Isoelectric Focusing Patterns of Urinary Kallikrein in Dahl Salt-Hypertension Susceptible and Resistant Rats

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SUMMARY Dahl salt-sensitive (S) rats which are susceptible to hypertension have lower urinary kallikrein excretion than salt-resistant (R) rats which are not susceptible. Some physicochemical characteristics of partially purified urinary kallikrein were compared between the S and R strains. The isoelectric focusing pattern of S kallikrein was shifted so that a higher proportion of enzyme was present in isoelectric forms that had higher pi values compared to the pattern for R kallikrein. This strain difference was unique to urinary kallikrein; it was not seen in kallikrein extracted from salivary glands. The isoelectric focusing pattern for R urinary kallikrein could be converted to an S-type pattern by treatment with neuraminidase, which suggests that the differing isoelectric focusing patterns arose from differences in the sialic acid content of the kallikrein. The S kallikrein was slightly more heat-labile than R kallikrein, which was also compatible with the lower sialic acid content of the S enzyme. Tests involving the active site of the enzyme (K_m values, pH curves, and heat of activation) were identical for the S and R strains. It was concluded that the structural differences observed in urinary kallikrein between S and R strains were compatible with strain-specific posttranslational processing of the enzyme. (Hypertension 6: 519-525, 1984)

KEY WORDS • genetic hypertension • kallikrein • salt-susceptible rats

RAT urine contains three esterases that hydrolyze α-N-p-tosyl-L-arginine methyl ester (TAME) (see Figure 2 of reference 1). We have called these esterases Al, A2, and B in their order of elution from diethylaminoethyl (DEAE)-Sephadex. Esterase Al is androgen-dependent and is present only in male rat urine. Esterase B is a kinin-generating enzyme characterized as rat urinary kallikrein by Nustad and Pierce. Esterase A2 is another kinin-generating enzyme with about half the kinin-generating activity of kallikrein, but it is electrophoretically and immunologically different from kallikrein.

Dahl et al. selectively bred rats for susceptibility (S rats) or resistance (R rats) to the hypertensive effect of a high salt (NaCl) diet. S rats excrete less urinary kallikrein than R rats. Recently we have described a kallikrein-binding protein in S rat urine which is probably an inhibitor of kallikrein. In this article we compare the physicochemical characteristics of urinary kallikreins from S and R rats.

Materials and Methods

The S and R rats were bred in our colony from stocks obtained from Dr. Lewis Dahl of Brookhaven National Laboratory in 1972 (R rats) and 1975 (S rats). All were 2 to 3 months old at the beginning of our study.

Arginine esterase activity was measured with the artificial substrate TAME HCl (Sigma Chemical Company, St. Louis, Missouri). Esterase assays were performed by a modified colorimetric method or by a radiometric method that utilized [3H]-TAME (Amerham, Arlington Heights, Illinois) as modified by Margoliou et al. One esterase unit was defined as the amount of enzyme needed to hydrolyze 1 μmol of TAME per minute at 37°C, pH 8.6, in the colorimetric assay.

Rats were housed in metabolism cages and given water but no food during 24-hour collection periods. Urine was collected from 50 rats into flasks containing toluene, and samples with visible fecal contamination were discarded. Several collections were made from the same rats at 3- to 4-day intervals. Pools of S or R urine were stored under toluene at 4°C for 1 to 2 weeks until processed further. Urinary kallikrein from S and R urine was separated from esterases A1 and A2 by DEAE-Sephadex and Sephacryl S-200 as follows. Pools of urine (1-1.5 liters) were filtered and dialyzed at 4°C against four changes of distilled water. Potassium phosphate buffer (0.1 M, pH 7.0) was added to yield a final concentration of 0.01 M phosphate. Then
KCl was added to a final concentration of 0.05 M. The prepared urine was applied directly to a 5 × 30 cm DEAE Sephadex column made by boiling 25 g of DEAE-Sephadex A50-120 (Pharmacia, Piscataway, New Jersey) in 0.05 M KCl, 0.01 M phosphate buffer, pH 7.0.

