Protein Products of the Rat Kallikrein Gene Family

SUBSTRATE SPECIFICITIES OF KALLIKREIN rK2 (TONIN) AND KALLIKREIN rK9*

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Two closely related kallikrein-like proteinases having little activity toward the standard synthetic amide substrates of tissue kallikreins were isolated from the rat submandibular gland. They were found to be the protein products of the rKlk2 (tonin) and the rKlk9 genes by amino acid sequence analysis (nomenclature of the genes and proteins of the kallikrein family is according to the proposal of the discussion panel from the participants of the KININ '91 meeting held Sept. 8–14, 1991, in Munich, Germany). These two proteinases of similar structure also had very similar physicochemical properties. They differed from other kallikrein-related proteinases in having high pH values of 6.20 (rK2) and 6.85 (rK9). Kallikrein rK2 was purified as a single peptide chain, whereas rK9 appeared as a two-chain protein after reduction. Their enzymatic properties were also very similar and differed significantly from those of other rat kallikrein-related proteinases. Unlike the five other kallikrein-related proteinases we have purified so far, kallikrein rK9 was not inhibited by aprotinin. rK9 also differed from rK2 by its tissue localization. The prostate gland contained only rK9 where it was the major kallikrein-like component.

The amino acids preferentially accommodated by the proteinase S3 to S2′ subsites were identified using synthetic amide and protein substrates. Unlike other kallikrein-related proteinases, rK2 had a prevalent chymotrypsin-like specificity, whereas rK9 had both chymotrypsin-like and trypsin-like properties. Both rK2 and rK9 preferred a prolyl residue in position P2 of the substrate and did not accommodate bulky and hydrophobic residues at that position, as did most of the other kallikrein-related proteinases. This P2-proline-directed specificity is necessary for processing the precursors of several biologically active peptides. Subsites accommodating residues COOH-terminal to the scissile bond were also important in determining the overall substrate specificity of these proteinases. rK2 and rK9 both showed a preference for hydrophobic residues in P2′. Other subsites upstream of the S3 subsite were found to intervene in substrate binding and hydrolysis. The restricted specificity of rK2 and rK9 is consistent with the presence of an extended substrate binding site, and hence with a processing enzyme function. Their P1 specificities enabled both proteinases to release angiotensin II from angiotensinogen and from angiotensinogen I, but rK9 was at least 100 times less active than rK2 on both substrates. The substrate specificities of rK2 and rK9 were correlated with key amino acids defining their substrate binding site. The predicted preferred sequence(s) around the cleavage site deduced from these data may be used to identify the biological substrate(s) of these proteinases.

The protein products of the tissue kallikrein gene family have not all been characterized. This is mainly because of the large number of genes in this family, particularly in the mouse and the rat, and the structural similarity of these genes (1-5). Although many of the kallikrein-related proteinases in the rat have been described, only a few have been correlated unambiguously with their corresponding gene or mRNA (6-9). Tissue kallikrein (rK1) and tonin (rK2), encoded by the genes rKlk1 (formerly rGK1) and rKlk2 (formerly rGK2), were the first to be characterized (6, 7). Four additional kallikreins have recently been characterized by amino-terminal sequence analysis and correlated with genes in the family. These include kallikreins rK7 and rK8, encoded by the the rKlk7 (formerly RSKG7) and rKlk8 (formerly rGK8) genes (8), kallikrein rK9, formerly reported as prostate specific protease (9), SEV (10) or KLP-S3 (11) which corresponds to the so far unidentified rKlk9 gene (8), and kallikrein rK10 (13), which is identical to antigen γ (14) and probably to TGF-kininogenase (15), for which no corresponding gene or mRNA has so far been described.

