Angiotensin-Producing Serum Enzyme II
Formation by Inhibitor Removal and Proenzyme Activation

ERWIN HAAS, LAVERA LEWIS, PAUL SCIPIONE, T. JOHN KOSHY, ANITA U. VARDE, AND LEA RENERTS

SUMMARY A highly active angiotensin-producing enzyme (enzyme II) was obtained from dog serum by acid treatment and fractionation to remove angiotensinase and converting enzyme, separate an inhibitor, and convert an inactive precursor (proenzyme II) to enzyme II. Proenzyme II was found to be converted to enzyme II by an endogenous activating enzyme identified as plasmin. Conversion was also caused by the interaction of bacterial streptokinase with human proactivator, by trypsin, and by an activator formed from liver tissue extract and dog serum. Neither plasma kallikrein nor the labile, human extrinsic tissue-type plasminogen activator induced activation. The inhibitor, which normally blocks the activation of proenzyme II, was unusually stable against high temperatures and extremes of pH, and it was not identical to any of the six known protease inhibitors of serum. Enzyme II was not identical to other angiotensin-producing enzymes such as enzyme I, renin, cathepsin D, pepsin, plasmin, tonin, or cathepsin G. Enzyme II reacted maximally at pH 4.7 and produced up to 2250 ng of angiotensin I/ml serum/hr from the substrate of dog serum (i.e., amounts 3200-fold higher than that produced by endogenous renin of normal dog serum). Since at pH 7.2, angiotensin I formation is still about 30 times higher than that of renin, enzyme II may be physiologically active under some conditions. (Hypertension 7: 938-947, 1985)

KEY WORDS • angiotensin I • serum • plasminogen • plasmin • prorenin • renin • kallikrein • activators • inhibitors • substrate

In the past few years we have observed two highly active angiotensin-producing enzymes different from renin: enzyme I, present in the serum of dogs, guinea pigs, rabbits, and rats after immunization with renin from the kidneys of hogs, cattle, dogs, rabbits, and humans,1 and enzyme II, obtained from the serum of normal dogs and in a higher concentration from the serum of nephrectomized dogs.2-4

Enzyme II is normally present in serum as an inactive precursor referred to as proenzyme II and is accompanied by its substrate, by an endogenous activating enzyme, and by an inhibitor of the activating enzyme. The purpose of the present study was not only to clarify the mechanisms involved in the activation of proenzyme II but also to establish optimum conditions for the formation of enzyme II as a prerequisite for its future isolation. We also attempted to ascertain that proenzyme II and enzyme II were distinct from endogenous prorenin, renin, or other known angiotensin-producing enzymes.

Materials and Methods

Highly purified human plasmin (specific activity = 21 CTA [Community for Thrombolytic Agents] units/mg protein) was provided by Dr. Daniel H. Liu (Rochester, MI, USA). Dr. Jack V. Pierce (Bethesda, MD, USA) and Dr. H. E. Karges (Behringwerke Marburg, West Germany) provided specimens of human plasma kallikrein; specimens from Dr. Karges had a specific activity of 5000 mU/mg protein. Cathepsin D, from bovine spleen, in lyophilized powder form (specific activity = 12 units/mg protein); human thrombin (specific activity = 3000 NIH [National Institutes of Health] units/mg protein); prothrombin, which was purified 2000-fold from bovine plasma; and streptokinase (specific activity = 48,000 units/mg protein) were purchased from Sigma Chemical Company (St Louis, MO, USA). Pepsin was obtained from hog.
stomach and had been crystallized three times (Nutritional Biochemicals Corp., Cleveland, OH, USA). Trypsin, which was obtained from bovine pancreas and had been twice crystallized, soybean trypsin inhibitor, and lima bean trypsin inhibitor were obtained from Worthington Biochemicals Corp. (Freehold, NJ, USA). Aprotinin (TrasyloL) was obtained from FBA Pharmaceuticals, Inc. (New York, NY, USA); benzamidine HCl was obtained from Calbiochem-Behring Corp. (San Diego, CA, USA).

The γ-globulin fractions of rabbit antisera prepared against α2-macroglobulin or against human plasminogen were products of Dako-Immunoglobulins, Ltd., Denmark. Synthetic human angiotensin I, synthetic tetradecapeptide renin substrate, and pepstatin were obtained from Beckman Biological and Fine Chemicals Division (Palo Alto, CA, USA). We are indebted to Dr. D. C. Rijken (Glaubius Institute, Leiden, The Netherlands) for a sample of purified human tissue-type plasminogen activator, which had been prepared according to the methods of Rijken and Collen.5

Fractionation Procedures

The serum of 15 adult dogs (10 female, 5 male; mean weight, 21 kg; range, 16–27 kg) that had undergone bilateral nephrectomy 2 days earlier was used to prepare proenzyme II, enzyme II, substrate, activating enzyme, and an inhibitor of the activating enzyme. After the dog had been sedated with acepromazine maleate and anesthetized with sodium pentobarbital (Nembutal), blood was collected through a cannula in a carotid artery. After 2 hours at room temperature, the serum was separated by centrifugation at 0 to 5 °C and then stored at −15 °C until processing from 1 day to 28 months later.

