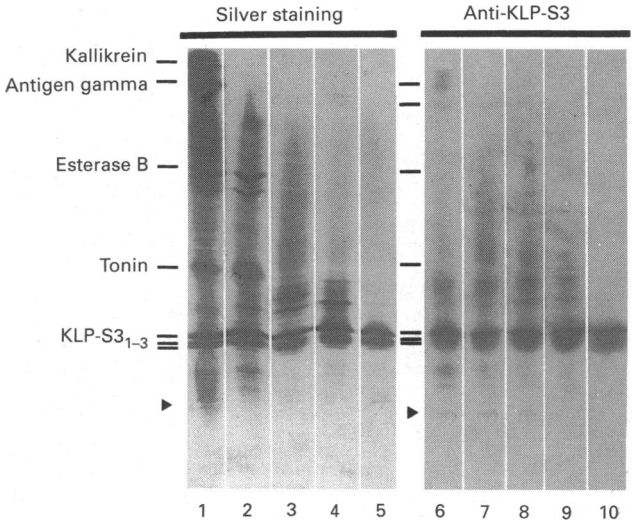

Fig. 5. Purification of SMG KLP-S3 monitored by IEF

The purity of pooled KLP-S3-fractions was monitored in IEF gels stained with silver (lanes 1–5), and the presence of KLP-S3 was confirmed by blotting and staining with the antiserum against KLP-S3 (lanes 6–10). The lanes contained: lanes 1 and 6, SMG homogenate; lanes 2 and 7, the KLP-S3 fraction from the Whatman column (Pooled Fr. 1 in Fig. 2); lanes 3 and 8, the KLP-S3 fraction from the MonoS column in Fig. 3; lanes 4 and 9, the KLP-S3$_{1-3}$ void fraction after chromatofocusing on the MonoP column (pH 7.4–4.0) (chromatogram not shown); lanes 5 and 10, Fr. 2 (Fig. 4) after a repeated run on the MonoS column, which gave a pure preparation of KLP-S3$_{1-3}$. The application points are marked by arrowheads.

showed 70% and 73% sequence identity with tissue kallikrein and tonin respectively (44 amino acid residues sequenced), whereas complete correspondence to the rat kallikrein-like mRNA S3 was observed.

Characterization of KLP-S3 enzyme activity

All chromogenic substrates, high-molecular-mass/low-molecular-mass kininogen, T-kininogen and angiotensin I were found to be poor substrates for KLP-S3 (Table 3). Highest activity was observed with S2288, and despite the finding that this substrate was not at all specific for KLP-S3 it was useful for monitoring column elution profiles. The low enzymic activity was not due to KLP-S3 being a proenzyme since only an 8% activation was detected after incubation with trypsin. KLP-S3 was inhibited by phenylmethanesulphonyl fluoride (Table 4), and was thus classified as a serine protease. KLP-S3 was very sensitive to

Table 1. Purification of SMG KLP-S3

For experimental details of the purification see the text. Since S2288 was a poor substrate for KLP-S3, purification factor was therefore not calculated. Specific angiotensin II-generating activity (angiotensin I as substrate) of purified tonin was 2772 units/mg. Recovery in pooled column fractions was calculated as the amount of protein as a percentage of protein applied to the column.

	Protein			S2288		Angiotensin I	
Purification step	Concn. (mg/ml)	Total (mg)	Recovery (%)	Total activity (units)	Sp. activity (units/mg)	Total activity (units)	Sp. activity (units/mg)
Whatman DE-52, pH 5.8:							
Applied	6.5	785		144000	185	297000	381
Pooled Fr. 1	3.6	142	27	5548	39	83120	577
After dialysis and filtration	2.8	101	71	3366	33	61560	611
First MonoS column:							
KLP-S3 fraction	0.9	17	17	60	66	120	7
MonoP column:							
KLP-S3 fraction	2.4	9	55	20.1	1.9	17.4	1.6
Second MonoS column							
KLP-S3 Fr. 1	1.0	1.8	19	6.7	3.7	1.8	1.0
KLP-S3 Fr. 2	1.6	5.2	55	20.1	4.0	6.1	1.2