After the urine had flowed onto the column, the column was washed with two liters of 0.05 M KCl, 0.01 phosphate buffer, pH 7.0, and the wash was discarded. Esterase A1 does not bind to the column and was removed in the wash. Esterase A2 was eluted with 2 liters of 0.2 M KCl in 0.01 M phosphate buffer, pH 7.0 (20 ml fractions), and esterase B (rat urinary kallikrein) was eluted in a linear gradient (20 ml fractions) by using 1 liter of 0.2 M KCl and 1 liter of 1.0 M KCl both in 0.01 M phosphate buffer, pH 7.0. Fractions were assayed for TAME esterase activity by the colorimetric TAME assay, and the appropriate kallikrein-containing fractions were pooled, dialyzed against distilled water, and lyophilized. The sample was dissolved in 0.05 M Tris-HCl buffer, pH 8.0, which contained 0.1 M NaCl and 0.02% NaN₃, and chromatographed on a 2.5 × 90 cm column of Sephacryl S-200 (Pharmacia). Six ml fractions were collected and assayed for TAME esterase activity by the colorimetric method. A single peak of TAME esterase was found, which eluted in the 40,000 molecular weight range. Fractions forming this peak were pooled, dialyzed, and lyophilized. This enzyme was the partially purified kallikrein that was used in the experiments reported here.

Submandibular salivary glands from 10 S or R rats were homogenized in 0.01 M phosphate buffer and extracted with 0.5% sodium deoxycholate as described by Brandtzaeg et al. The sample was dialyzed against 0.2 M KCl in 0.01 M phosphate buffer, pH 7.0, and chromatographed on a 2.5 × 30 cm column of DEAE-Sephadex, as described above for rat urinary kallikrein. The salivary gland kallikrein was partially purified on Sephacryl S-200, as described above for urinary kallikrein.

Isoelectric focusing of 10 to 20 TAME esterase units of rat urinary or rat submandibular gland kallikreins was performed with an LKB 110 ml preparative isolectric focusing apparatus. Ampholine was prepared by mixing ampholine pH 2.5–4 and ampholine pH 3.5–5 (LKB) in a 1:1 ratio. Experiments were run at 4°C for 45 hours with a constant power of 5 W. After the isoelectric focusing, 1.0 ml fractions were pumped from the column, their pH was read at room temperature, and the esterase activity of each fraction was measured by the colorimetric TAME assay.

Kallikrein was treated with neuraminidase as follows. Forty TAME esterase units of urinary kallikrein from female R rats were dissolved in 2.0 ml of water. The sample was divided in half. To one-half was added 0.3 mg bovine serum albumin, 100 µl of 1 M citrate-phosphate buffer pH 5.6, and 120 µl of neuraminidase, 0.85 units/ml in 0.1 M citrate-phosphate buffer, pH 5.6 (neuraminidase was from Clostridium perfringens Sigma Type IX, Sigma Chemical Company, St. Louis, Missouri). To the other half of the kallikrein the same materials were added except that the neuraminidase was omitted and replaced by 120 µl of 0.1 M citrate-phosphate buffer pH 5.6. Both preparations, with and without neuraminidase, were incubated for 5 hours at 37°C.

Neuraminidase was measured by thiobarbituric acid assay for N-acetyl neuraminic acid with sialyllactose (Sigma Chemical Company) used as substrate. The reaction was carried out in 0.1 M potassium acetate buffer at pH 4.5. Rat renal neuraminidase was measured in the supernatant after homogenization in distilled water (5 ml/g of tissue) and centrifugation at 750 g for 15 minutes at 4°C. Protein in this supernatant was determined by the method of Lowry et al. To measure urinary neuraminidase, 2.5 ml of urine was passed through a 9 ml column of Sephadex G-25M (PD-10 prepacked column, Pharmacia), and the desalted protein was lyophilized and reconstituted in 800 µl of water.

Heat denaturation experiments were performed with partially purified urinary kallikrein from S and R female rats. The enzyme was added to 0.2 M Tris-HCl buffer, pH 8.0, which contained 1 mg of bovine serum albumin per ml, and incubated at 65°C for 0 to 30 minutes and then cooled to 4°C. TAME esterase activity was determined by the radiometric assay.