There is increasing interest in these proteinases because several of them have different substrate specificities, despite their close structural relationship, suggesting that they could serve different biological functions. Kallikrein rK1, for example, is involved in processing kininogen from kininogens (16), whereas rK2 can release angiotensin II from angiotensinogen in vitro (17). We have recently shown that kallikreins rK7, rK8, and rK10 have substrate specificities which differ from those of rK1, particularly for residues in the P2 position (nomenclature of Schechter and Berger (see Ref. 18)), and could be involved in the processing of precursors other than kininogens and angiotensinogen (8, 13).

Kallikrein rK9, which has 84% amino acid identity with rK2 (12), has been reported to be a tonin-like enzyme having vasoconstrictive activity (10). These two proteinases therefore provide an excellent model for investigating structure-func-

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‡ Nomenclature of the genes and proteins of the kallikrein family is according to the proposal of the discussion panel from the participants of the KININ '91 meeting held Sept. 8–14, 1991, in Munich, Germany.
tion relationships in this protease family. We have determined their overall substrate specificity using synthetic amide and ester substrates as well as natural peptide and protein substrates. This specificity has been tentatively correlated with differences in the key amino acids which define the substrate binding site in the members of the kallikrein family.

EXPERIMENTAL PROCEDURES

RESULTS

Purification of Kallikreins rK2 and rK9—A rat submandibular gland extract was fractionated on a DEAE-A50 column equilibrated in 10 mM phosphate buffer, pH 6.35, 0.1 M NaCl. Fractions of the two main peaks of unbound material were assayed for their esterolytic activity using p-tosyl-L-arginine-methyl ester in the presence or absence of excess aprotinin. All fractions contained esterolytic activity, but part of it, under the first peak, was slightly inhibited by aprotinin (Fig. 1S, Miniprint Section). These fractions were pooled and fractionated on a Mono S column equilibrated in 25 mM sodium phosphate buffer, pH 6.0. The unbound material eluted as a single peak of aprotinin-inhibited esterolytic activity. The bound material, which also contained esterolytic activity, was eluted at 0.16 M NaCl and was not inhibited by aprotinin (Fig. 2S, Miniprint Section). The two peaks were concentrated and stored frozen until use.

NH₂-terminal Sequence Analysis—Both proteinases were analyzed for their NH₂-terminal sequence after reduction and pyridylethylation and fractionation on a C4 reverse phase chromatography column. The two peaks obtained after reduction of the Mono S-bound material corresponded to the NH₂-terminal sequence deduced from the nucleotide sequence of rKlk9 mRNA and to a segment starting at residue 90 (numbering based on the kallikrein rK1 sequence (5)) (Table I). These two segments correspond to the light and heavy chains of the molecule, a structural feature found in the related kallikreins rK7, rK8, rK10 (8, 13). According to the rules proposed for kallikrein nomenclature, this protein, which is identical to the prostatic protease (9), SEV (10), and KLP-S3 (11), will be called kallikrein rK9.

The unbound material eluted from the reverse phase chromatography column as a single peak after reduction-pyridylethylation, and its sequence corresponded to that of tonin or rK2.

Molecular Sizes and Isoelectric Points—The presence of one chain for rK2 and two chains for rK9 was confirmed by SDS-PAGE (Fig. 1). Kallikreins rK2 and rK9 appeared to be single chains of identical molecular mass 25 kDa prior to reduction with 2-mercaptoethanol, whereas rK9 had two chains of 20 and 14 kDa and rK2 a single band of 35 kDa after reduction (Table I). This unusual behavior of rK2 after reduction has also been reported for kallikrein rK1 (8).

Kallikrein rK2 and rK9 are probably the two most basic kallikreins present in the rat submandibular gland with pH values of 6.20 and 6.85, respectively, as determined from isoelectrofocusing on thin layer polyacrylamide gels using a pH 3.5–9.5 Ampholine gradient.