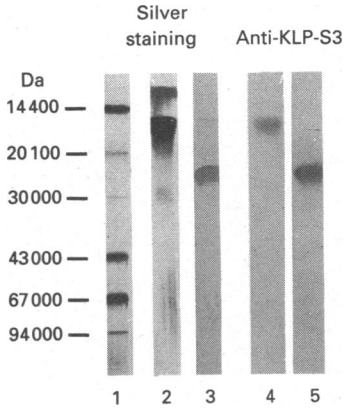

Fig. 6. SDS/PAGE of SMG KLP-S3

Purified SMG KLP-$S3_{1-3}$ preparations were run in SDS/PAGE without (lanes 3 and 5) and with (lanes 2 and 4) reduction with 2-mercaptoethanol in the Pharmacia Fast System as described in the Experimental section. The gels were stained with silver (lanes 1–3) or blotted on to a nitrocellulose membrane and stained with antiserum against KLP-S3 (lanes 4 and 5). Without 2-mercaptoethanol, one band was observed in the KLP-$S3_{1-3}$ preparation, corresponding to a molecular mass of 25800 Da. After reduction with 2-mercaptoethanol, two bands appeared, corresponding to molecular masses of 16500 and 13300 Da. After reduction with 2-mercaptoethanol, we also observed one additional faint band corresponding to a molecular mass of 29000 Da, which most probably represented residual KLP-S3 not split into two polypeptide chains. This was concluded from the fact that there were no indications of more than one band in the 25000–30000 daltons region without reduction with 2-mercaptoethanol, and because uncleaved proteins were observed to have a higher molecular mass with 2-mercaptoethanol than without. The latter was true for proteins that are not cleaved by reduction, such as for instance for tonin. This band did not stain with the antiserum, but because of its low concentration that would not have been expected. The light chain obtained by reduction had a molecular mass that was slightly lower than that of the smallest standard protein. The molecular mass of this band was therefore determined by extrapolating the standard curve. The light chain was also not visible when stained with antiserum, which may be due to altered immunoreactivity by alterations in the three-dimensional protein structure. On the other hand, traces of the 16500 Da band were observed also in the non-reduced enzyme preparation. It seems likely that this band represented autodegraded KLP-S3, since it reacted with the antiserum and since storage increased its intensity. Lane 1, low-molecular-mass standards; molecular masses are given to the left. For further details see the Experimental section.

inhibition by soya-bean trypsin inhibitor but not at all to inhibition by aprotinin (Table 4).

Immunological characterization of SMG KLP-S3 and its antibody

Several antisera were raised against SMG KLP-S3. Of these, the antiserum that gave a strong precipitin line against KLP-S3 with the least reactivity to other SMG proteins and that showed a pattern of immunological partial identity with cross-reacting proteins was used for further studies. Its immunoelectrophoretic reactivity pattern against SMG homogenate is shown in Fig. 8. In double immunodiffusion this anti-KLP-S3 serum showed reactivity against SMG tonin in a pattern of partial immunological identity with KLP-S3, but did not react with SMG kallikrein, esterase B or antigen gamma (Fig. 8). When tested against KLP-S3 (0.2 g/l), this antiserum showed a precipitin line after staining with Coomassie Blue up to a dilution of 1:8 (v/v). When tested in double immunodiffusion against anti-tonin serum, a precipitin line, although very faint, was observed against KLP-S3 in a pattern of immunological partial identity with SMG tonin (Fig. 8). No precipitin line was observed against KLP-S3 with antiserum against kallikrein (Fig. 8) or antigen gamma (results not shown).

Table 2. Amino acid composition of SMG KLP-S3 in comparison with antigen gamma and tissue kallikrein

The amino acid composition of antigen gamma is from Berg *et al.* (1991*a*) and that of tissue kallikrein from Ashley & MacDonald (1985*a*). Abbreviation: N.D., not determined.

