Purification and Partial Characterization of Canine Angiotensinogen

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SUMMARY A procedure is described to isolate angiotensinogen (renin substrate) from canine plasma. The isolation procedure resulted in an 800-fold purification with a rate of recovery of approximately 12%. The purified protein has a specific activity of 24 μg of angiotensin I/mg protein. The amino terminal amino acid sequence of canine angiotensinogen was found to be identical to that of the horse but to differ from that of human and rat angiotensinogens. Canine angiotensinogen was heterogeneous with respect to molecular weight and isoelectric point. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of pure angiotensinogen revealed two closely spaced bands with apparent molecular weights of 58,000 and 56,000. Chromatofocusing showed four isoforms: Peaks of pure angiotensinogen eluted at pH levels of 4.32, 4.23, 4.15, and 4.04. Isoelectric focusing confirmed the presence of four isoforms. Thus, the purification procedure identified two molecular weight forms and four isoforms of canine angiotensinogen. Isolation of the four isoforms will allow their characterization and the study of their physiological significance. (Hypertension 11: 21–27, 1988)

KEY WORDS • angiotensinogen • angiotensin • renin

The renin-angiotensin system plays a central role in the regulation of extracellular fluid volume and blood pressure. Angiotensins I and II are generated from angiotensinogen (renin substrate) by enzymatic processing by renin and converting enzyme. Plasma angiotensinogen is a glycoprotein of hepatic origin. Although plasma renin is believed to act on circulating angiotensinogen to generate angiotensin I, recent findings have hinted at a more complex organization of the renin-angiotensin system. Synthesis of angiotensinogen is not restricted to the liver, since messenger RNA for angiotensinogen has been localized in the brain and other organs. We have found a binding site for angiotensinogen in adrenoglo- merulosa cells. Angiotensin II can be generated directly from angiotensinogen by cathepsin G. Finally, angiotensinogen belongs to a superfamily of proteins, most of which are serine protease inhibitors, suggesting that angiotensinogen may act as a protease inhibitor. A variety of experimental approaches are needed to explore these issues, and pure angiotensinogen will be useful in some of these experiments.

Materials and Methods

Adult male mongrel dogs (Vrand, Millville, NY, USA) were anesthetized with pentobarbital and mechanically ventilated. Through flank incisions and retroperitoneal dissection, the renal arteries and veins were ligated and both kidneys removed. Through a femoral artery, a catheter was introduced into the aorta. Since the increase in the concentration of plasma angiotensinogen evoked by bilateral nephrectomy is completed in 8 hours, the dogs were maintained under anesthesia for this period. The animals were then exsanguinated by collecting blood through the femoral artery catheter. Blood was collected in bottles containing Na2 EDTA at a final concentration of 0.2%, and the plasma was separated by centrifugation. All procedures were done in accordance with the guidelines of the Institutional Animal Care and Use Committee.

Hog renin was obtained from Sigma Chemical (St. Louis, MO, USA) and had a specific activity of about 10 U/mg protein.

Assays

Proteins were measured by the method of Bradford, as detailed in the Bio-Rad kit (Bio-Rad Laboratories, Richmond, CA, USA). Bovine serum albumin
(radioimmunoassay grade, Sigma) was used as a standard. Angiotensinogen was determined by enzymatic assay. Samples were incubated at 37°C for 1 hour in 0.1 M citrate buffer, pH 6.5, containing 0.04 U of renin and 2.5 mM phenylmethylsulfonyl fluoride (PMSF). Liberated angiotensin I was measured by radioimmunoassay as described. 12

Purification of Angiotensinogen

The purification procedure consisted of five purification steps.

Step 1. Ammonium Sulfate Precipitation

At 4°C, plasma was diluted with equal volumes of distilled water in the presence of 2 mM PMSF and 5 mM sodium tetrathionate. Silicic acid (Aerosil 200, Degussa, Teterboro, NJ, USA), was added to the mixture, as described by Hilgenfeldt and Hackenthal. 13 Solid ammonium sulfate was then added, and a protein pellet between 35 and 55% saturation was obtained by centrifugation. The pellet was placed inside dialysis tubing and dialyzed for 18 hours against 5 L of water containing 10 mM Na2 EDTA, 2 mM PMSF, and 5 mM sodium tetrathionate. Next, the protein solution was dialyzed for 8 hours against 5 L of the buffer used in the second step.