Enzymatic Properties—pNPGB-titrated rK2 and rK9 were assayed for their amidolytic and proteolytic activities using a variety of synthetic fluorogenic substrates and native or denatured protein substrates. All the amidase substrates tested, corresponding to different combinations of P1, P2, and P3 residues (listed under "Experimental Procedures," Miniprint Section), were poorly hydrolyzed by both proteinases since most of the kₐ/Kₘ values were below 1 mm⁻¹ s⁻¹. This suggests that either rK2 and rK9 have little amidolytic activity or that subsites other than S1 and S2, and possibly S3, are critical for substrate binding. This possibility was investigated using protein substrates and two peptide substrates with intramolecularly quenched fluorescence, Abz-Phe-Arg-Ser-Arg-EDDnp and Abz-Phe-Arg-Leu-Val-Arg-EDDnp (19). The former was significantly hydrolyzed by both proteinases (Table II) though at a far lower rate than that reported for porcine or horse kallikrein (19). It was sensitive enough, however, to measure the activity of nanomolar concentrations of both proteinases.

Peptides and proteins, including reduced and pyridylethylated lysozyme, oxidized insulin B chain, and also substance P, renin tetradecapeptide substrate (angiotensigen 1–14), and human big endothelin-1 which may be related to the biological activity of these enzymes (both have been reported…
as proteinases with vasoconstrictive activity), were then used
to define the specificity of rK2 and rK9, and discriminate
between their activities. Both proteinases were incubated
under the same experimental conditions with the same
amount of each substrate. The enzyme/substrate molar ratios
varied between 1/15 and 1/23,000, depending on the substrate
used, but were kept constant for both enzymes. Cleavage
products were separated by reverse phase chromatography
on a C18 column and analyzed for their NH2-terminal sequence
to locate the preferential cleavage sites. As shown in Table
III, the two proteinases cleaved most of the substrates used
at different sites, indicating that they have different specific-
ities. Their overall specificity for P3 to P2' residues was
analyzed, taking into account all rK2 and rK9 cleavage sites
identified in the six peptide substrates as well as the frequency
of distribution of individual residues. Amino acids were classi-
cified into six groups as described under “Experimental Pro-
cedures” (Miniprint Section). Results are reported in Fig. 2.
Unlike other members of the kallikrein family, rK2 and rK9
were able to accommodate aromatic residues in position P1.
rK9 differed from rK2 in that it also accommodated basic
residues at that position. Both proteinases accommodated
negatively charged residues in P2 but showed a preference for
a prolyl residue at that position. Bulky and hydrophobic
residues, which are preferentially accommodated in S2 by
kallikreins K1 and several other members of the family, did
not fit the rK2 and rK9 requirements for hydrolysis. Both
proteinases also showed a preference for hydrophobic residues
in P2', which confirms the presence of an extended substrate
binding site on these proteinases.

**Angiotensin II Release from Rat Angiotensinogen and from Angiotensin I**—The results obtained using the renin substrate
tetradecapeptide showed that both enzymes cleaved at the
angiotensin II site. The direct generation of angiotensin II
from its protein precursor was further investigated by radio-
immunoassay using rat angiotensinogen as substrate. Though
both proteinases released angiotensin II but not angiotensin
I from the rat precursor, rK9 was about 300 times less efficient
than rK2 under the same experimental conditions. When
angiotensin I was used as a substrate, rK9 was about 100
times less efficient than rK2 to release angiotensin II as
revealed by reverse phase HPLC analysis on a C18 column.

**Inhibition by Proteinase Inhibitors**—The different suscep-
tibilities of rK2 and rK9 to serine proteinase inhibitors rep-
resent another way of discriminating between these two pro-
teinases. This feature was exploited to complete their purifi-
cation. Table IV shows the results obtained using several
representative serine proteinase inhibitors. Only soybean
trypsin inhibitor was as good an inhibitor of both proteinases,
with apparent Kᵢ values lower than 1 μM. Unlike all other
callikrein-related proteinases reported so far (8, 20), rK2 was
poorly inhibited by aprotinin and rK9 was insensitive to  this
inhibitor. Rat α1-PI, which does not inhibit kallikrein rK1,
binds both rK2 and rK9, although rather slowly. Neither rK2
nor rK9 were significantly inhibited by ovomucoid trypsin
inhibitor, lima bean trypsin inhibitor, or chymostatin.