To inactivate angiotensinase and converting enzyme,6 300 ml of serum was cooled to 0 °C and acidified to pH 2.5 with rapid stirring by the dropwise addition of approximately 12 ml of 2.5 N HCl. After 2 hours without stirring, the solution was adjusted to pH 6.4 with 8.2 ml of 2.5 N NaOH and stored for at least 1 day at −15 °C.

To obtain proenzyme II, 1.5 ml of 2.5 N HCl was added to the treated serum to adjust the pH to 5.4, 93 g of solid ammonium sulfate was slowly added to obtain a 1.9 M solution, the solution was stirred for 15 minutes, and then centrifuged for 10 minutes at 20,000 g, all performed at 25 °C. The resulting precipitate was suspended in 40 ml of water and dissolved during dialysis for 16 hours at 4 °C against distilled water. The solution (102 ml) was adjusted to pH 7.2 and 1% NaCl, stored frozen, and clarified by centrifugation; 0.35 ml of this proenzyme II preparation represents 1.0 ml of the original dog serum.

From the supernatant solution (321 ml containing 1.9 M ammonium sulfate) obtained after the separation of this precipitate, the inhibitor was precipitated and separated by adding 63 g of solid ammonium sulfate to 3.1 M, stirring for 15 minutes at 25 °C, and then centrifuging for 10 minutes at 20,000 g. This precipitate was suspended in 24 ml of water and dissolved during dialysis, and the resulting solution (128 ml) was neutralized and adjusted to 1% NaCl; 0.5 ml of this inhibitor solution represents 1.0 ml of the original dog serum.

The serum of seven normal (nephrectomized) dogs and human, bovine, and hog serum were processed by similar methods, including acid treatment and ammonium sulfate fractionation, in an attempt to obtain proenzyme II and inhibitor preparations from these species. The 0 to 1.9 M ammonium sulfate fraction from dog serum contains the proenzyme II and the activating enzyme, and since it was free of the inhibitor, enzyme II could be produced. In contrast, the same ammonium sulfate fraction from human, bovine, or hog serum still contains a rather high concentration of the inhibitor (discussed in Results); therefore, it was not possible to demonstrate the presence of proenzyme II or its activation to enzyme II for these three species.

Activation of Proenzyme II

For the endogenous activation to enzyme II, the proenzyme II preparations were stored at −15 °C (cryoactivation), or incubated at 38 °C in 0.05 M phosphate buffer, pH 7.2, for various periods (enzymatic activation), as indicated in Results.

The activation of proenzyme II was investigated using the following enzymes at concentrations normally available from serum or plasma: human plasmin at 3.4 CTA units/ml serum, prothrombin at 1 U/ml, thrombin at 250 NIH units/ml, and plasma kallikrein at 100 μg/ml serum.

An activator of proenzyme II was obtained by the interaction of a dog liver extract and dog serum. One hundred grams of minced liver tissue, which had been stored at −15 °C, was extracted twice, each time with 100 ml of water, by stirring for 15 minutes at 38 °C, continued incubation for 45 minutes at 38 °C, and centrifugation for 5 minutes at 20,000 g. The combined supernatant solutions (2.2 ml represents 1.0 g of the liver tissue) was stored frozen and clarified by centrifugation and filtration. The serum of three nephrectomized dogs was incubated with this aqueous extract (0.11 ml = 50 mg liver/ml serum) for 4 hours at 38 °C and pH 7.8. It was acidified for 2 hours at 0 °C, pH 2.5 (to remove angiotensinase), precipitated with 1.9 M ammonium sulfate (at 25 °C and pH 5.4), dialyzed against distilled water, neutralized, adjusted to 1% NaCl, and clarified by centrifugation; 0.6 ml of this activator preparation represents 1.0 ml of the original dog serum and 50 mg of the original liver tissue. This activator was obtained only by incubating the aqueous liver extract with the serum of nephrectomized dogs; it was not obtained when the liver extract was processed without the serum or with the pooled serum of eight normal, nonnephrectomized dogs.

