Purification of enzymes of the kallikrein gene family (rK8 and rK9) from the rat prostate

Helle SCHØYEN,* Irene WASSDAL,* Kim TOFT,† Merete ALMENDINGEN* and Torill BERG*‡

*Department of Physiology, Institute for Basic Medical Research, Medical Faculty, University of Oslo, Oslo, Norway,

and †Department of Bioanalysis and Biochemistry, Nycomed Imaging AS, Oslo, Norway)

The rat kallikrein family consists of multiple closely related proteins. A method for demonstration and identification of kallikrein-like proteins has been developed based on their differences in isoelectric point and their immunological similarity. The method, which involved separation in flat-bed isoelectrofocusing gels (pH range 3–9) and detection by immunoblotting using polyclonal antiserum against one of the family members, has been used in the present study to detect kallikrein-like proteins in the rat prostate. Nine immunoreactive kallikrein-like protein bands were detected with pI ranging from 5.30 to 8.35. Of these, six were completely purified and three were partially purified. Two proteins (pI 5.30 and 6.75–6.90) corresponded to protein bands in gels of rat submandibular-gland extracts, and were identified by partial amino acid sequence analysis as rK8 and rK9 respectively. In addition, sequence analysis revealed

complete sequence similarity between rK9 and the immunoreactive prostate proteins with pI 7.15, 7.25, 7.50 and 8.27. On the basis of this finding and immunological and biochemical characterization, we concluded that all the kallikrein-like proteins detected, except for rK8, represented isoenzymes of rK9. The molecular masses of the prostate rK9 isoenzymes (24600–29300 Da) were close to that of submandibular-gland rK9 (24600 Da), although differences were observed after reduction with mercaptoethanol. The prostate rK9 isoenzymes were, like submandibular-gland rK9, inhibited by soya-bean trypsin inhibitor but not by aprotinin, and were classified as serine proteases as they were inhibited by phenylmethanesulphonyl fluoride. rK8 (28700 Da) showed no activity with any of the substrates tested, and its inhibitory profile could therefore not be studied. No other enzymes of the kallikrein family were found in the rat prostate.

INTRODUCTION

A group of serine proteases has been shown to be encoded by a group of genes showing a high degree (74–86%) of sequence similarity [for a review, see Wines et al. (1991)]. This group of genes is referred to as the kallikrein gene family, as one of the genes encodes tissue kallikrein. Several proteins of the kallikrein family have been demonstrated in the rat submandibular gland (SMG). These include tissue kallikrein (rK1) (Brandtzæg et al., 1976), tonin (rK2) (Boucher et al., 1974), esterase B (rK7) (Khullar et al., 1986; Elmoujahed et al., 1990; Berg et al., 1992b), rK8, shown to be identical with P1 (Elmoujahed et al., 1990), rK9, formerly reported as prostatic protease (Winderickx et al., 1989), SEV (Yamaguchi et al., 1991) or KLP-S3 (Berg et al., 1992a), rK10 (Gutman et al., 1991), which is identical with antigen γ (Berg et al., 1987, 1991), and probably also proteinase B (Kato et al., 1987) and T-kininogenase (Barlas et al., 1987; Xiong et al., 1990).

Another organ in which kallikrein-like enzymes have been demonstrated is the prostate. In the human prostate, two kallikrein-like genes have been detected. One has been identified as the gene encoding 'prostate-specific antigen' (Lundwall and Lilja, 1987; Schedlich et al., 1987). In the rat prostate, two mRNAs, rKlk8 (formerly mRNA P1) and rKlk9 (formerly mRNA S3), have been sequenced (Brady et al., 1989; Winderickx et al., 1989).

Identification of kallikrein-like proteins can be difficult because of their similarities in substrate and inhibitor specificity, antigenic epitopes, amino acid sequence and molecular mass. However, as expected from their high degree of sequence homology, several of these proteins have been shown to be immunologically related,

whereas differences have been observed in their isoelectric point (Ørstavik et al., 1982; Berg et al., 1991, 1992a,b). Thus separation in flat-bed isoelectrofocusing (IEF) gels followed by immunoblotting has been found to be useful for detection and identification of known as well as new kallikrein-like proteins. In the present study, this method was used to detect kallikrein-like proteins in the rat prostate. After enzyme purification, the kallikrein-like nature of these prostate kallikrein-like proteins (pKLPs) was verified by partial amino acid sequencing, and the enzymes were identified as rK8 and rK9. No other proteins of the kallikrein family were detected.