Step 2. Blue Dextran—Sepharose Chromatography

As described by Tewksbury et al., 14 at 4°C, a blue dextran—Sepharose (Affi-gel blue, Bio-Rad) column (5 x 50 cm) was equilibrated with 0.05 M Tris HCl, pH 8.0, with 0.1 M NaCl, 10 mM EDTA, 2 mM PMSF, and 5 mM sodium tetrathionate. After application into the column, the protein solution was eluted with the same buffer into 16-ml fractions. The fractions with the highest specific activity were pooled and concentrated by ultrafiltration to 50 ml with a PM 30 Amicon membrane (Lexington, MA, USA). The protein mixture was then dialyzed for 18 hours at 4°C against 5 L of the buffer used for the third step.

Step 3. Cellulose-Ion Exchange Chromatography

As described by Tewksbury et al., 14 the protein mixture was introduced at 4°C into a diethylaminoethyl (DEAE)-Sephacel (Pharmacia Fine Chemicals, Uppsala, Sweden) column (5 x 30 cm) equilibrated with 0.05 M Tris HCl, pH 8.0, containing 0.1 M NaCl. Fractions of 15 ml were eluted with the same buffer, and the fractions with the highest specific activity were pooled. They were then concentrated to 50 to 100 ml with a PM 30 Amicon membrane and twice dialyzed against 6 L of distilled water for 8 hours. The protein mixture was aliquoted in tubes containing 12 μg of angiotensin I equivalents and lyophilized.

Step 4. Hydrophobic Interaction Chromatography

A prepacked Phenyl-Superose HR 5/5 column (Pharmacia) was equilibrated at room temperature with 0.05 M ammonium bicarbonate, pH 8.0, with 1.7 M ammonium sulfate. Aliquots (0.5-1.5 mg protein) of the protein mixture obtained from the DEAE-Sephadex chromatography were dissolved in 1 ml of 0.05 M ammonium bicarbonate, pH 8.0, with 1.7 M ammonium sulfate and were injected into the Phenyl-Superose column. Injection and elution of the sample were performed in the fast protein liquid chromatography (FPLC) system (Pharmacia). The column was eluted with a linear descending gradient of ammonium sulfate from 1.7 to 0 M in 0.05 M ammonium bicarbonate, pH 8.0. Fractions of 1 ml were obtained, and all fractions containing angiotensinogen were pooled and concentrated to dryness with a Speed Vac concentrator (Savant Instruments, Farmingdale, NY, USA).

Step 5. Chromatofocusing

The dried fractions containing angiotensinogen from the previous purification step were equilibrated with 0.025 M bis-Tris buffer, pH 6.3, by recovering the pellets with 2.5 ml of this buffer and eluting them with the same buffer through a PD-10 Sephadex 25 (Pharmacia) disposable column. The protein sample was then introduced with the FPLC system into a prepacked chromatofocusing column (Mono P HR 5/20, Pharmacia) equilibrated, at room temperature, with 0.025 M bis-Tris buffer, pH 6.0. A pH gradient was generated by eluting the column with 4% (vol/vol) Polybuffer 74 (Pharmacia), pH 4.0.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Protein samples were incubated at 90°C for 1 minute in 62.5 mM Tris HCl, pH 6.8, with 3% sodium dodecyl sulfate (SDS), 5% (vol/vol) 2-mercaptoethanol and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 5 to 15% gradient acrylamide gels. 15 To visualize proteins, gels were stained with Coomassie brilliant blue R-250 or the silver stain method of Merrill et al. 16

Isoelectric Focusing

The fraction with the highest specific activity from each one of the two peaks of angiotensinogen eluting from the Phenyl-Superose column were pooled. Then, 5 μg of angiotensinogen of this sample was radiiodinated with lactoperoxidase and glucose oxidase (New England Nuclear radioiodination system, Boston, MA, USA). After separation of 125I-angiotensinogen from free iodine by filtration with Sephadex G15 (Pharmacia), 125I-angiotensinogen was analyzed by isoelectric focusing.

Isoelectric focusing gels were prepared as follows: 4.85% acrylamide (ICN Biomedical, Costa Mesa, CA, USA), 0.15% bis-acrylamide (Bio-Rad), 6.33% (vol/vol) Pharmalyte 2.5 to 6.5 pH interval mixture (Pharmacia), 13% (vol/vol) glycerol, and 100 μl N,N'-tetramethylenediamine (Bio-Rad). After deaerating and adding 666 μl of ammonium persulfate (22.8 mg/ml), the solution was placed in glass tubes measuring 10 cm in height. After polymerization, the gels were placed in an electrophoresis apparatus cooled to 10°C. The lower (anode) chamber was filled with 0.01 M iminodiacetic acid, while the upper (cathode) cham-
Canine angiotensinogen was filled with 0.01 M histidine. An aliquot of 125I-angiotensinogen in 150 μl of 30% sucrose was placed on top of a gel. Gels with isoelectric focusing standards (low calibration kit, Pharmacia) were also prepared. Gels were focused at 250 V overnight and at 500 V for 1 hour at the end of the run. The gel containing 125I-angiotensinogen was sliced at 1-mm intervals, and the slices were eluted in individual tubes with 250 μl of 10 mM KCl. The tubes were counted in a gamma counter, and their pH was measured with a conventional pH electrode at 22°C.