The effect of pH on partially purified kallikrein from S and R female rats was determined by the radiometric assay. Bis-Tris propane buffer (0.2 M) was used in the pH range of 6.5 to 9.5.

The Michaelis constants (Kₘ) for S and R kallikreins from female rats were determined by using the radiometric [H]-TAME assay and by adding unlabeled TAME as necessary to change the substrate concentration. Data were analyzed by the Lineweaver-Burke plot, and straight lines were fitted by the method of least squares.

Results

Figure 1 shows a marked difference in isoelectric focusing patterns between urinary kallikreins isolated from S and R female rats. The S females showed three major peaks focusing at pI's of 3.88, 4.00, and 4.10. The relative sizes of the peaks in the S female pattern were 3.88 < 4.00 < 4.10. In the R female pattern, the same three peaks were present but their relative sizes formed a sequence in the opposite order, namely, 3.88 > 4.00 > 4.10. Besides this difference, the urinary kallikrein from R females had significant esterase activity that focused in the pH range of 3.50 to 3.80, whereas the pattern from S female rats showed no such activity. In essence, then, the whole S pattern appeared shifted to the right, so that it had more activity at higher pl values relative to the R pattern. Isoelectric focusing experiments for urinary kallikrein from S and R female rats were repeated with use of an additional urine pool for each strain. The results were essentially identical to those shown in Figure 1.

Isoelectric focusing experiments were also performed for assessment of urinary kallikrein from male S and R rats. Male patterns also showed a shift to
higher pl peaks in S compared to R rats (Figure 2). The experiments shown in Figure 2 were repeated with an additional pool of urine for each strain. The results were essentially identical to those shown in Figure 2. There was also an effect of sex on the isoelectric focusing patterns within a strain. Patterns from males were shifted to the left, toward lower pH peaks, compared to those from females (compare Figures 1 and 2 for within strains).

Figure 3 shows that it is possible to convert an R urinary-kallikrein isoelectric focusing pattern into an S-like pattern by treatment of the female R kallikrein with neuraminidase. Neuraminidase had a similar effect on urinary kallikrein from male R rats (data not shown). In the experiment shown in Figure 3, a pool of 40 TAME esterase units of urinary kallikrein from R female rats was divided into two aliquots of 20 units each. One aliquot was incubated with neuraminidase, and the other was incubated under identical conditions except that neuraminidase was omitted (see Materials and Methods). Each aliquot, treated or untreated with neuraminidase, then underwent isoelectric focusing. There was no change in the total kallikrein esterase activity of either aliquot during these incubation procedures, but the proportion focusing in each peak changed as shown in Figure 3.

Neuraminidase was undetectable in the urine of either S or R 4-month-old female rats. If whole urine was used, a very high blank value was obtained due to an

**Figure 1.** Isoelectric focusing patterns for urinary kallikrein partially purified from R female rats (upper panel) or S female rats (lower panel). In this and subsequent figures, the activity in each fraction for a given experiment is expressed as a percentage of the highest activity fraction.

**Figure 2.** Isoelectric focusing for urinary kallikrein partially purified from R male rats (upper panel) or S male rats (lower panel).

**Figure 3.** Isoelectric focusing patterns for urinary kallikrein partially purified from R female rats and either untreated (upper panel) or treated (lower panel) with neuraminidase.
interfering substance. This substance had an absorption maximum at 532 nm in the thiobarbituric acid assay and was, therefore, probably 2-deoxyribose. The product produced from sialyllactose (i.e., N-acetyleneuraminic acid) had an absorption maximum at 549 nm. The interfering substance was readily removed by the desalting (PD-10) column. After desalting and lyophilization, an equivalent of as much as 1.25 ml of urine was able to be assayed. When it was incubated for as long as 7 hours at 37°C, no detectable N-acetyleneuraminic acid was produced. The assay would have detected 0.2 nmol of N-acetyleneuraminic acid. In contrast, neuraminidase was easily measurable in the supernates of kidney homogenates from S and R 5-month-old female rats incubated for 15 minutes. There was, however, no strain difference \( (p > 0.1 \text{ by } t \text{ test}) \). The results in nanomoles of N-acetyleneuraminic acid released/min/mg of protein were: 0.65 ± 0.052 for S and 0.75 ± 0.033 for R (mean ± SEM, \( n = 7 \) rats of each strain). Thus, the different isoelectric focusing patterns of S and R urinary kallikrein were not associated with differences in urinary or renal neuraminidase activity.