EXPERIMENTAL

Reagents

Whatman DE-52 anion-exchange material was obtained from Whatman Chemical Separations (Maidstone, Kent, U.K.). Polybuffer 74 and 96 and CNBr-activated Sepharose 4B were from Pharmacia Biotech (Uppsala, Sweden). The chromogenic substrates S2266, S2288, S2302 and S2366 were purchased from Kabi Diagnostica (Stockholm, Sweden). BSA and soya-bean trypsin inhibitor (SBTI) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Phenylmethanesulphonyl fluoride (PMSF) was from Serva (Heidelberg, Germany), aprotinin (Trasylol) from Bayer (Leverkusen, Germany), trypsin (1:250) from Difco Laboratories (Detroit, MI, U.S.A.) and Aquacide from Calbiochem (La Jolla, CA, U.S.A.). Pig anti-rabbit IgG and peroxidase-antiperoxidase reagent were purchased from Boehringer (Mannheim, Germany), and 3,3'-diaminobenzidine tetrahydrochloride was obtained from Ega-Chemie (Steinheim am Albuch, Germany).

Abbreviations used: SMG, submandibular gland; IEF, isoelectrofocusing; pKLP, prostate kallikrein-like protein; SBTI, soya-bean trypsin inhibitor; PMSF, phenylmethanesulphonyl fluoride; PVDF, poly(vinylidene fluoride).

‡ To whom correspondence should be addressed.

Purified SMG rK1, rK2, rK7, rK9, rK10 and polyclonal rabbit antisera against these enzymes were obtained as previously described (Ørstavik et al., 1982; Johansen et al., 1987; Berg et al., 1987, 1991, 1992a,b). Except for that against rK10, these antisera showed reactivity against other members of the kallikrein family in a pattern of immunological partial identity to the homologous antigen.

SBTI-affinity chromatography

SBTI (395 mg) was coupled to CNBr-activated Sepharose 4B (15 g) as described by the manufacturer. Then 50 ml of coupled gel material and 381 mg of prostate gland protein, pre-equilibrated with 0.02 M sodium phosphate buffer (pH 7.0) containing 0.5 M NaCl, were incubated for 2 h at 37 °C by end-over-end rotation. The gel material was packed into a column at 4 °C and washed with equilibration buffer until the A_{280} approximated 0. Proteins adhering to the affinity column were eluted with 0.1 M sodium acetate buffer (pH 3.5) in 1 ml fractions into tubes containing 4 ml of 0.5 M Tris/HCl buffer (pH 8.5).

Tissue

Male Wistar rats, 3–4 months of age, were anaesthetized with pentobarbitone (Nembutal; 70 mg/kg; intraperitoneal injection). Ventral prostate gland and SMG were extirpated and frozen at –20 °C for later homogenization. Tissue homogenates were made in PBS (0.01 M sodium phosphate buffer, pH 7.4, containing 0.14 M NaCl) and, for the prostate, also in column start buffer (for details, see Figure 4 legend), in a Potter–Elvehjem homogenizer (20 strokes; 4 °C) at a ml volume/g gland wet weight ratio of 4:1 for prostate gland and 10:1 for SMG. The homogenates were centrifuged at 13000 *g* for 20 min at 4 °C, and the supernatants collected. For the SMG, the procedure was repeated on the precipitate, and the two supernatants were combined.

Assays

Amidolytic assays

The chromogenic substrates S2266 (H-D-Val-Leu-Arg-4-nitroanilide dihydrochloride), S2288 (H-D-Ile-Pro-Arg-4-nitroanilide dihydrochloride), S2302 (H-D-Pro-Phe-Arg-4-nitroanilide hydrochloride) and S2366 (Glu-Pro-Arg-4-nitroanilide hydrochloride) (usually used as substrates for glandular kallikrein, tissue plasminogen activator, Factor IX and plasma kallikrein respectively) were used to study enzyme activity. Enzyme solution (100 μ l) diluted with 800 μ l of assay buffer (0.05 M Tris/HCl, pH 8.4, for S2288, 0.2 M Tris/HCl, pH 8.2 and 8.0, for S2266 and S2302 respectively and 0.02 M Tris/HCl, pH 8.0, for S2366) and incubated for 5–120 min at 37 °C with 100 μ l of substrate (1.5, 1.5, 2 and 2 mM respectively). The reaction was stopped with 100 μ l of 50% (v/v) acetic acid, and A_{405} was measured (Amundsen et al., 1979). One unit is equal to the amount of enzyme hydrolysing 1 μ mol of substrate/min.