An efficient activator of proenzyme II also was obtained (see Results) by preparing the human plasminogen activator according to Müllertz:7 human proactivator (human serum euglobulin, 9 mg/ml) was incubated with streptokinase (200 U/ml) for 1 hour at 38 °C in 0.05 M phosphate buffer, pH 7.2.
Assay Procedures

Enzyme II

The assay of enzyme II is based on the rate of angiotensin I formation with the endogenous renin substrate that had been precipitated together with proenzyme II from the serum of the nephrectomized dogs (Figure 1). After incubation for 1 to 2 hours at 38 °C in 0.05 M acetate buffer (pH 4.7), 5 × 10⁻³ M phenylmercuric acetate, and 1 × 10⁻² M dipropyl fluorophosphate (total volume = 2.0 ml), the solution was cooled to 0 °C, diluted with 1 ml of saline, 0.9% NaCl, and adjusted to pH 4.7. Angiotensin I was assayed by the pressor action induced in the pentolinium-treated, anesthetized rat8 or by radioimmunoassay with the ¹²⁵I-labeled angiotensin I kit (New England Nuclear, Boston, MA, USA) using synthetic angiotensin I (Beckman) as a standard. A unit was defined as the amount of enzyme II that produced 1 ng of angiotensin I per hour at pH 4.7. Blanks were determined and subtracted in every assay, so that the point at the y axis was always an experimental point too. In five preparations of proenzyme II, these blanks ranged from 8 to 26 U of enzyme II per milliliter of serum.

Substrate was not limiting in the assay of enzyme II (Figure 1), since 1) the rate of angiotensin I formation remained constant during the 100-minute incubation, while the substrate concentration fell from the original level of 1.8 to 0.5 μM; and 2) the addition of exogenous substrate to raise its concentration about twofold increased the rate of angiotensin formation only about 10% (from 579 ng/hr/ml serum to 639 ng/hr/ml serum). Thus, for incubation periods of from 1 to 2 hours (Figure 1) the concentration of enzyme II, and not of substrate, was rate-determining.

The pressor product of the action of enzyme II is the decapeptide angiotensin I, and not the octapeptide angiotensin II, as shown by three criteria:

1. The radioimmunoassay uses antibodies specific for angiotensin I with less than 0.008% of cross-reactivity to angiotensin II according to information supplied by the manufacturer, New England Nuclear.
2. The angiotensin I values obtained by this radioimmunoassay closely correspond to those from bioassay in the rat (coefficient of correlation = 0.9988).
3. The product can be identified using the countercurrent distribution system of Skeggs et al. After 15 transfers in the system sec-butyl alcohol/0.05 M sodium phosphate (0.1 M NaCl; pH 7.0), 65% of the pressor activity was found in tubes 4 to 9 and only 18% in tubes 0 to 3, a distribution corresponding to that of angiotensin I.

Substrate of Enzyme II

The experimental conditions for the assay of enzyme II (Figure 1) also served to determine the concentration of the substrate. The incubation at 38 °C and pH 4.7 was varied between 4 and 8 hours, depending on the concentration of the endogenous enzyme II, to achieve the complete conversion of the substrate to angiotensin I. In five proenzyme II preparations, after conversion by enzyme II, the substrate was equivalent to a mean angiotensin I concentration of 1520 ng/ml serum (range, 566–2400 ng/ml serum). Thus, essentially identical concentrations of substrate were obtained by both procedures, which suggests that enzyme II substrate is identical to renin substrate. This was confirmed by the results of sequential incubations (i.e., no more angiotensin I was formed when renin was added to a substrate preparation that previously had been completely digested by enzyme II).

Enzyme I, Dog Renin, Cathepsin D, and Pepsin

Enzyme I, dog renin, cathepsin D, and pepsin were assayed by measuring the angiotensin I formed when they were incubated for 10 minutes to 16 hours with dog serum protein renin substrate (angiotensinogen) or tetradecapeptide renin substrate at 38 °C, pH 4.7, in the presence of phenylmercuric acetate and dipropyl fluorophosphate. Similar conditions for the assay of these four enzymes had been employed previously with protein renin substrate from hog plasma and with tetradecapeptide renin substrate.1

A unit of enzyme I or II was defined as the amount of enzyme that produced 1 ng of angiotensin I per hour. A unit of renin, the Goldblatt or International unit, is the quantity that, when injected intravenously or intra-arterially into an unanesthetized, trained dog, raises the direct mean femoral artery blood pressure 30 mm Hg.10

The substrate for the assay of enzyme I, renin, cathepsin D, and pepsin, prepared from the serum of bilaterally nephrectomized dogs, was treated (2 hr at 0 °C and pH 2.5) to inactivate angiotensinase and converting enzyme and then subjected to dialysis and lyophilization. Dog renin produced angiotensin I at the same rate with each of two substrates prepared either by lyophilization or by fractionation with ammonium sulfate.8
Inhibitors of Activating Enzymes

