Summary

A series of acetyl-peptidyl-amides containing the amino acid sequence around the Arg-Ser kallikrein cleavage site of bovine kininogen were synthesized and tested for their ability to inhibit both the kinin-releasing activity and the amidase activity of purified human urinary kallikrein. The substrate analogues were competitive inhibitors for human urinary kallikrein and the heptapeptides (P4-P3), hexapeptides (P3-P3), and pentapeptides (P2-P3) gave Kᵣ values of 140, 64, and 18 μM respectively, while the tetrapeptides (P1-P3), tripeptides (P1-P3) and dipeptides (P2-P3) had little or no inhibitory activity. The effective analogues had neither kinin-like nor kinin-blocking activity on the rat uterus either before or after exposure to human urinary kallikrein. The effective human urinary kallikrein inhibitors were further examined for their effect on other serine proteases, including human plasma kallikrein, plasmin, complement components (C1s, C1r), bovine coagulation factors (IIa, IXa, and Xa), elastase, and trypsin. These peptides showed little inhibition of the circulating serine proteases but yielded a Kᵣ for the nonspecific protease trypsin in the μM range. These results should provide the basis for the development of highly specific tissue kallikrein inhibitors to aid in elucidating the in vivo role(s) of tissue kallikreins.

Key Words: kinogen • kinin • complement • coagulation • plasmin

The capacity of urinary kallikrein (a glandular kallikrein EC 3.4.21.35) to release biologically active kinin peptides from kininogen substrates has been known for many years, and during the past decade numerous studies have examined a role of the kallikrein-kinin system in the intrarenal regulation of blood pressure. Recent reports have described other potentially relevant functions of urinary (glandular) kallikrein or kallikrein-like proteases in the processing of prohormones or proenzymes such as preprorenin, proinsulin, atriopeptigen, tissue plasminogen activator, and nerve and epidermal growth factors. In further examination of the biological importance of these findings, it will be necessary to conduct in vitro and in vivo studies with specific kallikrein inhibitors. To date, such inhibitors have not been identified. The kinin analogues available, which have been studied mostly as end-organ antagonists, also have some kinin-like activity, and the protease inhibitors used such as aprotinin, benzamidine, aromatic diamidines, and peptides of arginine chloromethyl ketones are not specific for glandular kallikreins and may possess intrinsic undesirable biological activity such as induction of hypotension. In the present study, we have synthesized a series of substrate analogues based on the amino acid sequence of bovine kininogen around the site at which cleavage first occurs and tested their capacity to inhibit human urinary kallikrein (HUK) as well as a number of other serine proteases with kallikrein-like properties.

Materials and Methods

Peptides were synthesized according to the method previously described for renin inhibitors with p-methyl benzhydrylamine resin (Peninsula Laboratories, San Carlos, CA). L-Valine [2,3-¹H] (ICN Pharmaceuticals, Irvine, CA) was incorporated in the P2' position (see Table 1) as a tracer. Hydrogen fluoride cleavage of the peptides from the solid support was followed by acetic acid extraction, filtration on Sephadex G-15, and isocratic elution from an Ultrasphere ODS column (Beckman, Palo Alto, CA) by high-performance liquid chromatography (HPLC). The resultant peptides satisfied the criteria of purity in amino acids.
acid analysis, HPLC, thin-layer chromatography, and A_{280}/A_{210} ratio analysis.

The HUK was highly purified by ultrafiltration, aprotinin-CH-Sepharose affinity chromatography, and Sephadex G-100 gel filtration. Rat urinary kallikrein was a gift from Dr. Narendara Oza, and porcine pancreatic kallikrein was provided by Bayer (Leverkusen, FRG). Single chain, functionally active, human low-molecular-weight kininogen (LMW-Kg) was purified to apparent homogeneity by QAE-Sephadex chromatography, reverse ammonium sulfate gradient solubilization, phenyl Sepharose chromatography, Sephadex G-200 gel filtration, and removal of the remaining contaminants by passage over Affi-Gel Blue and zinc.

The peptides were examined for inhibition of the capacity of HUK to cleave p-nitroaniline from D-Val-Leu-Arg-pNA (S-2266, Kabi, Stockholm, Sweden) at pH 9.0 and 37°C and to release biologically active kinin from LMW-Kg at pH 7.8 and 37°C. Kinin was determined on the estrous rat uterus calibrated with bradykinin, the concentration of which had been determined by amino acid analysis. Pentapeptide (KKI-4), hexapeptide (KKI-5), and heptapeptide (KKI-6) also were examined to see if they served as substrates for HUK. These peptides were incubated with the enzyme at pH 9.0 and 37°C for 1 hour, and the resultant peptide fragments were analyzed by HPLC.