	Composition (residues/molecule)		
Amino acid	KLP-S3	Antigen gamma	Tissue kallikrein
Asp/Asn	27	35	18/13
Thr	19	14	11
Ser	18	18	13
Glu/Gln	22	30	15/10
Pro	15	19	16
Gly	22	21	24
Ala	13	12	9
Val	15	15	16
Met	6	5	5
Ile	12	11	13
Leu	21	24	22
Tyr	7	12	8
Phe	5	7	6
His	5	6	6
Lys	16	17	12
Cys	6	8	10
Arg	5	5	3
Trp	N.D.	N.D.	7
Total	234	259	237

In isoelectrofocusing with immunoblotting of SMG homogenate, the anti-KLP-S3 serum showed strong reactivity against all KLP-S3 isoenzymes (Figs. 1 and 5). Weak reactivity was also observed against other SMG proteins (Figs. 1 and 5). KLP-$S3_{1-3}$ was stained by antiserum against SMG kallikrein and more strongly by the anti-tonin serum (Fig. 1), but only very faintly by antiserum against the T-kininogenase antigen gamma (results not shown).

Quantification of KLP-S3 in SMG homogenates

By single radial immunodiffusion, the concentration of KLP-S3 in the SMG was found to be 47 ± 3 μg/mg of protein. Purified preparations of cross-reacting enzymes that are present in the SMG in high concentrations, i.e. tonin, tissue kallikrein, antigen gamma and esterase B, did not give a precipitin ring, and thus in this organ did not interfere with assay specificity.

Effect of KLP-S3 on isolated aortic rings

KLP-S3 was without effect on contracted aortic rings (0.01–100 nM) but demonstrated a small, but statistically significant, concentration-dependent contractile action on four out of four relaxed tissues (1–100 nM; Figs. 9*a* and 9*b*). The maximum contraction was $15 \pm 4\%$ ($P < 0.05$) of the response to phenylephrine (1 μM). The vasoconstrictor effect of KLP-S3 was not blocked by the angiotensin II antagonist DuPont753 (0.4 μM); with 10 nM-KLP-S3, a concentration close to its [A_{50}], statistically significant contractions of $5.2 \pm 0.6\%$ and $7.2 \pm 1.2\%$ were observed in the absence and in the presence of the antagonist respectively ($P > 0.05$; Fig. 9*c*). In control experiments, angiotensin II concentration–response curves were displaced dextrad,