**DISCUSSION**

In spite of their close structural similarities, with homolo-
gies of 72–89% (5), the proteinases of the rat tissue kallikrein

### Table II

| Substrate                   | Enzyme | Km  | Kcat | kcat/Km |
|-----------------------------|--------|-----|------|---------|
| Pro-Phe-Arg-MCA             | rK2    | 75  | 0.27 | 3.60    |
|                             | rK9    | 160 | 0.25 | 1.56    |
|                             | rK1    |     |      | 171**   |
| Z-Phe-Arg-MCA               | rK2    | 105 | 0.21 | 2.00    |
|                             | rK9    | 50  | 0.05 | 1.00    |
|                             | rK1    |     |      | 57**    |
| Boc-Leu-Ser-Thr-Arg-MCA     | rK2    | 65  | 0.20 | 3.00    |
|                             | rK9    | 48  | 0.06 | 1.25    |
| Boc-Ala-Gly-Pro-Arg-MCA     | rK2    | 61  | 0.22 | 3.60    |
|                             | rK9    | 146 | 0.20 | 1.40    |
| Abz-Phe-Arg-Ser-Arg-EDDnp   | rK2    |     |      | 21.00   |
|                             | rK9    |     |      | 5.90    |
| Abz-Phe-Arg-Leu-Val-Arg-EDDnp| rK2 |     |      | 0.64    |
|                             | rK9    |     |      | 0.28    |

*Ref. 8.

### Table III

**Cleavage sites in various peptide and protein substrates hydrolyzed by kallikreins rK2 and rK9**

| Substrate                             | Kallikrein rK2 | Kallikrein rK9 |
|---------------------------------------|----------------|----------------|
| PE lysozyme                           |                |                |
| Phe Gly Arg PECys Glu                 | 3–7            | Met Lys Arg His Glu 12–16 |
| Arg Gly Tyr Ser Leu                   | 21–25          | Arg Gly Tyr 19–22 |
| Asn Ser Arg Trp Trp                   | 59–63          | Trp Asn Arg Asn Thr 43–47 |
|                                      |                | Asn Ser Arg Trp Trp 59–63 |
|                                      |                | PECys Ala Lys Lys Ile 94–98 |
|                                      |                | Arg Asn PECys Lys 112–116 |
|                                      |                | Trp Ile Arg PECys PECys 123–127 |
| Oxidized insulin B chain              |                |                |
| Val Asn Glu Ala Leu Tyr Gly Glu Arg Gly Phe | 20–24          |
| Rat angiotensinogen                   |                |                |
| His Pro Phe His Leu                   | 6–10           | His Pro Phe His Leu 6–10 |
|                                      |                | His Pro Phe His Leu 6–10 |
| Renin substrate tetradecapeptide      |                |                |
| His Pro Phe His Leu                   | 6–10           | His Pro Phe His Leu 6–10 |
| Big-endothelin-1 (human)              |                |                |
| Val Pro Tyr Gly Leu                   | 29–33          | Val Pro Tyr Gly Leu 29–33 |
| Substance P*                         |                |                |
| Gln Glu Phe Phe Phe Gly               | 5–9            |                |
|                                      |                |                |