The effect of inhibitors in blocking activation of proenzyme II to enzyme II was determined by activating the proenzyme II by incubation for 6 hours at 38 °C and pH 7.2 in the presence of various concentrations of each inhibitor. The enzyme II produced was determined subsequently (as in Figure 1) from the concentration of angiotensin I produced during incubation for 1 hour at 38 °C, pH 4.7. For example, the assay of the inhibitor from normal human serum is shown after logarithmic transformation (Figure 2). This method has the advantage of yielding a straight-line plot with a correlation coefficient of —0.995, which relates to the linear transformation of the data. The concentration of the inhibitor required for 50% inhibition of the activating enzyme (ID₅₀) was calculated 1.2 mg protein/ml (Figure 2). In contrast, the inhibitor had no effect on enzyme II, even at 26 mg/ml (i.e., at concentrations normally present in the serum of humans or dog).

Interference with the Bioassay of Angiotensin I

The intravenous injection of human plasma kallikrein or antibody γ-globulin to human plasminogen or α₂-macroglobulin caused a profound fall of the rat’s blood pressure. Therefore, angiotensin I could not be assayed by its pressor action in the rat in the studies concerned with the attempted activation of proenzyme II by kallikrein or inhibition by the antibody γ-globulins. The radioimmunoassay of angiotensin I was employed instead.

Results

Activation of Proenzyme II

Endogenous Activation of Proenzyme II

Considerable amounts of angiotensin-producing enzymatic activity appeared spontaneously during storage in some samples of fractionated serum containing proenzyme II. Thus, proenzyme II preparations were obtained from the serum of five dogs (Dogs 1–5) 2 days after bilateral nephrectomy. Storage at −15 °C, (cryoactivation) for 0.5 to 4 months resulted in activation and conversion to enzyme II (mean concentration [±SE] = 716 ± 84 U/ml serum; range, 600–1050 U/ml serum). These five proenzyme preparations apparently contained sufficient plasmin to permit activation at low temperature.

Other proenzyme II preparations were not activated by frozen storage even up to 26 months, but they could be activated by subsequent incubation at 38 °C and pH 7.2. Thus, the serum of eight nephrectomized dogs (Dogs 6–13) was processed to obtain proenzyme II preparations, and these were activated by storage at −15 °C for 0 to 26 months followed by incubation at 38 °C for 2 to 48 hours. Mean concentration of enzyme II was 724 ± 223 U/ml serum (range, 350–2250 U/ml serum). The serum of a nonnephrectomized dog also was collected by exsanguination under anesthesia and processed to yield 228 U of enzyme II per milliliter of serum. Only small samples of blood were taken from seven normal, intact, unanesthetized dogs (Dogs 14–20); mean concentration of enzyme II was 155 ± 25 U/ml serum (range, 110–196 U/ml serum).

The complementary effects of long-term cryoactivation at −15 °C and short-term heat activation at 38 °C are illustrated in Table 1 for the proenzyme II preparation obtained from serum of Dog 11. Without cold storage, activation for 48 hours at 38 °C was required to achieve complete conversion to enzyme II (Table 1). In contrast, after frozen storage for 17 and 26 months, the subsequent incubation at 38 °C required for full activation could be shortened to 8 hours and 2 hours, respectively.

Proenzyme II and enzyme II appear to be stable at 38 °C, since no loss was observed by prolonging the incubation of proenzyme II from 18 to 48 hours or by extending the incubation of enzyme II from 8 to 18 hours (Table 1).

Activation of Proenzyme II by Five Procedures

Proenzyme II from five nephrectomized dogs, which had not been stored frozen and was recalcitrant to endogenous activation, could be activated by four other procedures using physiological levels of plasmin, plasmin generated by plasminogen activator, trypsin, or an activator from dog liver–dog serum (Table 2). As a result of prolonged incubation enzyme II was slowly degraded by the plasmin (72% loss in 30 hr at 38 °C, pH 7.2). Separate incubation either with euglobulin or
Activation of Proenzyme II in the Presence of the Endogenous Activating Enzyme

Serum Inhibitor

The incubation of proenzyme II with the dog liver-dog serum activator, and in 6 hours at 38 °C and pH 7.2 it produced 306 U of enzyme II per milliliter of serum. Since this amount is within the range (278-388 U/ml serum) of the five proenzyme II preparations after fractionation to remove the inhibitor (Table 2), this method apparently circumvents the inhibitor.

No Activation of Proenzyme II by Plasma Kallikrein

Because various investigators have observed that inactive prorenin is converted to enzymatically active renin by low concentrations of kallikrein (6-8 µg/ml),11,12 we investigated whether plasma kallikrein also participates in the endogenous activation of proenzyme II. Our results indicate that the endogenous activating enzyme is not plasma kallikrein for the following reasons:

1. The normal concentration of plasma kallikrein is about 30 µg/ml,13 but three different preparations of authentic, highly purified plasma kallikrein failed to activate proenzyme II even when added at 110 µg/ml.