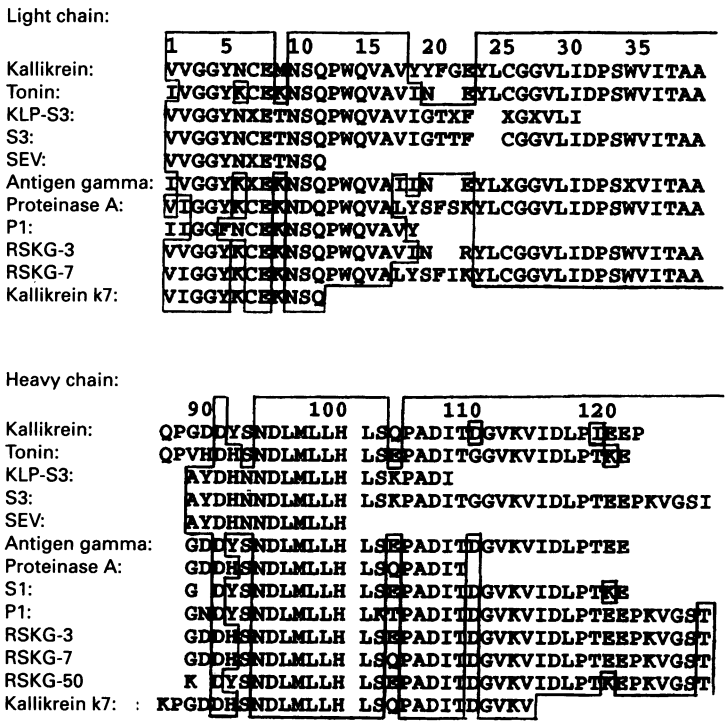

Fig. 7. Partial amino acid sequence of SMG KLP-S3

Partial amino acid sequences of the two fragments (heavy chain and light chain) of SMG KLP-$S3_{1-3}$ obtained in SDS/PAGE after 2-mercaptoethanol treatment are compared with those of other kallikrein-like enzymes [proteins: tonin (Lazure *et al.*, 1987), the T-kininogenase antigen gamma (Berg *et al.*, 1991*a*), which has the same sequence as kallikrein k10 (Gutman *et al.*, 1991), and with the exception of one amino acid also proteinase B (Kato *et al.*, 1987), both shown to have T-kininogenase activity, proteinase A (Kato *et al.*, 1987), kallikrein k7 (Elmoujahed *et al.*, 1990) and SEV (Yamaguchi *et al.*, 1991); the mRNAs: kallikrein, S1 and S3 (Ashley & MacDonald, 1985*a*) and P1 (Brady *et al.*, 1989), which has the same sequence as kallikrein k8; the genes: RSKG-3, RSKG-7 (Chen *et al.*, 1988) and RSKG-50 (Shai *et al.*, 1989)]. Complete correspondence was found between the sequenced amino acid residues of KLP-S3 and SEV as well as the mRNA S3. X, unidentified amino acid. Open spaces are used to enable alignment of identical sequences in the different proteins.

Table 3. Substrate-specificity of SMG KLP-S3 in comparison with other SMG kallikrein-like enzymes

S2288, S2266, S2366 and S2302 are mostly used for tissue plasminogen activator, tissue kallikrein, Factor IX and plasma kallikrein respectively. The values for rat T-kininogen and dog high-molecular-mass/low-molecular-mass kininogen (HMW/LMW kininogen) are from Berg *et al.* (1991*a*). For further details see the Experimental section.

	S2288 (units/mg)	S2266 (units/mg)	S2366 (units/mg)	S2302 (units/mg)	Angiotensin I (units/mg)	T-kininogen (ng of kinin/h per mg)	HMW/LMW kininogen (μg of kinin/h per mg)
SMG KLP-S3	4.0	0.6	1.2	0.3	1.2	0	0
Tissue kallikrein	357	458	60	297	0.1	158	42200
Antigen gamma	336	305	76	380	0.2	7424	10
Esterase B	149	188	21	61	22	0	31
Tonin	2.2	1.6	0.9	1.4	2772	16	11

in parallel, 2.30 ± 0.07 ($P < 0.001$) log units by this concentration of the antagonist (Fig. 9*c*). From this result, the pK_B (negative logarithm of the equilibrium dissociation constant) of the antagonist was estimated to be 8.7 (95% confidence limits 8.4 and 9.0). Thus the dose added in the KLP-S3 experiments was more than 100 times higher than the equilibrium dissociation constant. Tonin (0.05 and 0.5 μM) had no effect on relaxed tissue although the tissues showed the expected contraction on subsequent addition of phenylephrine (1 μM). Vehicle controls (5% of bath volume) showed no effect on vascular tension.

DISCUSSION

A new member of the kallikrein family has been identified, isolated and characterized in these studies. The enzyme, which we have named KLP-S3, was classified as a kallikrein-like protein by virtue of being recognized by antisera against other members of the kallikrein family such as tissue kallikrein, tonin and esterase B. Partial amino acid sequencing showed 70% and 73% sequence identity with kallikrein and tonin respectively, which supports its kallikrein-like nature. Furthermore, the sequenced

Table 4. Sensitivity of SMG KLP-S3 to protease inhibitors in comparison with other SMG kallikrein-like enzymes

For details see the Experimental section.

	Phenylmethanesulphonyl fluoride		Soya-bean trypsin inhibitor		Aprotinin	
	Protein concn. (μM)	I_{50} (mM)	Protein concn. (μM)	I_{50} (μM)	Protein concn. (μM)	I_{50} (μM)
KLP-S3	1.0	0.73	2.7	6.8	2.7	0%*
Tonin	1.0	2.67	2.7	58	2.7	6%*
Kallikrein	0.03	1.27	0.02	24%*	0.02	0.002
Antigen gamma	0.03	1.33	0.02	43	0.2	0.24
Esterase B	0.03	0.91	0.02	2.5	0.2	0.34

* Percentage inhibition at highest concentration of inhibitor: for phenylmethanesulphonyl fluoride 22.7 mM, for soya-bean trypsin inhibitor 455 μM and for aprotinin 86 μM final concentration.