* Cleavage sites of substance P by rK2 were obtained from Ref. 32.
family can be relatively easily separated from the same tissue homogenate on the basis of their different electric charges. This was first exploited by Brandtzaeg et al. (21), who used anion-exchange chromatography to fractionate a rat submandibular gland homogenate into four kallikrein-containing pools. DEAE-A50-unbound material was used to purify four kalli-  

kreins to proteinase inhibitors have also been used to achieve their purification. In the present study, rK2 and rK9 were separated during their purification by their different sensi-

titivities to aprotinin. Previously, rat kallikreins rK7 and rK8 were separated by taking advantage of the fact that rK8 is insensitive to soybean trypsin inhibitor (8). Purified rK9 occasionally showed microheterogeneity in some preparations, depending on the starting material and the purification procedure used. Similar microheterogeneity has been reported for kallikrein rK10 (13) and shown to be due in part to alternative cleavage of the initial peptide chain in the region of the kallikrein loop (22), generating the light and heavy chains of this molecule, and to a variable glycosylation of the light chain. Such a process can be expected for kallikrein rK9, which is the only other member of the family to have two potential cleavage sites after Arg residues in this region, one giving rise to an NH2-terminally blocked heavy chain starting with a Gln residue and therefore unidentifiable by sequence analysis (13).

The amino acid sequences of rK2 and rK9 are 84% identical, one of the highest percentages of homology between proteinases of the kallikrein family (12). This is most significant, as this similarity includes the key amino acid residues thought to be involved in the interaction with the substrate (Table IV). As a consequence, the two proteinases could be expected to have similar functions. In accordance with this hypothesis, Yamauchi et al. (10) reported that kallikrein rK9, like rK2 (7, 17), has vasoconstrictive activity in vitro. However, the mechanism by which this activity occurs seems to be different (10). We demonstrated by radioimmunoassay that both proteinases specifically release angiotensin II from its precursor and this was confirmed using a synthetic renin substrate (angiotensinogen fragment 1-14) and angiotensin I which were cleaved at their angiotensin II releasing site. However, the total amount of angiotensin II released from both substrates by rK9 was far lower than that released by rK2 under the same experimental conditions, suggesting a different biologic activity for each proteinase in spite of their similar specificity.

rK2 and rK9 are similarly regulated by androgens, but their tissue localizations differ significantly. Both are found in the submandibular gland, but only rK9 is present in the rat prostate, where it is the main kallikrein-like component. The level of rK9 mRNA in this tissue has been reported to decrease dramatically upon castration (9, 23), whereas it is affected less, and to the same extent as rK2 mRNA, in the submandibular gland (23). Kallikrein-related proteinases have also been identified in the human and dog prostate glands (24, 25). The key amino acids of the human prostate, reported as prostate-specific antigen, differ from those in rat kallikreins rK9 and rK2. In particular Asp183, which is highly conserved throughout the kallikrein family, is replaced by a Ser residue in human prostate-specific antigen (26). Other critical resi-

family can be relatively easily separated from the same tissue homogenate on the basis of their different electric charges. This was first exploited by Brandtzaeg et al. (21), who used anion-exchange chromatography to fractionate a rat submandibular gland homogenate into four kallikrein-containing pools. DEAE-A50-unbound material was used to purify four kallikreins, including kallikreins rK2 and rK9. The other two kallikreins, rK7 and rK8, were purified and characterized as described previously (8). The different sensitivities of kalli-

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titivities to aprotinin. Previously, rat kallikreins rK7 and rK8 were separated by taking advantage of the fact that rK8 is insensitive to soybean trypsin inhibitor (8). Purified rK9 occasionally showed microheterogeneity in some preparations, depending on the starting material and the purification procedure used. Similar microheterogeneity has been reported for kallikrein rK10 (13) and shown to be due in part to alternative cleavage of the initial peptide chain in the region of the kallikrein loop (22), generating the light and heavy chains of this molecule, and to a variable glycosylation of the light chain. Such a process can be expected for kallikrein rK9, which is the only other member of the family to have two potential cleavage sites after Arg residues in this region, one giving rise to an NH2-terminally blocked heavy chain starting with a Gln residue and therefore unidentifiable by sequence analysis (13).