2. Plasma prekallikrein is readily activated to kallikrein by 20% acetone in 4 hours at 24 °C, and pH 7.5,14 while proenzyme II was not activated to enzyme II under the same conditions.

3. Precipitation with casein activates serum prekallikrein to kallikrein,15 but it failed to activate proenzyme II.

4. Lima bean trypsin inhibitor, 50 µg/ml, completely blocked the endogenous activating enzyme, while plasma kallikrein retained its full activity, even at a 1000-fold higher concentration of the inhibitor.16

Human tissue-type plasminogen activator is normally synthesized in the vascular wall and released into the circulation, where it becomes proteolytically active only after binding to fibrin.2 Its normal plasma level of about 7 ng/ml can be increased more than tenfold after numerous in vivo stimuli such as stress, exercise, shock, and venous occlusion.17 The addition of even larger amounts (30-300 ng/ml of serum) failed to activate proenzyme II, even after incubation for 6 hours at 38 °C and pH 7.2.

As another indication of their nonidentity, the endogenous activating enzyme was inhibited at rather low concentrations, 1.7 µg/ml of aprotinin or 9 µg/ml of soybean trypsin inhibitor (Table 3). In contrast, t-PA lost no activity even when these inhibitors were added at 1000 µg/ml.17

The concentration of blood clotting proteins was investigated in two proenzyme II preparations from the serum of nephrectomized dogs, which after activation contained 536 U and 2250 U of enzyme II per milliliter of serum, respectively. Direct assays of these two proenzyme II preparations in Dr. Oscar D. Ratnoff's laboratory (Department of Medicine, Case Western Reserve University, Cleveland, OH, USA) yielded the following values, where 100% represents the titer in pooled normal human plasma: Hageman factor, 32% and 1.2%; factor XIIa, 1% and less than 1%; plasma

**TABLE 1. Endogenous Activation of Proenzyme II (from Dog II) During Frozen Storage and After Incubation at 38 °C**

<table>
<thead>
<tr>
<th>Storage at -15 °C (mo)</th>
<th>Activation at 38 °C, pH 7.2 (hr)</th>
<th>Enzyme II (U/ml serum)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>26</td>
</tr>
<tr>
<td>0</td>
<td>6</td>
<td>23</td>
</tr>
<tr>
<td>0</td>
<td>18</td>
<td>20</td>
</tr>
<tr>
<td>0</td>
<td>48</td>
<td>673</td>
</tr>
<tr>
<td>17</td>
<td>0</td>
<td>268</td>
</tr>
<tr>
<td>17</td>
<td>1</td>
<td>454</td>
</tr>
<tr>
<td>17</td>
<td>2</td>
<td>576</td>
</tr>
<tr>
<td>17</td>
<td>8</td>
<td>671</td>
</tr>
<tr>
<td>17</td>
<td>18</td>
<td>690</td>
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<tr>
<td>26</td>
<td>0</td>
<td>338</td>
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<td>26</td>
<td>1</td>
<td>661</td>
</tr>
<tr>
<td>26</td>
<td>2</td>
<td>744</td>
</tr>
<tr>
<td>26</td>
<td>8</td>
<td>703</td>
</tr>
</tbody>
</table>

**TABLE 2. Five Procedures for the Enzymatic Activation of Proenzyme II to Enzyme II**

<table>
<thead>
<tr>
<th>Activation of proenzyme II at 38 °C</th>
<th>Mean enzyme II formation (U/ml serum)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hr</td>
<td>pH</td>
</tr>
<tr>
<td>6</td>
<td>7.2</td>
</tr>
<tr>
<td>6</td>
<td>7.2</td>
</tr>
<tr>
<td>4</td>
<td>4.7</td>
</tr>
<tr>
<td>6</td>
<td>7.2</td>
</tr>
</tbody>
</table>

Range, from serum samples of five dogs, is in parentheses.

with streptokinase alone failed to activate proenzyme II. Thus, the interaction of bacterial streptokinase with human proactivator (egulobulin) was required to generate the plasminogen activator of Mullertz that converted the canine plasminogen into plasmin, resulting finally in the conversion of proenzyme II to enzyme II. The incubation of proenzyme II with the dog liver–dog serum activator (Table 2) produced enzyme II by a mechanism shown by inhibition studies (see Tables 4 and 5) to be different from that of plasmin.