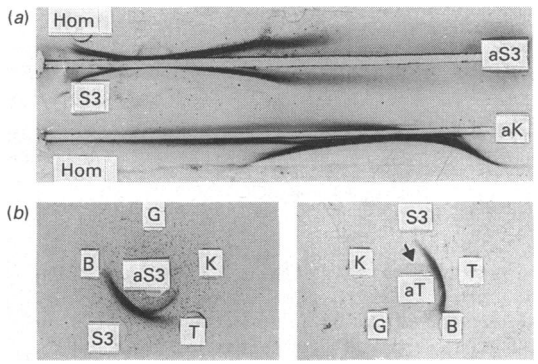

Fig. 8. Immunological characterization of SMG KLP-S3 and its antiserum in relation to other kallikrein-like enzymes

(*a*) SMG homogenate (Hom) and KLP-$S3_{1-3}$ (S3) were run in immunoelectrophoresis against polyclonal rabbit antisera against rat SMG KLP-S3 (aS3) and tissue kallikrein (aK). aS3 gave one precipitin line in SMG homogenate, which ran parallel to purified KLP-S3. A tail on the precipitin line indicates some cross-reactivity with other SMG proteins. No reaction was observed against KLP-S3 with anti-kallikrein serum. (*b*) The immunological relation between KLP-S3 and other kallikrein-like enzymes was tested in double immunodiffusion against anti-KLP-S3 serum (aS3) and anti-tonin serum (aT). Partial identity was observed between tonin and KLP-S3 with both antisera. Anti-KLP-S3 serum did not precipitate tissue kallikrein, antigen gamma or esterase B. Key: G, SMG antigen gamma (1.6 g/l); K, SMG kallikrein (0.5 g/l); B, SMG esterase B (0.2 g/l); T, SMG tonin (0.1 g/l); S3, KLP-$S3_{1-3}$ (0.1 and 1.6 g/l for anti-KLP-S3 serum and anti-tonin serum respectively). The arrow indicates the faint line of partial identity of KLP-S3 against the anti-tonin serum.

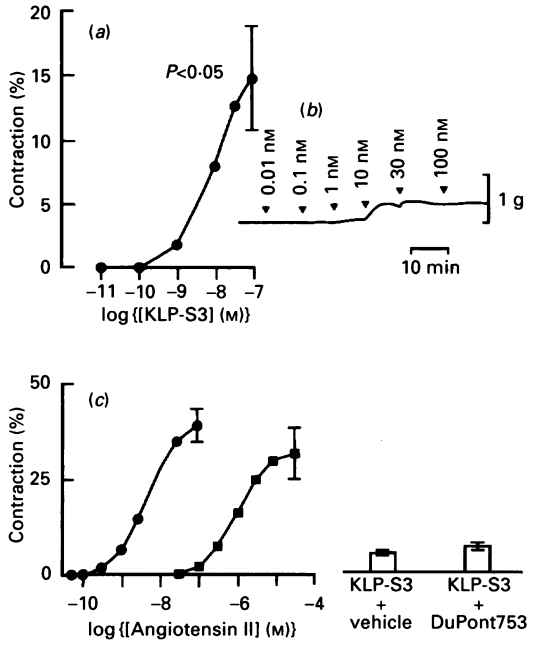

Fig. 9. Vasoconstrictor activity of SMG KLP-S3

(*a*) The vasoconstrictor effect of SMG KLP-S3 was tested on relaxed (●) isolated rat aorta ($n = 4$). The effect is shown as contraction in percentage of the response to phenylephrine (1 μM). The contractile effect of KLP-S3 was significantly different from vehicle controls ($P < 0.05$). (*b*) Record of the contractile effect of KLP-S3 on a relaxed isolated rat aortic ring. Tension on a gram scale is indicated to the right. The molar concentration of KLP-S3 is shown on the recording. (*c*) Angiotensin II concentration–response curves in the absence (●, $n = 4$) and in the presence (■, $n = 4$) of the angiotensin II antagonist DuPont753 (0.4 μM). Also shown on the same scale are the effects of KLP-S3 (10 nM) in the absence ($n = 4$) and presence ($n = 4$) of DuPont753 (0.4 μM).