The KKI-6, KKI-5, and KKI-4 peptides were tested for inhibition of other serine proteases related to glandular kallikreins. The esterase activity of complement components C1s and C1r (provided by Dr. David Bing) was measured with Z-Gly-Arg-SBzl (Enzyme Systems Products, Livermore, CA) as the substrate. The amidolytic activity of human polymorphonuclear leukocyte elastase (provided by Dr. Philip Stone) was measured with Suc-Ala-Ala-Ala-pNA (Sigma, St. Louis, MO) as the substrate. Bovine trypsin (Sigma, St. Louis, MO), human plasmin (Kabi), and bovine coagulation factors IIa, IXa, and Xa (provided by Dr. Bruce Fure) were measured with Z-Lys-SBzl (Vega Biochemical, Tucson, AZ) as the substrate. The chromogenic substrate S-2302 (D-Pro-Phe-Arg-pNA, Kabi) was used for human plasma kallikrein (Kabi). The effect of KKI peptides on the kaolin-activated partial thromboplastin time was examined as described, using serial dilutions of normal plasma to correct plasma prekallikrein deficient plasma (George King Bio-Medical Inc., Overland Park, KS).

Results

The inhibition of HUK by substrate analogue peptides is summarized in Table 1. Results with the chromogenic substrate assay indicate that KKI-4, KKI-5, and KKI-6 are effective urinary kallikrein inhibitors. The tetrapeptide KKI-3 (P1-P3) was the minimum length sequence to elicit any detectable inhibition. In the kinin-generating assay, K, values in the micromolar range were also obtained with KKI-4 and -5. The K, for the heptapeptide (KKI-6) was not determined as mixed inhibition was obtained. A representative Dixon plot (Figure 1) indicates the competitive inhibition of HUK obtained with KKI-5 in the amidolytic and kinin-generating assays. In the latter assay, the rat uterus did

![Figure 1](https://example.com/figure1.png)

**Table 1** Inhibition of the Action of Human Urinary Kallikrein on Synthetic (S-2266) or Natural (Low-Molecular-Weight Kininogen) Substrates by Kinogen Substrate Analogues

<table>
<thead>
<tr>
<th>KKI</th>
<th>P1</th>
<th>P10</th>
<th>P9</th>
<th>P8</th>
<th>P7</th>
<th>P6</th>
<th>P5</th>
<th>P4</th>
<th>P3</th>
<th>P2</th>
<th>P1</th>
<th>P1'</th>
<th>P1''</th>
<th>P2'</th>
<th>P3'</th>
</tr>
</thead>
</table>
| KKI-1 | Ac-Val-Gln-NH₂ | >10³ | —
| KKI-2 | Ac-Ser-Val-Gln-NH₂ | >10³ | —
| KKI-3 | Ac-Ser-Val-Gln-NH₂ | 1240 | —
| KKI-4 | Ac-Phe-Arg-Ser-Val-Gln-NH₂ | 18 | 146
| KKI-5 | Ac-Pro-Phe-Arg-Ser-Val-Gln-NH₂ | 64 | 57
| KKI-6 | Ac-Ser-Pro-Phe-Arg-Ser-Val-Gln-NH₂ | 140 | —

Arrows indicate the cleavage sites of glandular kallikreins
LMW-Kg = low-molecular-weight kininogen
*Not examined
†Yielded mixed inhibition
not respond to substrate analogues at molar concentrations 250-fold greater than the assay range for kinin, nor was kinin itself inhibited by the analogues at these concentrations. Incubation of the analogues with HUK in the relative concentrations used in the kinetic studies did not lead to the development of kininlike activity or to any inhibition of the response to kinin. The HPLC analysis of the substrate analogue inhibitors after incubation for 1 hour with HUK indicated that the peptides probably are cleaved at a single peptide bond. A radioactive peak corresponding to a fragment including P2' (radiolabeled valine) and another unlabeled peak were detected in proportion to the decrease in the size of the peak representing the parent peptide. K_m values on the order of 100 to 200 μM were obtained for these reactions.