**Activation of Proenzyme II in the Presence of the Serum Inhibitor**

When the fractionation with ammonium sulfate was omitted in the preparation of proenzyme II from dog serum, the serum inhibitor of the endogenous activating enzyme was not removed. Therefore, the subsequent endogenous activation of proenzyme II was prevented, and only an insignificant amount of enzyme II, 20 U/ml serum, was produced even during frozen stor-
prekallikrein, 126% and 1.5%; plasma kallikrein, 1.4% and less than 1%; plasminogen, 41% and 30%; plasmin, 39% and 2.3%.

The chemical and thermal stability of dog liver–dog serum activator was investigated, first as a prerequisite for its future isolation and second to distinguish it from other known activating enzymes. A standard test sample of proenzyme II served to estimate the rate of activation of proenzyme II to enzyme II by this activator. Thus, the effects of chemical treatment leading to the partial or complete destruction of the activator were estimated by the diminished rate of enzyme II formation. The dog liver–dog serum activator was inactivated as follows: 84% loss after 30 minutes at pH 3 and 70 °C; 74% loss after 2 hours at pH 7.5 and 70 °C; 23% loss after 2 hours at pH 7.5 and 50 °C. These results (i.e., profound inactivation of the dog liver–dog serum activator) permit its classification as labile. Plasminogen activators from blood and various tissues have been classified as either acid-stable, remaining fully active after 30 minutes at 70 °C (pH 3.0) or labile, destroyed in 2 hours at 70 °C (pH 7.5).18 By comparison, the human plasminogen activator of Müllertz17 was even more labile (i.e., completely inactivated in 30 min at 50 °C at pH 7.5 or at pH 3), while plasmin was found to be acid-stable (30 min at pH 3 and 70 °C).

Inhibition of the Endogenous Activating Enzyme and Exogenous Plasmin

Studies with enzyme inhibitors and specific antibodies further confirmed the identity of the endogenous activating enzyme with plasmin. These inhibitors were investigated under the assay conditions shown in Figure 2. A 50% inhibition of both the endogenous activating enzyme and the added plasmin was induced by the concentrations (shown in Table 3) of the four known group-specific serine protease inhibitors20 and of the human serum inhibitor. The five inhibitors at similar or even at twofold to 26-fold higher concentrations had no effect on enzyme II, which retained full activity in its reaction with the substrate to produce angiotensin I.

Robbins and Summaria20 have reported that up to 80% of the proteolytic activity of plasmin is neutralized by specific antibody γ-globulin prepared against human plasminogen or plasmin. As shown in Table 4, incubation of proenzyme II for 2 hours at 0 °C and pH 7.2 with such specific antibodies, at a precipitating plasminogen concentration of 0.3 mg/ml, inhibited the formation of enzyme II in three procedures. Therefore, in those three procedures proenzyme II was activated to enzyme II by the proteolytic action of plasmin. In contrast, the antisera did not inhibit the activation of proenzyme II by trypsin or by the dog liver–dog serum activator (Table 4), which suggests that different mechanisms of activation not involving plasmin are available and warrant further investigation. The antiserum to plasminogen and plasmin neither inhibited nor enhanced the reaction of enzyme II with the substrate to produce angiotensin I.

Serum Inhibitor of the Activating Enzyme

Endogenous activating enzyme and added plasmin were inhibited 50% by the addition of 1.2 and 1.7 mg/ml, respectively, of the inhibitor from human serum (Table 3). Semipurified preparations of this unknown inhibitor have been obtained from the serum of dogs, hogs, cattle, and humans. Since it is precipitated at 25 °C and pH 5.4 in the region between 1.9 and 3.1 M ammonium sulfate and is not dialyzable, it is presumably a protein. It is remarkably stable, with complete recovery after 10 minutes at 100 °C at pH 7.0 or pH 1.0 or after 24 hours at 25 °C and pH 12. This unusual stability distinguishes our inhibitor from the following five known inhibitors in serum: the polyclonal α1-trypsin inhibitor, the α1-chymotrypsin inhibitor, the trypsin inhibitor of Laskowski, the kallikrein inhibitor of Werle (all reviewed by Vogel et al.21), and the plasmin inhibitor of Hedner and Nilsson,22 all of which are particularly unstable to acid and to heat.

It is also distinct from α1-macroglobulin, the other polyclonal inhibitor present in human serum because 1) it has been shown to be more soluble requiring a higher concentration of ammonium sulfate for precipitation (i.e., 1.9–3.1 M vs 1.2–1.8 M for α1-macroglobulin),21 and 2) the action of our inhibitor from 1 ml of human serum was not abolished by incubation (18 hr at 4 °C, pH 7.5) with 6 mg of the γ-immunoglobulin fraction of antisera to α1-macroglobulin, which represents a 2.3-fold excess over the concentration of α1-macroglobulin in normal human serum.23

The inhibitor from normal human serum almost completely blocked activation of proenzyme II by the endogenous activating enzyme, human plasmin, or trypsin (Table 5). It did not inhibit activation by the