Trypsin activation

This was achieved by incubating about 5 μ g of protein with trypsin (0.3 g/l final concentration; 22 °C; 20 min) in a total volume of 30 μ l of PBS. Trypsin activity was subsequently inhibited by aprotinin (13 μ M in 30 μ l; 22 °C for 30 min), and activity towards S2288 was tested as above. Equivalent volumes containing buffer, trypsin and aprotinin and enzyme, buffer and

inhibitor were used as blank and 100% activity respectively. At this concentration, aprotinin reduced homogenate S2288 activity by 25%. For purified rK8, activation was also tested using SBTI (450 μ M in 30 μ l of PBS) to stop trypsin activity.

Protein

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

Enzyme inhibition

Enzyme preparations (50 μ l) were dialysed against PBS (24 h, 4 °C) and then incubated for 24 h at 22 °C with 10 μ l of PMSF (250 mM dissolved in propanol), SBTI (45 μ M in PBS) or aprotinin (1 mM in PBS) [1:1 (v/v) 30 min, 22 °C]. To test prostate total protein sensitivity to SBTI, prostate-gland homogenate (0.53 g/l in PBS) was incubated as above, using different concentrations of SBTI (0.9–90 μ M). S2288 enzyme activity was subsequently tested as described above. Equivalent volumes of enzyme incubated with inhibitor solvent and PBS incubated with inhibitors were used as 100% activity and blanks respectively.

Flat-bed IEF and SDS/PAGE with Immunoblotting

Tissue homogenate and enzyme fractions were subjected to IEF and SDS/PAGE using the Pharmacia Phast System as described in the manufacturer's (Pharmacia Biotech) manual. For IEF, PhastGel (IEF 3–9) was used with the Pharmacia IEF calibration kit (pH 3–10) as standard. For SDS/PAGE, PhastGel gradient 8–25 and the Pharmacia electrophoresis calibration kit (low-molecular-mass proteins) were used, and the proteins were pretreated with 2.5% (w/v) SDS with or without 5% (v/v) 2-mercaptoethanol for 2 min at 100 °C. The gels were stained by the silver-staining technique as described in the manufacturer's manual or blotted on to Immobilon poly(vinylidene difluoride) (PVDF) membranes and immunostained as previously described (Berg et al., 1991). Antisera against rat SMG rK1, rK2, rK7, rK9 and rK10 (1:1000–5000, v/v) were used as primary antisera, followed by pig anti-rabbit IgG and peroxidase-antiperoxidase reagents. Staining was developed with 3,3'-diaminobenzidine tetrahydrochloride.

Determination of partial N-terminal amino acid sequence

Purified prostate proteins were subjected to SDS/PAGE in Excel™ Gel (SDS gradient 8–18) and subsequently electroblotted on to PVDF membranes in a Pharmacia Multiphor II Novablot System as described by the manufacturer. The membranes were stained with Coomassie Blue and the protein bands were cut out and used for N-terminal amino acid sequencing in an automatic 477A Protein/Peptide Sequencer with an on-line 120A phenylthiohydantoin amino acid analyser from Applied Biosystems (Foster City, CA, U.S.A.) (Cornwell et al., 1988).

Purification procedures

Column elution profiles were monitored by determining A_{280} and for rK9 also by enzyme activity with S2288. At each step the pKLPs were visualized in flat-bed IEF gels stained with silver, and kallikrein-like immunoreactive proteins were identified after blotting on to PVDF membrane and staining with antiserum against SMG rK7 or rK9. Column fractions were concentrated with Aquacide (4 °C) and dialysed against PBS or the next column start buffer (24 h at 4 °C). The f.p.l.c. system was from Pharmacia Biotech, and was operated at 22 °C. Further details on the purification procedures are given in the Results section and Figure legends.