Amino Terminal Sequence Analysis
A 500-pmol sample of angiotensinogen was dissolved in 150 μl of 30% trifluoroacetic acid and loaded onto an Applied Biosystems 470A gas phase sequencer equipped with a Model 120A PTH analyzer (Applied Biosystems, Foster City, CA, USA) and sequenced to 15 cycles following directions recommended by the manufacturer.

Results
As shown in Table 1, pure canine angiotensinogen was obtained after an 800-fold purification by the five-step purification procedure. The angiotensin I content of the final preparation was the theoretical maximum of 24 μg/mg protein of pure angiotensinogen. In four runs, the angiotensin I content of the final preparation averaged 23.8 ± 1.2 (SD) μg/mg protein. The elution profiles of the protein mixture through the Affi-gel blue and DEAE-Sephacel columns are shown in Figures 1 and 2, respectively. In both instances, canine angiotensinogen eluted as a single peak. Of note is that, although canine angiotensinogen, like rat17 and human18 angiotensinogen, is an acidic protein (as will be discussed), it eluted from DEAE-Sephacel with an isocratic elution at pH 8.0. Isocratic elution in DEAE-Sephacel resulted in a very broad peak of angiotensinogen, but it increased the purity of the preparation four-fold. Phenyl-Superose (Figure 3) proved very effective in purifying canine angiotensinogen (see Table 1), with excellent recovery of the injected sample (85%). Moreover, Phenyl-Superose separated angiotensinogen into two peaks (see Figure 3), suggesting that this protein contains two forms with different hydrophobicity.

SDS-PAGE analysis of the protein mixture after each of the first four purification steps is shown in Figure 4. Proteins with an apparent molecular weight of approximately 57,000 were successively enriched after each step. The protein sample after Phenyl-Superose chromatography (see Figure 4, Lane 4) re-

Table 1. Purification of Canine Angiotensinogen

<table>
<thead>
<tr>
<th>Step</th>
<th>Protein (mg)</th>
<th>μg Ang I/mg protein</th>
<th>Total Ang I (μg)</th>
<th>Recovery (%)</th>
<th>Purification factor</th>
<th>Purity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nephrectomized dog plasma</td>
<td>72,240</td>
<td>0.03</td>
<td>2110</td>
<td>100</td>
<td>1</td>
<td>0.1</td>
</tr>
<tr>
<td>1. Ammonium sulfate</td>
<td>10,880</td>
<td>0.10</td>
<td>1069</td>
<td>51</td>
<td>3</td>
<td>0.4</td>
</tr>
<tr>
<td>2. Affi-gel blue</td>
<td>261</td>
<td>2.16</td>
<td>564</td>
<td>27</td>
<td>72</td>
<td>9</td>
</tr>
<tr>
<td>3. Diethylamineethyl-Sephacel</td>
<td>33</td>
<td>8.85</td>
<td>292</td>
<td>14</td>
<td>295</td>
<td>38</td>
</tr>
<tr>
<td>4. Phenyl-Superose</td>
<td>1.0*</td>
<td>18</td>
<td>—</td>
<td>85†</td>
<td>600</td>
<td>77</td>
</tr>
<tr>
<td>5. Mono P</td>
<td>0.2*</td>
<td>24</td>
<td>—</td>
<td>100†</td>
<td>800</td>
<td>100</td>
</tr>
</tbody>
</table>

Summary of one of four purification runs. The recovery estimates of Steps 1 through 3 refer to the overall recovery of the initial angiotensinogen. Steps 4 and 5 were performed by injections of protein aliquots into the Phenyl-Superose and Mono P columns run in the fast protein liquid chromatography system.

Ang I = angiotensin I.

*Amount of aliquot.

†The recovery estimates of these two steps refer to the individual recoveries of the angiotensinogen injected into each column.
Figure 2. Elution profile from cellulose-ion exchange chromatography. Protein was loaded on a diethylaminoethyl-Sephascel column in 0.05 M Tris HCl buffer, pH 8.0 (see Materials and Methods for details). Column was run with the same buffer at a flow rate of 150 ml/h and eluted into 15-ml fractions.