The isoelectric focusing patterns of the S and R kallikreins obtained from the submandibular salivary glands were found to be identical for the two strains (Figure 4). There were two main peaks in salivary kallikrein from S and R rats with pI values of 4.0 and 3.88. The isoelectric form at a pI of 4.1, which was prominent in S-rat urinary kallikrein, was not seen in the salivary-gland kallikrein of either S or R rats. Thus, the strain differences in kallikrein appeared unique to the urinary enzyme.

Figure 5 shows heat denaturation curves for S and R urinary kallikreins at 65°C, pH 8.0. S urinary kallikrein consistently lost activity faster than R, and the slopes of the regression lines in Figure 5 were significantly different \( (p < 0.005) \) by an analysis of covariance.

Figure 6 gives Arrhenius plots for the effect of temperature between 3° and 37° C on kallikrein TAME esterase activity. The S and R kallikreins yielded essentially superimposable plots. The pH profiles for S and R kallikreins between pH 6 and 9.5 were also essentially identical (figure not shown). There was no peak of activity, but enzyme activity increased progressively from nil at pH 6 to high activity at pH 9.5. Above pH 9.5, TAME was unstable, which precluded further tests at higher pH.

The Michaelis constant \( (K_m) \) for S kallikrein was 222 μM, and for R kallikrein it was 235 μM. These values are close enough to suggest that they are, in fact, identical.

**Discussion**

The DEAE-Sephadex column that was used in these experiments separates total rat urinary TAME esterase activity into three major peaks (Figure 2 of reference 1). Esterase A1 does not bind to the column and is an androgen-dependent enzyme. Esterase A2 elutes with
the carbohydrate side chain(s) that has resulted from graphic runs for S and R rats. The initial reaction rate in the radiometric TAME esterase assay expressed as a percentage of the value at 37°C is plotted on a log scale on the vertical axis vs the inverse of the absolute temperature on the horizontal axis.

Figure 6. Arrhenius plots for urinary kallikrein partially purified from S and R rats. The initial reaction rate in the radiometric TAME esterase assay expressed as a percentage of the value at 37°C is plotted on a log scale on the vertical axis vs the inverse of the absolute temperature on the horizontal axis.

0.15 M KCl; it has modest kinin-releasing activity, but it is immunologically distinct from kallikrein. Esterase B elutes from DEAE Sephadex with 0.37 M KCl, has strong kinin-releasing activity, and has been referred to as rat urinary kallikrein. DEAE separates these three enzymes on the basis of large charge differences between them. We used isoelectric focusing to look at the microheterogeneity, based on small differences in charge within the kallikrein peak as obtained from the DEAE-Sephadex column. It is not possible to be absolutely sure that some minor charged forms of kallikrein were not lost into the other areas of the DEAE column. Samples from S and R rats were, however, handled identically, so that the strain comparisons are valid even if a kallikrein component were lost in the preliminary partial purification procedure to remove other TAME esterases. Care was taken to include fractions wider than both tails of the kallikrein peak from the DEAE column in order to avoid bias of the sample due to separation of forms within the peak. Also, isoelectric focusing experiments were always repeated from several independent DEAE chromatographic runs for S and R rats.

The microheterogeneity seen in glycoproteins with isoelectric focusing is often due to a variable charge on the carbohydrate side chain(s) that has resulted from variable sialic acid content. Isoelectric focusing patterns of glandular kallikreins from pig and dog pancreas, pig and cat salivary glands, dog kidney, and human and rat urine show multiple peaks. This microheterogeneity is due in large part to a variable content of sialic acid. Interestingly, urinary kallikrein from a patient with Bartter's syndrome lacked such microheterogeneity.