amino acid residues of KLP-S3 showed complete correspondence to the comparable segment of the kallikrein-like mRNA S3 (Ashley & MacDonald, 1985*a*), indicating that KLP-S3 was encoded by the gene corresponding to S3. This finding prompted us to call this enzyme kallikrein-like protein (KLP) S3, in order to fit the nomenclature of the kallikrein protein family with that of the kallikrein gene family. In addition, as is the case with tissue kallikrein and other known members of the kallikrein family, KLP-S3 was classified as a serine protease since it was inhibited by the specific serine-protease inhibitor phenylmethanesulphonyl fluoride. KLP-S3 has so far only been detected in the rat. However, since the sequence of an enzyme such as tissue kallikrein differs across species (Wines *et al.*, 1991), demonstration of KLP-S3 in other species probably will depend upon the determination of its biological substrate.

The members of the rat kallikrein family that so far have been identified have all been shown either to release vasoactive peptides such as kinins and angiotensin II or to activate enzymes such as plasma and urinary prokallikrein (Kamada *et al.*, 1990). In the present study, we found that this new member of the family, i.e. KLP-S3, induced a statistically significant vascular response by constricting isolated rat aortic rings, further strengthening the hypothesis that several of the rat kallikrein family members may

be involved in vasomotor regulation. Moreover, the vasoconstrictor enzymes KLP-S3 and tonin were not sensitive to inhibition by aprotinin whereas tissue kallikrein, antigen gamma and esterase B were all easily inhibited. The former two show vasoconstrictor action whereas the latter three are directly or indirectly related to vasorelaxation.

Recently a phenylmethanesulphonyl fluoride-sensitive enzyme (SEV) with vasoconstrictor effect on rabbit aortic rings has been demonstrated in the rat SMG (Yamaguchi *et al.*, 1991*a*,*b*). On the basis of the complete identity in partial amino acid sequence, it is most likely that SEV is identical with KLP-S3. However, of the 24 amino acid residues that were determined in SEV, only seven were in a variable region and of these only three were not detected in other kallikrein-like proteins. Since not all members of the kallikrein family have yet been sequenced, the comparison between the two could have been done with total confidence if the SEV sequence had included the first hypervariable region of the light chain. This may be important, since minor differences were observed between the two proteins such as in their pI: SEV was reported to have a pI of 7.3, i.e. slightly higher than KLP-S3, and with no report of microheterogeneities in charge as for KLP-S3 (pI for KLP-$S3_{1-3}$ 6.75–6.95). This difference may be of relevance since we have recently purified another SMG protein with a pI of 7.1 in our system and which in preliminary studies shows weak vasoconstrictor activity (T. Berg, R. Hull & V. P. Gerskowitch, unpublished work). This protein shows immunological partial identity with kallikrein, and migrates very similarly to KLP-S3 in SDS/PAGE both with and without 2-mercaptoethanol. However, further characterization of biological activity as well as amino acid sequencing of this protein in comparison with KLP-S3 and SEV awaits purification of more substance. Because of these considerations, the possibility that KLP-S3 may be different from SEV cannot yet be totally ignored.

The nomenclature of kallikrein-like proteins, mRNAs and genes is complex and probably will remain confusing until a complete map of all elements has been established. Often the same protein has been given different names by different groups, and gene, mRNA and protein nomenclatures differ. In addition, the nomenclatures are based in part on numbering clones or fractions as they were obtained during the experimental procedure, and in part on biological functions, even though the physiological role of these enzymes is poorly understood. Since the substrate for KLP-S3 is not yet known, and since vasomotor activity is the only biological effect demonstrated so far, we cannot exclude the possibility that there may also be other functions for this enzyme. The name kallikrein-like protein S3 where S3 refers to the previously sequenced kallikrein-like mRNA S3 (Ashley & MacDonald, 1985*a*) provides a link between gene technology and protein chemistry while still indicating that it is a protein and not a gene or mRNA. Furthermore, if a differentiation between kallikrein-like protein, gene or mRNA is needed, the abbreviation can reflect this by using KLP, KLG or KLR respectively. This name is also neutral with respect to organ, which may be advantageous since kallikrein-like mRNA S3 has been demonstrated in the prostate as well as in the SMG (Brady *et al.*, 1989). Kallikrein-like protein S3 therefore for several reasons seemed preferable when selecting a name for this protein, and a similar nomenclature may prove useful also for other products of the kallikrein gene family. When a biological function or substrate has been established, this knowledge can be included so that for instance KLP-S3 could be called the vasoconstrictor (or the substrate name with the addition of 'ase') KLP-S3.