Revealed two main, closely spaced bands with apparent molecular weights of 58,000 and 56,000 and a few contaminant proteins. This finding suggests that canine angiotensinogen has two molecular weight forms. To determine whether the different molecular weight forms of angiotensinogen corresponded to the two peaks of this protein eluting from the Phenyl-Superose column, SDS-PAGE analysis of separated samples from these two peaks was performed. As shown in the inset in Figure 3, both molecular weight forms of angiotensinogen were present in both peaks.

In the final purification step, the fractions containing angiotensinogen that eluted from Phenyl-Superose chromatography were chromatofocused in a shallow pH gradient generated by eluting the Mono P column with 4% Polybuffer 74, pH 4.0. As shown in Figure 5, four isoforms of angiotensinogen eluted from the column (arrows). The pH at which the four peaks of angiotensinogen eluted averaged 4.32 ± 0.06 (SE), 4.23 ± 0.06, 4.15 ± 0.06, and 4.04 ± 0.07 (n = 6). Each of these peaks yielded the maximal theoretical content of angiotensin I of pure angiotensinogen (24 μg of angiotensin I/mg protein). Figure 6 shows silver staining of an SDS-PAGE gel of the four isoforms of canine angiotensinogen. Note that the high molecular weight form (58,000) eluted in the four peaks (see Lanes 1–4 in Figure 6), whereas the low molecular weight form (56,000) eluted in the two peaks with higher isoelectric points (see Lanes 1 and 2 in Figure 6).

Although chromatofocusing revealed four isoforms of canine angiotensinogen, the exact isoelectric points of these forms may differ somewhat from their elution pH, because nonspecific interactions with the column may result in elution of the protein at a pH that differs from its isoelectric point. Indeed, isoelectric focusing of purified 125I-angiotensinogen showed four isoforms (Figure 7), but their isoelectric points (4.69, 4.60, 4.54, and 4.48) were about 0.4 pH U higher than the pH at which the four isoforms eluted during chromatofocusing.

The overall recovery of the first three purification steps was 14%. The last two purification steps, performed with prepacked columns and small protein samples, gave excellent rates of recovery: about 85% for the Phenyl-Superose column and about 100% for the Mono P column. From these values an overall rate of recovery for the purification procedure of approximately 12% can be estimated.

Analysis of the NH₂-terminal sequence of the first 15 amino acids of angiotensinogen revealed the following sequence:

1 5 10 15
Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser-Lys

Discussion

The five-step procedure described here allowed the isolation of canine angiotensinogen. This protein was purified from plasma obtained from dogs that had undergone bilateral nephrectomy because nephrectomy increases angiotensinogen concentration in plasma and decreases plasma renin activity. The purification strategy

Figure 3. Elution profile of hydrophobic interaction chromatography. Protein samples in 0.05 M ammonium bicarbonate, pH 8.0, with 1.7 M ammonium sulfate were loaded into a Phenyl-Superose HR 5/5 column equilibrated with the same buffer. Injection and elution of the sample were performed in the fast protein liquid chromatography system. The column was eluted with a linear descending gradient of ammonium sulfate into 1-ml fractions (see Materials and Methods for details). Inset shows sodium dodecylsulfate-polyacrylamide gel electrophoresis analysis of one fraction from each of the two different peaks eluting from the Phenyl-Superose column. Migration of molecular weight standards is shown to the left of the gel. Gel stained with Coomassie blue.
used ammonium sulfate fractionation followed by chromatography with blue dextran-Sepharose and ion-exchange chromatography, two purification steps described by Tewksbury et al. to purify human angiotensinogen and used by Bouhnik et al. to isolate rat angiotensinogen. The final two steps that we used differed notably from other purification procedures and made use of the FPLC system and prepacked columns. Hydrophobic interaction chromatography with Phenyl-Superose proved very effective. Tewksbury et al. have shown that human angiotensinogen binds to Phenyl-Superose, and canine angiotensinogen bound tightly to this gel. This finding indicates that canine angiotensinogen possesses a hydrophobic region that is exposed in 1.7 M ammonium sulfate. Moreover, elution from Phenyl-Superose separated canine angiotensinogen into two peaks, suggesting the existence of two molecular forms of different hydrophobicity. Finally, since angiotensinogen of several species is known to contain several isoforms, chromatofocusing was used as the next purification step. This procedure yielded four distinct peaks containing the maximal theoretical content of angiotensin I of pure angiotensinogen (approximately 24 μg of angiotensin I/mg protein) and showed no contaminant bands when examined by SDS-PAGE (see Figure 6).