Figure 1 Detection of prostate and SMG kallikrein-like proteins by IEF and immunoblotting

Prostate-gland and SMG homogenates were run in IEF gels at a pH range from 3 to 9. The gels were stained with silver (S) or after blotting on to a PVDF membrane, with antiserum against SMG kallikrein (arK1), tonin (arK2), rK9 (arK9) or rK7 (arK7). Several immunoreactive protein bands were detected in both organs. The previously identified SMG kallikrein-like proteins are indicated. The immunoreactive proteins in the prostate were tentatively named pKLPs (prostate kallikrein-like proteins) and were numbered consecutively according to their pI as indicated (pKLP1–9). Only pKLP1 and 2 were found in both organs. Subsequent amino acid sequencing (Figure 8) and biochemical characterization showed that these two could be identified as rK8 and rK9 respectively, and that pKLP3–9 represented isoenzymes of rK9 present only in the prostate gland. Three additional very weak immunoreactive bands corresponding to pI values of 7.75, 7.68 and 8.67 were observed, particularly with the anti-rK9 antiserum. The presence of these immunoreactive bands was inconsistent, and characterization of these proteins was thus not further pursued. For further details, see the Experimental section.

RESULTS

Identification of immunoreactive kallikrein-like proteins in prostate-gland homogenate

When prostate-gland homogenate was subjected to IEF with immunoblotting, using antiserum against rK1, rK2 or rK9, several immunoreactive bands with pI ranging from 6.75 to 8.35 were detected. The strongest reaction was observed with the antiserum against rK9 (Figure 1), and one of the bands (a double band at pI 6.75 and 6.90) migrated identically with SMG rK9. When antiserum against rK7 was used, an additional immunoreactive band at pI 5.30 was detected, corresponding to a similar band in SMG homogenate (Figure 1). No reactivity was detected with antiserum against rK10 (not shown). These immunoreactive proteins, identified by their pI values (Table 1), were tentatively named prostate kallikrein-like proteins (pKLPs) 1–9, as indicated in Figure 1.

Purification of pKLPs

When enzyme activity was tested in homogenates prepared in PBS individually from ten rats, S2288 was found to give the highest activity (317 ± 25 munits/mg of protein; mean $\pm$ S.E.M.). Trypsin activation did not result in increased S2288 activity, suggesting that proenzymes were not present. As S2288 is also known to be cleaved by all SMG kallikrein-like proteins so far

tested (Berg et al., 1992a), we used activity with this substrate in addition to IEF and immunoblotting, to monitor enzyme purification. The purification procedure is shown in Figure 2 and is demonstrated by IEF and immunoblotting in Figure 3.

When prostate-gland homogenate was applied to a DE-52 Whatman anion-exchange column (Figure 4), pKLP1–9 were detected exclusively in the void volume.

Amidolytic activity in prostate-gland homogenate was found to be almost totally inhibited by SBTI; at a protein concentration of 0.26 g/l, a maximum inhibition of 85% was obtained at

Table 1 Isoelectric point and molecular mass of kallikrein-like proteins from the prostate (pKLPs) and the SMG

Isoelectric point was measured by IEF and molecular mass by SDS/PAGE. Kallikrein-like immunoreactivity was confirmed by immunoblotting. For details, see the Experimental section. The values for SMG rK1, rK2, rK7, rK9 and rK10 are from Berg et al. (1991, 1992a,b).

	Isoelectric point	Molecular mass (Da)		
		Non-reduced	Reduced	Reduced
Prostate				
rK8 (pKLP1)	5.30	28 700	16 500	14 500
rK9 isoenzymes				
pKLP2	6.75–6.85	24 600	16 500	12 900
pKLP3	7.08	29 100	16 600	13 800
pKLP4	7.15	28 000	16 700	—
pKLP5	7.25	28 000	16 700	—
pKLP6	7.50	29 200	16 400	—
pKLP7	7.61	—	Not available	—
pKLP8	8.27	28 600	28 600	—
pKLP9	8.35	29 300	29 300	—
SMG				
rK1 (kallikrein)	3.50–3.85	25 400–28 200	31 400	—
rK10 (antigen γ)	3.95–4.25	28 400	19 000	10 000
rK7 (esterase B)	5.15	25 100	17 200	13 000
rK2 (tonin)	6.05–6.15	25 700	29 200	—
rK9 (KLP-S3)	6.75–6.95	24 600	16 200	12 600

Figure 2 Overview of the purification of pKLPs

Pure preparations were obtained of pKLP1, 2, 4, 5, 6 and 8. pKLP1 was found to be identical with rK8, and pKLP2–9 to represent isoenzymes of rK9. The SBTI-affinity fraction thus contained a pure preparation of the prostate rK9 isoenzymes.