The mechanism by which KLP-S3 induced vasoconstriction is as yet not clear. However, release of angiotensin II did not seem to be involved since a high concentration of an angiotensin II antagonist (DuPont753) did not preclude the vasoconstrictor response. Furthermore, the specific angiotensin I catalytic activity of KLP-S3 was only 0.04% of that of tonin, a kallikrein-like enzyme that generates angiotensin II directly from angiotensinogen as well as angiotensin I (Boucher *et al.*, 1974). Moreover, the vasoconstrictor response to KLP-S3 differed from that of tonin, which has been shown to have a vasoconstrictor effect only on precontracted isolated rat aortic vessels (Garcia *et al.*, 1981). The absence of an independent effect of tonin on relaxed tissue was confirmed in the present study. These results indicate that the mechanisms for vasomotor action of KLP-S3 are distinguishable from that of tonin. Since KLP-S3 is a proteolytic enzyme, one may hypothesize that, in common with other members of the kallikrein family, vasomotion is induced through the release of a vasoactive peptide. We are investigating the possibility that endothelin, released from vascular-wall pro-endothelin, may be a possible candidate.

The three KLP-S3 protein bands detected in flat-bed isoelectrofocusing appeared to represent isoenzymes of the same protein since microheterogeneities in charge were detected, these possibly being due to differences in carbohydrate moieties. This was concluded from the observation that all three bands reacted strongly with the antiserum against KLP-S3 and from the failure to obtain separation by all chromatographic procedures tested. Since no other peptide sequences were detected during the *N*-terminal analysis, the results further confirm that the protein bands obtained after IEF represented isoenzymes of KLP-S3.

As we have observed previously with tissue kallikrein, antigen gamma, esterase B and tonin (Johansen *et al.*, 1987; Berg *et al.*, 1991*a*,*b*), the isoelectric point of KLP-S3 was significantly different from these other kallikrein-like enzymes. Thus isoelectrofocusing in flat-bed gels proved a most efficient method for the separation and identification of various members of this closely related enzyme family. Furthermore, by exploiting their immunological similarity, the kallikrein-like nature could be verified by subsequent blotting and immunostaining, which was how we discovered KLP-S3. For this, it was necessary to use a polyclonal antiserum without removing reactivity against common antigenic epitopes by pre-absorption. We have previously used a similar principle to identify antigen gamma as a kallikrein-like protein (Berg *et al.*, 1987), where antigen gamma was defined as one of the SMG proteins that in immunoelectrophoresis reacted with anti-kallikrein serum in a pattern of partial immunological identity to SMG kallikrein. However, flat-bed isoelectrofocusing gives a far better resolution and separation of the different kallikrein-like enzymes than immunoelectrophoresis. Moreover, the technique was sensitive and thus allowed protein identification during the purification procedure without prior concentration, and cross-reacting antigens were normally easier to detect than with precipitating techniques.

In conclusion, a new member of the rat kallikrein family, KLP-S3, has been discovered, purified and provisionally characterized. Partial amino acid sequencing indicated complete correspondence to the kallikrein-like mRNA S3, providing a rationale for naming the enzyme kallikrein-like protein (KLP) S3. Since a physiological substrate for this new enzyme is not yet known, specific identification was based on its immunological similarity to other kallikrein-like enzymes following separation from other such enzymes by IEF. KLP-S3 represented about 5% of the total protein in the submandibular gland. In functional studies KLP-S3 caused a moderate contraction of rat aorta *in vitro*.