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dues such as His\(^{30}\), Gly\(^{29}\), and Ala\(^{31}\) in rK2 and rK9 are replaced by Ser, Trp, and Gly, respectively, in human prostatespecific antigen, indicating that the proteinases do not have the same specificity and therefore serve different functions.

rK9 can be readily distinguished from the other kallikreins in the rat submandibular gland by its resistance to aprotonin inhibition. There is, as yet, no structural feature which can explain this property of rK9. Both kallikreins rK2 and rK9 are strongly inhibited by soybean trypsin inhibitor, a property they share with rK7 and rK10 (8, 13) but which makes them different from kallikreins rK1 and rK8, which are not susceptible to this inhibitor. The way in which the activities of the kallikrein family proteinases are regulated by physiological protease inhibitors is not yet well understood; only rK1 significantly modifies blood pressure when injected in minute amounts into the circulation (16). Whether or not this is the fact that all the kallikreins except rK1 are inhibited by α1-PI remains questionable, given the low rate at which this reaction occurs \textit{in vitro}. However, an inhibitor of tissue kallikrein which irreversibly binds rat rK1 has been recently described (27). This kallikrein-binding protein corresponds to the negative acute phase reactant SPI-1 (28, 29), but its ability to inactivate other kallikrein-related proteinases remains to be investigated.

The relationship between the structure of kallikreins and their substrate specificity was investigated using a variety of peptide and protein substrates. Unlike other kallikrein proteinases, both rK2 and rK9 hydrolyze amide fluorogenic substrates very slowly, but they are the only two that have chymotrypsin-like specificity in addition to trypsin-like specificity. These two proteinases hydrolyzed the commonly used kallikrein substrates Pro-Phe-Arg-MCA and Z-Phe-Arg-MCA 10–100 times slower than kallikreins rK1, rK7, rK8, and rK10 (8, 13). Intramolecularly quenched fluorogenic peptide substrates were used to study the P1' and P2' specificity of proteinases. Such substrates have been recently developed by Chagas et al. (19) to measure tissue kallikrein activity. One of them, Abz-Phe-Arg-Ser-Arg-EDDnp, was hydrolyzed much faster than methyl-coumarylamine peptide substrates by both rK2 and rK9. However, the main substrate cleavage site, as identified by high performance liquid chromatography analysis, was not at the expected Arg-Ser bond found for rK1 and rK10, but at the amide bond involving the second Arg residue of the peptide substrate. This means that substrates upstream of S1 and S2 in the proteinase are important for defining the unusual specificity of rK2 and rK9. There is also evidence from structural studies that rK2 has a specificity for residues far from the scissile bond (30). The low activity of rK2 and rK9 toward the amide bond of the fluorogenic methycoumarylamide substrates may thus be explained by the reduced length of the peptide chain, which generally has more than 3 residues. Protein substrates are therefore appropriate tools for investigating the substrate specificity of rK2 and rK9, and possibly discriminating between their activities. The P3 to P2' overall specificity of rK2 and rK9, as defined by the cumulative data for the cleavage sites in all the protein substrates used, shows a striking resemblance between the two proteinases. The main difference is in their P1 specificity: rK9 has both trypsin-like and chymotrypsin-like specificity cleaving after aromatic as well as basic residues, whereas rK2 is more strictly chymotrypsin-like, although still able to accommodate basic residues in P1. rK9 is therefore more related to the other members of the kallikrein family. On the other hand, both proteinases preferentially accommodate a prolyl residue in position P2, whereas a bulky, hydrophobic residue is generally preferred by other kallikreins (8, 13). It has been suggested that a proline-directed arginyl cleavage is important in the processing of peptide precursors (31). rK9, which obeys this specificity, could therefore be a good candidate for the processing of such precursors.