The overall purification from plasma was 800-fold with a recovery rate of about 12%. This rate was made possible by the excellent recoveries of the two chromatographic steps used with the FPLC system (Phenyl-Superose and Mono P columns), and despite that, recovery rates of the first three purification steps were, by design and in order to maximize the rise in specific activity, quite low.

Analysis of the NH₂-terminal of canine angiotensinogen revealed the sequence of the first 15 amino acids. Skeggs et al. reported the first 14 amino acids of the amino terminal of horse angiotensinogen, and their sequence was identical to the one reported here. In contrast, angiotensinogens from humans and rat have a different NH₂-terminal amino acid sequence. In humans, the 11 amino acid is a valine residue, and in the rat, the 12 amino acid is a tyrosine. Thus, although the first 10 amino acids of angiotensinogen (i.e., angiotensin I) are conserved in many mammalian species, variability in the sequence is frequently found after Position 10.

When analyzed by SDS-PAGE, canine angiotensinogen showed two closely spaced bands with apparent molecular weights of 58,000 and 56,000. Hence, canine angiotensinogen appears to have a molecular weight similar to that for angiotensinogen purified from hog, humans, and rat. A previous estimation of the molecular weight of canine angiotensinogen yielded a value of 80,000, but the method used for determination was gel filtration and the preparation studied contained 2 μg of angiotensin I/mg of protein (about 10% angiotensinogen).

Two closely associated molecular weight forms of angiotensinogen have also been found in humans and rat. Studies by Campbell et al. in hepatocytes and hepatoma cell lines suggest that variable glycosylation is the basis of the molecular weight heterogeneity of angiotensinogen. Moreover, the molecular weight heterogeneity of angiotensinogen does not appear to be due to variations in its amino acid sequence, since this protein appears to be encoded by a single gene in the rat and human genomes.

Chromatofocusing and analytical isoelectric focusing revealed four isoforms of canine angiotensinogen. Isoelectric heterogeneity has also been found in rat, human, and rabbit angiotensinogens. While both chromatofocusing and isoelectric focusing showed four isoforms of canine angiotensinogen, the isoelectric points obtained by the latter technique were considerably higher than the pH at which angiotensinogen eluted from the Mono P column and more in agreement with values obtained in other species. This finding indicates that during chromatofocusing the different isoforms of angio-
Figure 5. Chromatofocusing. Protein samples in 0.025 M bis-Tris buffer, pH 6.3 (see Materials and Methods for details), were loaded into a Mono P column equilibrated with the same buffer. Injection and elution of the sample were performed in the fast protein liquid chromatography system. The column was eluted with a pH gradient generated by a 4% solution of Polybuffer 74, pH 4.0, into 1-ml fractions. Isoforms are indicated by arrows.

Figure 6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of angiotensinogen peaks from chromatofocusing. Samples containing 0.11 µg of angiotensin I (5 µg of angiotensinogen) from the four peaks of angiotensinogen of the Mono P elution were prepared as described in Materials and Methods and subjected to SDS-PAGE. Lane 1. Sample from peak eluting at pH 4.32. Lane 2. Sample from peak eluting at pH 4.23. Lane 3. Sample from peak eluting at pH 4.15. Lane 4. Sample from peak eluting at pH 4.04. Migration of molecular weight standards is shown to the left of the gel. Gel stained with silver stain.

Figure 7. Isoelectric focusing profile of 125I-angiotensinogen. Electrofocusing was conducted in acrylamide gels, as detailed in Materials and Methods. The pH was measured in 1-mm slices eluted in 10 mM KCl. Angiotensinogen eluted at a lower pH than their isoelectric points.

The isoelectric distribution of the two molecular weight forms of canine angiotensinogen closely resembles that of rat angiotensinogen. The higher molecular weight angiotensinogen (58,000) showed more acidic isoelectric points than the lower molecular weight angiotensinogen (56,000). The exact origin of the isoelectric heterogeneity of angiotensinogen remains unresolved. Although isoelectric heterogeneity of glycoproteins is frequently due to differences in sialic acid content, neuraminidase treatment of angiotensinogen has been reported both to decrease and to have little effect on its isoelectric heterogeneity.
The particular functions of the different molecular size and isoelectric forms of angiotensinogen are unknown. Since the purification procedure described here allows separate isolation of four isoelectric forms, it may provide a necessary initial step to the study of their function.

Acknowledgments

I thank Dr. Steven Birken and Mary Ann Gawinowicz for the sequence analysis. Peter Wynne provided technical assistance, and Margaret Coleman provided patient secretarial assistance.

References

Purification and partial characterization of canine angiotensinogen.
J A Oliver

Hypertension. 1988;11:21-27
doi: 10.1161/01.HYP.11.1.21
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1988 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

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