Figure 3 Purification of pKLP1 (rK8) and pKLP2-9 (rK9) visualized in IEF gels stained with silver and after immunoblotting

Purification of pKLP1 (rK8) and pKLP2-9 (rK9) was followed in IEF gels stained with silver (a) or antiserum against SMG rK7 (b) or rK9 (c) after blotting on to a PVDF membrane. Lane 1, prostate-gland homogenate; lane 2, anion-exchange column void volume (Figure 4); lane 3, fraction bound to anion-exchange (Figure 4); lane 4, protein fraction not bound to the SBTI-affinity column; lane 5, fraction bound to the SBTI-affinity column containing all rK9 isoenzymes; lane 6, rK8 fraction from first Mono P (7.4-4.0) column (Figure 5); lane 7, rK8 fraction from second Mono P (7.4-4.0) column; lane 8, purified rK8 after Mono S cation-exchange chromatography (Figure 6).

Figure 4 Anion-exchange chromatography of prostate-gland extract

Pooled prostate glands from 38 rats (7.8 g wet weight) were homogenized in column start buffer. After centrifugation, the supernatant was dialysed against the same buffer (24 h, 4 °C), filtered and pH and ionic strength were adjusted to equal that of the column start buffer. The void-volume and bound proteins were pooled into two fractions as indicated on the chromatogram. Of the total S2288 amidolytic activity applied to the column (13 units), 8% was recovered in the void volume, representing 75% of the activity eluted from the column. Immunoreactive proteins were observed only in the void volume (Figure 3). The details were as follows: column, DE-52 Whatman anion-exchange column (4 °C); column volume, 50 ml; start buffer, 0.05 M ammonium acetate, pH 5.8; elution gradient, 0.05-1.0 M ammonium acetate gradient (pH 5.8), 100 ml of each; flow rate, 14 ml/h; fraction volume, 10 ml; 381 mg of protein was applied to the column. For further details, see the Experimental section. —, A_{280} ; ∇ , amidolytic activity; — — —, conductivity.

11 μ M SBTI (IC_{50} 1.4 μ M). The Whatman column void fraction was therefore run on an SBTI-affinity column and pKLP1 was

Figure 5 Purification of pKLP1 (rK8) in the unbound fraction from the SBTI-affinity column by Mono P chromatofocusing (pH 7.4-4.0)

The unbound protein fraction from the SBTI-affinity column was dialysed against column start buffer and applied to the Mono P column, equilibrated against the same buffer. Fractions were collected as indicated on the chromatogram. The pKLP1 (rK8) fraction was further purified by a second run on the same column (chromatogram not shown). The details were as follows: column, f.p.l.c. Mono P HR5/20; start buffer, 0.05 M imidazole (pH 7.4); elution buffer, polybuffer 74 (pH 4.0) (1:10, v/v); flow rate, 0.5 ml/min; fraction volume, 1 ml; 1.5 mg of protein (10 ml) was applied to the column per run; repeated four times. For further details, see the Experimental section. —, A_{280} ; — — —, pH gradient.

detected exclusively in the unbound protein fraction, whereas pKLP2-9 were found in the bound fraction, containing no other proteins (Figure 3).

pKLP1 in the unbound protein fraction from the SBTI-affinity column was run twice through a Mono P chromatofocusing column (pH range 7.4-4.0) using the f.p.l.c. system (Figure 5). A

Figure 6 Purification of pKLP1 (rK8) by Mono S cation-exchange chromatography

The pKLP1 (rK8) fraction from the second run on the Mono P column was given a final purification step by cation-exchange chromatography on a Mono S column. Two protein peaks were eluted from the column, but IEF and immunoblotting showed that both peaks represented pKLP1. The two peaks were therefore pooled as indicated on the chromatogram. The details were as follows: column, f.p.l.c. Mono S HR5/5; start buffer, 0.05 M ammonium acetate (pH 4.0); elution buffer, 0.5 M ammonium acetate, pH 4.0; flow rate, 1.0 ml/min; fraction volume, 1 ml; 2.7 mg of protein (2 ml) was applied to the column. For further details, see the Experimental section. —, A_{280} ; ---, gradient.