The differing specificities of both rK2 and rK9 from those of other members of the family agree well with the unusual structural features of these proteinases, and explain why they cannot accommodate bulky, hydrophobic residues in P2, as do most of the other proteinases of the family. This specificity is thought to be due to the presence of a hydrophobic sandwich between Tyr\(^{30}\) and Trp\(^{29}\) of the kallikreins, which traps hydrophobic P2 residues (22). Kallikreins rK2 and rK9 are the only members of the family to have a glycol residue in position 205, and Tyr93 is replaced by a histidyl residue. The results reported here confirm the importance of these residues for determining the size, shape and ionic character of the substrate binding pocket of kallikreins. The Gly/Ser residue at position 217 in most members of the kallikrein family may also be important for substrate binding specificity. This residue is replaced by Ala in rK2 and rK9, and also in rK7, which has the same histidyl residue in position 93 (5), and has been shown to have a P2 specificity not restricted to bulky, hydrophobic residues (8).

This approach, which allows investigation of the structure-substrate specificity relationship of these two closely related proteinases, may provide a method for elucidating their biological function. However, there is, to date, no evidence that the vasoconstrictor activity reported \textit{in vitro} for both rK2 and rK9 (10, 11, 17) is of biological relevance. With the notable exception of rK1, which is a physiological kinin-releasing enzyme, the function of all other members of the rat kallikrein family also remains to be established. Identifying the physiological substrates of these proteinases therefore represents a considerable challenge.

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Substrate Specificities of Rat Kallikreins rK2 and rK9

EXPERIMENTAL PROCEDURES

A novel fluorescent-based assay was developed for the detection of rat kallikrein 2 and kallikrein 9. This assay was used to measure the substrate specificity of these kallikreins.

Supplementary Material: Protein Products of the Rat Kallikrein Gene Family: Substrate Specificities of Kallikreins 2 (rK2) and 9 (rK9)

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Fig. 15: Aminopeptidase Chromatography on DEAE-A50

The eluted aminopeptidase was applied to a DEAE-Sephadex A50 column (2 x 40 cm), equilibrated with dialysis buffer (10 mM sodium phosphate buffer, pH 7.4, 0.1 M NaCl). The elution was monitored by absorbance at 280 nm. Fractions were collected at a flow rate of 0.5 ml per min. The absorbance at 280 nm was recorded continuously.

Fig. 16: Cationic Aminopeptidase Chromatography on Mono S

The peak obtained by DEAE-A50 chromatography was dialyzed against 25 mM sodium phosphate buffer, pH 7.4. The aminopeptidase activity was assayed as described above in a flow cell of 1 cm width. The aminopeptidase activity of each fraction (0.5 ml) was assayed by incubating the sample with 25 mM sodium phosphate buffer, pH 7.4, and measuring the absorbance at 280 nm. The absorbance at 280 nm was recorded continuously.

Fig. 17: Effect of Inhibitors on Aminopeptidase Activity

The aminopeptidase activity was assayed in the presence of different inhibitors. The inhibitory effect of each inhibitor was determined by measuring the absorbance at 280 nm. The absorbance at 280 nm was recorded continuously.

Fig. 18: Effect of pH on Aminopeptidase Activity

The aminopeptidase activity was assayed at different pH values. The absorbance at 280 nm was recorded continuously.

Fig. 19: Effect of Temperature on Aminopeptidase Activity

The aminopeptidase activity was assayed at different temperatures. The absorbance at 280 nm was recorded continuously.

Fig. 20: Effect of Ionic Strength on Aminopeptidase Activity

The aminopeptidase activity was assayed at different ionic strengths. The absorbance at 280 nm was recorded continuously.
Substrate Specificities of Rat Kallikreins rK2 and rK9

Electrophoretic Procedure.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was carried out at 12%, under reduced conditions with 0.1 M diisopropylfluorophosphate (DFP) added to the gel and stacking gels. The gels were stained with Coomassie Brilliant Blue R-250 and dried. The gel was then scanned using densitometry to determine the amount of protein present in each lane.

Exponentially with Flavoprotein Substrate.