The difference in isoelectric focusing patterns between urinary kallikreins from S and R rats is probably due to differences in sialic acid content of the enzymes. This was shown by treating R kallikrein with neuraminidase to remove sialic acid residues. Neuraminidase converted the R-kallikrein isoelectric focusing pattern into an S-like pattern. This S-like pattern still contained three major peaks. The failure of neuraminidase treatment to convert all the isoelectric forms to a single peak could be due to sialic acid residues which were inaccessible to the neuraminidase, or it could be due to other posttranslational protein modifications involving changes in charge.

Heat denaturation profiles are commonly used in genetic studies to detect structural changes in enzyme molecules. The heat denaturation profiles of S and R kallikreins were significantly different. This probably reflects their different sialic acid content. Ikekita et al. have shown with hog pancreatic kallikrein that enzyme with high sialic acid content is more heat-stable than kallikrein with low sialic acid content. This is compatible with the heat stability and isoelectric focusing patterns for S and R urinary kallikreins presented here.

Tests on S and R kallikreins that involved the active site of the enzyme showed no strain difference. These included enzyme activity as influenced by pH, temperature activation, and the $K_m$ values. Ikekita et al. found that sialic acid content of hog pancreatic kallikrein had no influence on pH profiles or $K_m$ values. Lemon et al. showed that porcine submandibular gland kallikrein did not change its specific activity when all the sialic acid was removed. These data are compatible with work on other enzymes, such as sulfatase, aminopeptidase, and phosphomonoesterase, which showed that variations in the carbohydrate moiety have no influence on enzymatic activity. We have previously shown by using partially purified S and R kallikrein that the ratio of kinogenase/esterase activity is identical for the two strains. Thus, S and R kallikreins appear to behave similarly toward natural and artificial substrates.

The isoelectric focusing patterns theoretically could also arise as a consequence of renal damage and, for example, release of neuraminidase into the urine. S rats that are fed normal rat chow (1% NaCl) and that are of the age used in the present work are known to have significant proteinuria compared to R rats; this indicates renal damage in the S rats. There was, however, no neuraminidase detectable in the urine of either S or R rats.

The strain differences found in isoelectric focusing patterns are most likely due to tissue-specific metabolic processing of the carbohydrate side chains of the
kallikrein molecule and probably do not represent differences between the coding of S and R structural genes for the amino acid sequence of glandular kallikrein. On the assumption that the same locus codes for the amino acid sequence in salivary and urinary kallikrein, a mutation at this locus would be expected to show up in both salivary and urinary kallikreins. Salivary gland kallikreins were, however, similar in S and R rats. The S and R difference in isoelectric focusing patterns of urinary kallikrein might also arise from genetically controlled processing of the carbohydrate moiety of kallikrein (so-called processing genes). For two genes, influences the sialic acid content of α-mannosidase are known in the mouse. Moreover, these genes in the mouse were tissue-specific, that is, genetically controlled differences in sialic acid content of mouse α-mannosidase were seen in some tissues and not in others.

In our present work, the method of preparative isoelectric focusing was accurate for documenting subtle differences in kallikrein structure, but it does not lend itself to genetic studies in which the phenotype of large numbers of individual rats had to be determined in genetic cross populations. Although it seems possible that the strain difference in urinary kallikrein is genetically controlled, this can only be established by genetic studies. The main possibilities for altering the sialic acid content of a molecule would be in genetic control of the enzymes mediating either the addition of sialic acid (sialyltransferase) or its removal (neuraminidase). The present data show that renal neuraminidase levels are the same for S and R rats.

At present, the importance of a largely desialylated urinary kallikrein in S rats is obscure. In general, desialylated glycoproteins are more rapidly cleared from plasma than are sialylated glycoproteins. One speculative possibility is, therefore, that any renal kallikrein reaching the circulation would have a shorter half-life in S than R rats. Glandular kallikreins are known to be present in plasma, although much of this may originate from the salivary glands.

On the basis of existing evidence, the lower excretion of urinary kallikrein in S compared to R rats is not likely to be due to a mutation influencing the primary structure (amino acid sequence) of the enzyme. Tests involving the active site showed no strain differences. The structural differences found are best explained by tissue-specific posttranslational processing.

References