Figure 7 Mono P chromatofocusing (pH 9.3–6.0) of the SBTI-affinity fraction for the separation of pKLP2–9 (rK9 isoenzymes)

Proteins bound to the SBTI-affinity column were further separated on a Mono P column. Fractions were collected as indicated (1–5), and the various proteins present were visualized by IEF and immunoblotting. Fraction 1 contained pKLP8, 2 contained pKLP8 contaminated with pKLP7 and pKLP6, 3 pKLP5, 4 pKLP4, and 5 pKLP3 and some pKLP2. pKLP2 was purified by a second run on the same column, and pKLP4, 5, 6 and 8 by separate runs on a Mono S cation-exchange column (chromatograms not shown). Details were as follows: column, f.p.l.c. Mono P HR5/20; start buffer, 0.075 M Tris/HCl (pH 9.3); elution buffer, Polybuffer 96 (pH 6.0) (1:10, v/v); flow rate, 0.4 ml/min; fraction volume, 0.75 ml; 1.6 mg of protein (1.2 ml) was applied to the column. For further details, see the Experimental section. —, A_{280} ; ●, amidolytic activity; ---, pH gradient.

Light chain :	1	5	10	15	20	25	30	35
rKLK1 (kallikrein) :	VGGYNCEMNSQPWQVAVYVFGYLCGGVLIDPSWVITAA							
rK2 (tonin) :	VGGYKCEKNSQPWQVAVIN EYLCGGVLIDPSWVITAA							
K10 (antigen γ) :	IVGGYKCEKNSQPWQVAVIN EYLCGGVLIDPSWVITAA							
rKLK12 (RSK-3) :	VGGYKCEKNSQPWQVAVIN RYLCGGVLIDPSWVITAA							
rKLK7 (RSK-7) :	VGGYKCEKNSQPWQVAVYVFIKLYLCGGVLIDPSWVITAA							
rK7 (esterase B) :	VGGYKKEKNSQPWQV							
rKLK8 (P1) :	IIGGFNCEKNSQPWQVAVY							
pKLP1 :	IIGGFNAEKNSQPQVAVXXIXFEPQXGGV							
rKLK9 (S3) :	VGGYCNCEMNSQPWQVAVIGTTF CGGVLDIPSWVITAA							
pKLP2 :	VGGYCNKETNSQ							
pKLP4 :	VGGYCNKETNSQPWQVAVIGTTF XGGVLI							
pKLP5 :	VGGYCNKETNSQPQVAVIGTTF XGGVLI							
pKLP6 :	VGGYCNKETNSQPWQVAVIGTTF XGGVLI							
pKLP8 :	VGGYCNKETNSQPQVAVIGTTF							
Heavy chain :	90	100	110	120				
rKLK1 (kallikrein) :	QPGDDYSNDMLLLH LSQPADITDGVKVIDLPTEEP							
rK2 (tonin) :	QPVVDHSDNDMLLLH LSEPADITGGVKVIDLPTKE							
rK10 (antigen γ) :	GDDYSNDMLLLH LSEPADITDGVKVIDLPTKE							
rKLK12 (RSK-3) :	GDDHSDNDMLLLH LSEPADITDGVKVIDLPTPEPKVGST							
rKLK7 (RSK-7) :	GDDHSDNDMLLLH LSQPADITDGVKVIDLPTPEPKVGST							
rKLK3 (RSK-50) :	K DYSNDMLLLH LSEPADITDGVKVIDLPTPEPKVGST							
rK7 (esterase B) :	KPGDDYSNDMLLLH							
rKLK3 (S1) :	G DYSNDMLLLH LKTPADITDGVKVIDLPTPEPKVGST							
rKLK8 (P1) :	GNDYSNDMLLLH LKTPADITDGVKVIDLPTPEPKVGST							
rKLK9 (S3) :	AYDHNNDMLLLH LSKPADITGGVKVIDLPTPEPKVGS							
pKLP2 :	AYDHNNDMLLLH L							
pKLP4 :	AYDHNNDMLLLH LSXPADI							
pKLP6 :	AYDHNNDMLLLH LSKPADITGGVXXIDL							

Figure 8 Partial N-terminal amino acid sequence of prostate rK8 (pKLP1) and rK9 isoenzymes (pKLP2–9)

Partial acid sequences of the fragments (light and heavy chains) of pKLPs obtained by SDS/PAGE after 2-mercaptoethanol treatment are compared with those of other members of the kallikrein family (Ashley and MacDonald, 1985; Lazure et al., 1987; Chen et al., 1988; Brady et al., 1989; Elmoujahed et al., 1990; Berg et al., 1991, 1992b; Gutman et al., 1991). Complete sequence similarity was found between pKLP1 and the rKLK8 gene (P1), whereas the other pKLPs were similar to rKLK9 (S3). X, unidentified amino acid. Open spaces were used to enable alignment of identical sequences in the different proteins.

pure preparation was obtained after a final run on an f.p.l.c. Mono S cation-exchange column (Figures 3 and 6).