The stability of rK2 and rK9 was assessed spectrophotometrically using a panel of synthetic peptide-MCA substrates. Each substrate was prepared at 1 mM in 50 mM Tris buffer (pH 7.4) and incubated at 37°C with no enzyme or in the presence of 0.1 U of rK2 or rK9 for 30 min. The reaction was terminated by adding the substrate and the absorbance at 340 nm was recorded continuously using a UV/VIS spectrophotometer with a cuvette containing 340 nm and monitored at 340 nm and intensity was measured for 400 min and a 5-s interval-4-s interval solution. The stability of the ketone carbonyl, the activity of both kallikreins towards Pro-Arg-MCA, Z-Phe-Arg-MCA, Boc-Arg-MCA, Boc-Leu-Arg-MCA, Boc-Leu-Pro-Arg-MCA, Boc-Leu-Pro-Arg-MCA with Pro-Arg-MCA, and Boc-Leu-Pro-Arg-MCA with Pro-Arg-MCA was measured using these substrates for 30 min and 30 U of rK2 or rK9 was added to the solution. The absorbance at 405 nm was then determined every 5 min for 400 min and the values determined from linear least squares. The values of K ≈ 400 were also determined under the same conditions as for the following substrates: Boc-Leu-Pro-Arg-MCA, Boc-Leu-Pro-Arg-MCA, Boc-Leu-Pro-Arg-MCA, Boc-Leu-Pro-Arg-MCA, and Boc-Leu-Pro-Arg-MCA.

In order to determine the relative contributions of the two substrates in this study, the following substrates were used to determine the contribution of Ala28-Arg of Boc-Leu-Pro-Arg-MCA and no substrates determined from the values of absorbance at 405 nm and the concentration of Boc-Leu-Pro-Arg-MCA.

Hydrolysis of peptides and proteins substrates.

The peptides and proteins substrates were hydrolyzed with rK2 and rK9 at 37°C in 0.1 M phosphate buffer (pH 7.4) at a final concentration of 1 mg/ml. The reaction mixture without enzymes or proteases was incubated in a shaking water bath at 37°C and aliquots were taken at various time points. The reaction was stopped by adding 50 μl of 1 M NaOH. The absorbance was read at 405 nm using a spectrophotometer.

Reverse Phase HPLC Separation of the Peptides.

The peptides derived from rK2 and rK9 digestion of PE were separated using a C18 reversed phase column using a Biorad-HPLC pump and a Biorad HPLC detector. The elution was monitored at 214 nm using a diode array detector. The peptides were eluted using a linear gradient of 0-60% B over 40 min.

Amino acid sequence analysis.

The amino acid sequence analysis was performed using an Applied Biosystems 47Amino acid sequencer with the chemical and program recommended by the manufacturer. The results were compared to the sequence of rK2 and rK9.

Table 1. Properties of rK2 and rK9 from the analysis of their amino acid content.

| Amino Acid | rK2 | rK9 |
|------------|-----|-----|
| Asp | 15 | 14 |
| Glu | 18 | 18 |
| Arg | 12 | 12 |
| Lys | 16 | 16 |
| Ser | 20 | 20 |

Inhibitor spectra of rK2 and rK9.

The inhibition constants (K_i) were determined as previously reported (44). The inhibition spectra were measured under conditions of varying substrate concentration over the range of 0.1-100 μM. The inhibition of rK2 and rK9 was measured over a range of 10-100 μM substrate. The inhibition constants were determined from the inhibition curve (46).


g = k_i [I] + (1/k_i) [I] i

with k_i being the inhibitory rate constant.

The slope of the graph was obtained by using a non-linear least squares method. The results were then plotted according to Lineweaver and Burk (47).


g = k_i [I] + (1/k_i) [I] i

with [I] being the inhibitor concentration.

K_i = k_i/[I] (1)

K_i = k_i/[I] (2)

*Grimm, D., Honecker, B., Juns, M., Barabas, G., Pfeifer, Z., Rues, B., Moertel, J., and van der Velden, B. (1983) J. Biol. Chem. 258, 11493-11502.*

**Masur, C. and colleagues (1984) J. Biol. Chem. 259, 13051-13061.**