![Table 3. Inhibition of the Endogenous Activating Enzyme of Serum and of Added Plasmin by Five Inhibitors](https://hyper.ahajournals.org/)

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Proenzyme II activation (ID50%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aprotinin</td>
<td>1.7 µg/ml 2 µg/ml</td>
</tr>
<tr>
<td>Soybean trypsin inhibitor</td>
<td>9 µg/ml 9 µg/ml</td>
</tr>
<tr>
<td>Lima bean trypsin inhibitor</td>
<td>12 µg/ml 11 µg/ml</td>
</tr>
<tr>
<td>Benzamidine HCl</td>
<td>0.8 mM 0.7 mM</td>
</tr>
<tr>
<td>Human serum inhibitor</td>
<td>1.2 mg/ml 1.7 mg/ml</td>
</tr>
</tbody>
</table>

-ID50% = inhibitory dose, 50%.

![Table 4. Effect of Antidbody to Plasminogen on the Activation of Proenzyme II by Five Procedures](https://hyper.ahajournals.org/)

<table>
<thead>
<tr>
<th>Proenzyme II activation</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endogenous</td>
<td>78</td>
</tr>
<tr>
<td>Human plasmin</td>
<td>86</td>
</tr>
<tr>
<td>Human plasminogen activator</td>
<td>100</td>
</tr>
<tr>
<td>Trypsin</td>
<td>0</td>
</tr>
<tr>
<td>Dog liver–dog serum activator</td>
<td>0</td>
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</tbody>
</table>
TABLE 5. Effect of Human Serum Inhibitor* on the Activation of Proenzyme II by Four Procedures

<table>
<thead>
<tr>
<th>Proenzyme II activation</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endogenous</td>
<td>94</td>
</tr>
<tr>
<td>Human plasmin</td>
<td>90</td>
</tr>
<tr>
<td>Trypsin</td>
<td>84</td>
</tr>
<tr>
<td>Dog liver–dog serum activator</td>
<td>0</td>
</tr>
</tbody>
</table>

*Obtained from 1 ml of normal pooled human serum.

Table 6.

Inhibition of Five Angiotensin I-Producing Enzymes by Pepstatin

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Enzyme concentration (per ml)</th>
<th>Incubation at 38 °C, pH 4.7 (hr)</th>
<th>Pepstatin concentration (ID$_{50%}$, ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzyme II</td>
<td>200 U</td>
<td>16</td>
<td>1</td>
</tr>
<tr>
<td>Cathepsin D</td>
<td>20 μg</td>
<td>16</td>
<td>17</td>
</tr>
<tr>
<td>Pepsin</td>
<td>5 μg</td>
<td>16</td>
<td>28</td>
</tr>
<tr>
<td>Dog renin</td>
<td>1 × 10$^{-3}$ U</td>
<td>16</td>
<td>60</td>
</tr>
<tr>
<td>Enzyme I</td>
<td>30 U</td>
<td>16</td>
<td>67</td>
</tr>
</tbody>
</table>

Assay conditions: 0.5 μM dog substrate; 5 × 10$^{-5}$ M phenylmercuric acetate; 1 × 10$^{-3}$ diisopropyl fluorophosphate.

Inhibitors of Enzyme II

The endogenous activating enzyme was inhibited by the five inhibitors (Table 3), by the antiserum to plasminogen (Table 4), by 5 × 10$^{-5}$ M phenylmercuric acetate, by 0.001 M diisopropyl fluorophosphate and by 0.02 M pyrophosphate. In contrast, none of these inhibitors had an effect on enzyme II, which remained fully active in its reaction with the substrate to produce angiotensin I. Even large amounts of the inhibitor, obtained from 1 ml of human serum (13 mg/ml) or from 2 ml of dog serum (26 mg/ml), failed to inhibit enzyme II.

Diisopropyl fluorophosphate has no effect on carboxyl proteases, but it inhibits various serine proteases (e.g., trypsin, chymotrypsin, cathepsin G, and plasmin) by reacting specifically with the serine residue of the active site of these enzymes. Therefore, since enzyme II retained full activity even after prolonged incubation with 1 mM diisopropyl fluorophosphate, it is not a serine protease.

Inhibition of Enzyme II by Pepstatin

Cathepsin D, pepsin, and renin are classified as carboxyl proteases, in which the hydrolysis of peptide bonds is catalyzed by two or more carboxyl groups. Pepstatin is a noncovalent powerful inhibitor of all carboxyl proteases and has little or no effect on other classes of proteases such as Serine, or metallo, or thiol.