pKLP2–9 in the SBTI-affinity fraction were further separated on a Mono P chromatofocusing column (pH range 9.6–6.0) (Figure 7). The pKLP2-containing fraction was purified to homogeneity by a second run on the Mono P column. The fractions containing pKLP4, 5, 6 and 8 were further purified by separate runs on a Mono S cation-exchange column. Further purification of pKLP3, 7 and 9 was not attempted because of the low protein concentration. An impure preparation of pKLP9 was, however, obtained after anion-exchange chromatography and Mono P (pH 9.3–6.0) chromatofocusing without the SBTI-affinity-chromatography step. For pKLP4–6 only the 16700 and 16400 Da band was detected.

Determination of molecular mass of pKLPs

The molecular masses of pKLP1–9, as obtained by SDS/PAGE, are given in Table 1. After reduction with 2-mercaptoethanol, pKLP1–6 migrated as two bands in SDS/PAGE, whereas pKLP8 and 9 migrated as single bands.

Partial N-terminal amino acid sequence

The partial N-terminal amino acid sequences of the purified pKLPs are shown in Figure 8 together with those of other members of the kallikrein family. Complete sequence similarity between pKLP1 and the rat kallikrein-like mRNA rKLK8 (formerly P1) was observed, and pKLP1 was thus identified as rK8. Furthermore, complete sequence similarity was also observed between pKLP2, 4, 5, 6 and 8 and rat SMG rK9, thus indicating that all these proteins represent isoenzymes of rK9.

Table 2 Specific activity with S2288 and sensitivity to protease inhibitors of prostate rK8 (pKLP1) and rK9 isoenzymes (pKLP2–9)

Protein (1.5–3.0 μ M) was incubated at 22 °C with 42 mM PMSF (20 h), 23 μ M SBTI (30 min) or 0.5 mM aprotinin (30 min) (final concentrations). Inhibitor profile was tested using S2288 as substrate which was not a very good substrate for rK9, but of those tested, it gave the highest activity. Owing to this low activity, the variations between the different isoenzymes should not be emphasized, but all enzymes showed low S2288 activity and sensitivity to PMSF and SBTI and were not inhibited by aprotinin. This is similar to rK9 from rat SMG (Berg et al., 1992a). For details, see the Experimental section.

	Specific activity (munits/mg)	Inhibition (%)		
		PMSF	SBTI	Aprotinin
rK8 (pKLP1)	0	—	—	—
rK9 isoenzymes				
pKLP2	1.5	89	90	3
pKLP3*	3.1	78	—	—
pKLP4	1.0	73	99	—
pKLP5	1.1	75	81	0
pKLP6	3.1	79	95	7
pKLP7*	0.2	82	97	7
pKLP8	0.1	62	92	0

* Semipurified preparations.

Substrate and inhibitor characteristics of pKLPs

Specific activity of pKLP2–9 with S2288 is shown in Table 2. Of the four substrates tested, the highest activity was observed with S2288 (results not shown). The enzymes were classified as serine proteases as they were inhibited quite strongly by PMSF almost completely. They were also inhibited by SBTI, but not by aprotinin (Table 2). From these results, combined with the similarity in molecular mass and immunoreactivity, and the similarity in amino acid sequence for pKLP2, 4, 5, 6 and 8, we concluded that pKLP2–9 all represent isoenzymes of rK9. The SBTI-bound fraction thus contained a pure preparation of all rK9 isoenzymes, and its specific activity with S2288, i.e. 1.5 units/mg, was comparable with that of the separate isoenzymes (Table 2).

rK8 showed no activity against any of the four chromogenic substrates tested, and sensitivity to inhibitors could therefore not be investigated. Enzyme activity was also not observed after trypsin activation.

As rK9 was the only enzyme found to cleave the S2288 substrate, purification was calculated for rK9 (Table 3). As the method used for identification of rK8, i.e. IEF, is not quantitative, a purification factor was not calculated for this protein.