The inhibitory effect of KKI-4, KKI-5, and KKI-6 on other arginine esterases was examined with synthetic substrates, and the results are summarized in Table 2. With the exception of trypsin and glandular kallikreins from two other species, these proteases, including plasma kallikrein, showed little inhibition by the peptides examined. The minimal inhibition of the coagulation factors is consistent with the finding that the partial thromboplastin time was not affected by these peptides at a concentration of 60 μM, which indicates no significant inhibition of the intact clotting cascade. In addition, preliminary findings indicate that KKI-5 is a poor inhibitor of angiotensin-converting enzyme in vitro and had no effect on the in vivo pressor response to angiotensin I.

### Discussion

In contrast to previous reports in which sequence analogues of bovine kininogen were examined as kallikrein substrates, the present study examined the capacity of sequence analogues to inhibit the action of human glandular kallikrein on both natural and synthetic substrates. The amino acid sequence of bovine kininogen was used as the model, as an unequivocal sequence using cyanogen bromide-generated fragments or cDNA-derived data, rather than isolated light chain analysis, was not available for human kininogens. The data obtained indicate that the tetrapeptide is a poor inhibitor with a K_m in the mM range, while the pentapeptides, hexapeptides, and heptapeptides exhibited potent inhibition of HUK, especially of its kinin-generating activity (see Table 1). The difference in the K_values obtained in the kinin-releasing assay and in the amidolytic assay may be due to the fact that release of kinin from LMW-Kg requires that kallikrein act at two sites, and the inhibitor may have a multiplier effect.

As indicated in Table 2, the inhibitory analogues had a higher degree of specificity for glandular kallikreins than do the previously described kallikrein inhibitors. Minimal or no detectable inhibition was observed for the activated forms of plasma enzymes in the complement, coagulation, and fibrinolytic systems and for polymorphonuclear leukocyte elastase. It is interesting that human plasma kallikrein, which shares high-molecular-weight kininogen as a natural substrate with glandular kallikrein, was only poorly inhibited by these substrate analogues. This result suggests that human plasma kallikrein has less affinity for the bovine kininogen sequence Ser-Val-Gln (P1'–P3') than for the proposed Ser-Ser-Arg (P1'–P3') in human kininogens. Furthermore, the extension of analogues to P3' seems to provide higher specificity than was seen with Pro-Phe-Arg-CH2Cl and D-Phe-Phe-Arg-CH2Cl. In addition to the lack of inhibition noted for eight other serine proteases (see Table 2), no kinin-like or kinin-blocking activity of these substrate analogues was observed, either with the intact peptides or after their exposure to HUK. The inhibition seen with trypsin (see Table 2) was predicted because of the low substrate specificity of this enzyme.

Thus, the substrate sequence analogues examined here appear to be more highly specific than those previously described and lack product mimicking or inhibitory activities. To clarify the role of glandular

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Species</th>
<th>KKI-4 (μM)</th>
<th>KKI-5 (μM)</th>
<th>KKI-6 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urinary kallikrein</td>
<td>Human</td>
<td>18</td>
<td>64</td>
<td>140</td>
</tr>
<tr>
<td>Pancreatic kallikrein</td>
<td>Rat</td>
<td>—</td>
<td>15</td>
<td>—</td>
</tr>
<tr>
<td>Plasma kallikrein</td>
<td>Human</td>
<td>704</td>
<td>1255</td>
<td>276</td>
</tr>
<tr>
<td>Complement Clr</td>
<td>Human</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Complement Cls</td>
<td>Human</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Factor IXa</td>
<td>Bovine</td>
<td>689</td>
<td>1056</td>
<td>ND</td>
</tr>
<tr>
<td>Factor IIa</td>
<td>Bovine</td>
<td>1065</td>
<td>560</td>
<td>570</td>
</tr>
<tr>
<td>Plasmin</td>
<td>Human</td>
<td>ND</td>
<td>1200</td>
<td>414</td>
</tr>
<tr>
<td>Elastase</td>
<td>Human (PMN)</td>
<td>—</td>
<td>500</td>
<td>—</td>
</tr>
<tr>
<td>Trypsin</td>
<td>Bovine</td>
<td>60</td>
<td>30</td>
<td>39</td>
</tr>
</tbody>
</table>

ND = no inhibition detected. PMN = polymorphonuclear leukocyte. — = not examined.
kallikreins in regulation of blood pressure, renal electrolyte handling, and processing of prohormones and proenzymes, biological stability will also be required. Modulation of amino acid residues in the substrate analogues presented here should permit the synthesis of inhibitors with high specificity and stability. The present study has provided the rationale for further development of specific glandular kallikrein inhibitors.

References
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