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REFERENCES

Amundsen, E., Putter, J., Friberger, P., Knos, M., Larsbraten, M. & Claeson, M. (1979) Adv. Exp. Med. Biol. **120A**, 83–95

Ashley, P. L. & MacDonald, R. J. (1985*a*) Biochemistry **24**, 4512–4520

Ashley, P. L. & MacDonald, R. J. (1985*b*) Biochemistry **24**, 4520–4527

Barlas, A., Gao, X. & Greenbaum, L. M. (1987) FEBS Lett. **218**, 266–270

Berg, T., Holck, M. & Johansen, L. (1987) Hoppe-Seyler's Z. Physiol. Chem. **368**, 1455–1467

Berg, T., Wassdal, I., Mindroui, T., Sletten, K., Scicli, G., Carretero, O. A. & Scicli, A. G. (1991*a*) Biochem. J. **280**, 19–25

Berg, T., Wassdal, I. & Sletten, K. (1991*b*) J. Histochem. Cytochem., in the press

Boucher, R., Asselin, J. & Genest, J. (1974) Circ. Res. **34/35**, Suppl. **I**, I-203–I-209

Brady, J. M., Wines, D. R. & MacDonald, R. J. (1989) Biochemistry **28**, 5203–5210

Brandtzæg, P., Gautvik, K. M., Nustad, K. & Pierce, J. V. (1976) Br. J. Pharmacol. **56**, 155–167

Chen, Y.-P., Chao, J. & Chao, L. (1988) Biochemistry **27**, 7189–7196

Cornwell, G. G., Sletten, K., Johansson, B. & Westermark, P. (1988) Biochem. Biophys. Res. Commun. **154**, 648–653

Elmoujahed, A., Gutman, N., Brillard, M. & Gauthier, F. (1990) FEBS Lett. **265**, 137–140

Furchgott, R. F. & Zawadzki, J. V. (1981) Nature (London) **288**, 373–376

Garcia, R., Schiffrin, E. L., Thibault, G., Boucher, R. & Genest, J. (1981) Can. J. Physiol. Pharmacol. **59**, 790–793

Gutman, N., Moreau, T., Alhenc-Gelas, F., Baussant, T., El-Moujahed, A., Akpona, S. & Gauthier, F. (1988) Eur. J. Biochem. **171**, 577–582

Gutman, N., Elmoujahed, A., Brillard, Monegier, M., Du Sorbier, B. & Gauthier, F. (1991) Eur. J. Biochem. **197**, 425–429

Johansen, L., Bergundhaugen, H. & Berg, T. (1987) J. Chromatogr. **387**, 347–359

Kamada, M., Furuhata, N., Yamagushi, T., Ikekita, M., Kizuki, K. & Moriya, H. (1990) Biochem. Biophys. Res. Commun. **166**, 231–237

Kato, H., Enjyoji, K.-i., Miyata, T., Hayashi, I., Oh-ishi, S. & Iwanaga, S. (1987) J. Biochem. (Tokyo) **102**, 1389–1404

Khullar, M., Scicli, G., Carretero, O. A. & Scicli, A. G. (1986) Biochemistry **25**, 1851–1857

Lazure, C., Leduc, R., Seidah, N. G., Thibault, G., Genest, J. & Chretien, M. (1987) Biochem. Cell Biol. **65**, 321–337

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275

Mancini, G., Carbonaro, A. O. & Heremans, J. F. (1965) Immunochemistry **2**, 235–254

Ørstavik, T. B., Carretero, O. A., Hayashi, H., Scicli, A. G. & Johansen, L. (1982) J. Histochem. Cytochem. **30**, 1123–1129

Ouchterlony, Ö. (1958) Prog. Allergy **5**, 1–78

Shai, S.-Y., Woodley-Miller, C., Chao, J. & Chao, L. (1989) Biochemistry **28**, 5334–5343

Sletten, K., Husebekk, A. & Husby, G. (1987) Scand. J. Immunol. **26**, 79–84

Weeke, B. (1973) Scand. J. Immunol. **2**, Suppl. **1**, 15–35

Wines, D. R., Brady, J. M., Southard, E. M. & MacDonald, R. J. (1991) J. Mol. Evol. **32**, 476–492

Xiong, W., Chen, L.-M. & Chao, J. (1990) J. Biol. Chem. **265**, 2822–2827

Yamaguchi, T., Carretero, O. A. & Scicli, A. G. (1991*a*) Hypertension **17**, 101–106

Yamaguchi, T., Carretero, O. A. & Scicli, A. G. (1991*b*) J. Biol. Chem. **266**, 5011–5017

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