DISCUSSION

Although kallikrein-like proteins seem to have one preferred natural substrate, overlap in substrate as well as inhibitor specificity has been observed for members of the rat SMG kallikrein family (Berg et al., 1992). Because of similarities in their amino acid sequences, these enzymes also share antigenic epitopes. Identification of kallikrein-like proteins is therefore hampered by their close relationship, particularly when the natural substrate is not known. In rat SMG, we observed that kallikrein-like enzymes differed in their pI values and thus could easily be separated by IEF (Berg et al., 1991, 1992a,b). After separation, three-dimensional structures common to kallikrein-like enzymes could be demonstrated by an antiserum with reactivity against common antigenic epitopes. By this method we were able in the present study to detect and purify rK8 and rK9 from the rat prostate. rK8 appeared as a single band during flat-bed IEF whereas rK9 appeared as several bands showing large heterogeneity in charge. Heterogeneity in charge has not been observed for kallikrein-like enzymes of rat SMG, including rK9, but sequence analysis demonstrated that these immunoreactive proteins of the rat prostate in fact represent isoenzymes of rK9. The observed heterogeneity in charge was independent of the purification procedure used (H. Schøyen and T. Berg, unpublished work), storage or freezing and thawing, and was never observed for rK9 from SMG using similar procedures. In addition, proteins differing in charge had similar molecular mass. Therefore degradation of the protein was not a likely explanation for the differences in pI. Differences in carbohydrate moieties could, however, induce charge heterogeneity, and could also possibly explain why two of the isoenzymes were less susceptible to cleavage by reduction with 2-mercaptoethanol. Varied post-translational handling of rK9 seemed to be specific for the prostate, as only minor charge heterogeneities were observed for SMG rK9. Owing to the low protein concentration of pKLP3, 7 and 9, we were unable to obtain enough material for partial amino acid sequencing of these enzymes. However, they all reacted strongly with the antiserum against rK9, and the substrate specificity and patterns of inhibition sensitivity to PMSF, aprotinin and SBTI of these semipurified preparations did not differ from those of the sequenced rK9 isoenzymes. It is therefore most likely that these proteins also represent isoenzymes of rK9. This is probably also true for those proteins that we did not attempt to purify because of their low concentration and inconsistent presence, as they were most clearly observed with the anti-rK9 serum. This conclusion is in agreement with observations at the mRNA level, where only rKlk9 and rKlk8 were demonstrated (Brady et al., 1989; Winderickx et al., 1989).

In the human prostate, prostate-specific antigen has been

Table 3 Purification of rK9 isoenzymes (pKLP2–9) by anion-exchange and SBTI-affinity chromatography quantified by protein and activity with S2288

Purification of a collected fraction of rK9 isoenzymes was achieved by anion-exchange chromatography on a Whatman column, where rK9 was eluted in the void volume, followed by SBTI-affinity chromatography, where rK9 isoenzymes adhered to the column. The homogenate prepared from the anterior prostate gland from 38 rats. For further details, see the Experimental section.

	Enzyme activity			Protein		
	Concentration (units/ml)	Total (units)	Specific activity (units/mg)	Concentration (mg/ml)	Total (mg)	Recovery (%)
Prostate homogenate	4.88	171	0.44	11.0	386	100
Anion-exchange column void volume	0.37	14	0.17	2.2	81	21
SBTI-affinity column bound fraction	0.56	15	1.45	0.4	10	3

identified by sequence analysis as a member of the kallikrein gene family (Watt et al., 1986; Lundwall and Lilja, 1987). In contrast with prostate-specific antigen (Watt et al., 1986), rK9 was not inhibited by aprotinin, and therefore probably does not correspond to prostate-specific antigen. We were not able to test for inhibitor specificity for rK8 as this protein did not cleave any of the substrates tested. Prostate-specific antigen has been shown to break down the major protein of the seminal clot, i.e. the high-molecular-mass seminal-vesicle protein, causing liquefaction of coagulated seminal fluid (Lilja and Laurell, 1984; Lilja, 1985). The main cleavage product is similar to the histidine-rich portion of high-molecular-mass kininogen (Han et al., 1975, 1976; Lilja and Jeppson, 1985), and has been suggested to act as an inhibitor, inhibiting the release of pituitary follicle-stimulating hormone (Seidah et al., 1984). Whether rK8 in the rat prostate has a similar role remains to be investigated. For rK9, a vasoconstrictory action has been demonstrated on isolated aortic rings via an angiotensin-II-independent mechanism (Yamaguchi et al., 1991; Berg et al., 1992b). Whether this effect has any physiological significance in the rat prostate has not been examined.

In conclusion, immunoreactive kallikrein-like proteins were purified from the rat prostate. After partial amino acid sequence analysis, only two proteins of the rat kallikrein family, i.e. rK8 and rK9, were detected. rK9 was observed as several isoenzymes with large heterogeneities in charge.

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