Enzyme II was strongly inhibited by pepstatin, which identifies it as a carboxyl peptidase (Table 6). Comparison with other angiotensin-producing enzymes showed that enzyme II was the most susceptible to pepstatin (ID$_{50}$ = 1 ng/ml = 1.6 × 10$^{-9}$ M), which distinguished it from the other carboxyl-peptidases, cathepsin D, pepsin, dog renin, or enzyme I (Table 6).

Reaction of Enzyme II as a Function of pH

The activity of enzyme II was determined in the range between pH 3.5 and 7.2 and it was found to react maximally at pH 4.7 (Figure 3).

Discussion

The present study of a second highly active, non-renal, angiotensin-producing enzyme, enzyme II, obtained from the serum of normal and nephrectomized dogs, served mainly to distinguish enzyme II from each of the other enzymes (Table 7; opposite).

Reaction of Enzyme II, Dog Renin, Enzyme I, Pepsin, and Cathepsin D with Dog Angiotensinogen and with Tetradecapeptide Renin Substrate

Comparing the angiotensin formation with dog angiotensinogen and tetradecapeptide renin substrate by each of the five enzymes revealed profound differences (i.e., the relative rates ranged from 1:5 for dog renin to 1:6000 for cathepsin D), and those differences distinguish enzyme II from each of the other enzymes (Table 7; opposite).

**Figure 3.** The pH dependence of angiotensin I formation by enzyme II (from Dog 2) at a concentration of 525 U/ml with 1.0 μM dog substrate, which had been incubated for 2 hours at 38 °C in 0.05 M acetate-phosphate buffer.
Table 7. Rate of Reaction of Five Angiotensin-Forming Enzymes with Dog Angiotensinogen and Tetradecapeptide Renin Substrate

<table>
<thead>
<tr>
<th>Enzyme concentration (1.0 μM)</th>
<th>Substrate</th>
<th>Incubation at 38 °C, pH 4.7 (hr)</th>
<th>Angiotensin I formation ng/ml Rate TRS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog renin</td>
<td></td>
<td>16.0</td>
<td>220</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.0</td>
<td>325</td>
</tr>
<tr>
<td>Enzyme I</td>
<td></td>
<td>16.0</td>
<td>235</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.33</td>
<td>124</td>
</tr>
<tr>
<td>Enzyme II</td>
<td></td>
<td>1.0</td>
<td>1050</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.33</td>
<td>216</td>
</tr>
<tr>
<td>Pepsin</td>
<td></td>
<td>16.0</td>
<td>170</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.167</td>
<td>92</td>
</tr>
<tr>
<td>Cathepsin D</td>
<td></td>
<td>16.0</td>
<td>216</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.167</td>
<td>264</td>
</tr>
</tbody>
</table>

TRS = tetradecapeptide renin substrate.

The enzyme concentration and incubation time have been selected in each case to be within the range where the rate of angiotensin I formation is a linear function of the enzyme concentration and incubation time (see Figure 1 and refs. 1 and 6).

Comparison of Enzyme II to Other Angiotensin-Producing Enzymes

Nonidentity of Enzyme II with Renin

The following experimental observations suggest that enzyme II is distinct from renin in several ways. The residual renin in the serum of dogs, 2 days after bilateral nephrectomy, produced angiotensin I at a rate of 0.10 ng/ml/hr.26 Enzyme II, also obtained from dog serum after nephrectomy, produced up to 2250 ng angiotensin I/ml/hr, a 22,500-fold higher rate than the endogenous renin. The substrate requirements of enzyme II differed from those of renin. For example, after acid treatment of the dog substrate for 1 hour at 38 °C and pH 2.5, it continued to be fully active as the substrate for enzyme II, but it retained only 3% of its reactivity as the substrate for dog renin. The relative rate of angiotensin formation with dog substrate and with tetradecapeptide renin substrate was 1:12 for enzyme II and 1:5 for dog renin (Table 7). Enzyme II had its peak activity at pH 4.7 (Figure 3), while dog renin produced angiotensin I in a broad range between pH 5.5 to 7.5.27 Enzyme II was inhibited by pepstatin at a very low concentration (ID_{50} = 1 ng/ml), while 60 ng/ml was required for the equal inhibition of renin (Table 6). Renin is inactivated irreversibly by antirenin in 2 minutes,1 while enzyme II retained its full activity even after incubation with antirenin for 16 hours at 38 °C. Enzyme II was found to be unusually stable in acid solution (i.e., it retained 90% of its enzymatic activity after treatment for 60 min at 38 °C and pH 2.5), while renin was 98% inactivated. Prorenin is activated to renin by plasma kallikrein at relatively low concentrations, 6 to 8 μg/ml,6 while plasma kallikrein, even at 110 μg/ml, failed to induce the activation of proenzyme II to enzyme II. Thus, proenzyme II is not identical to prorenin.

Nonidentity of Enzyme II with Enzyme I

Enzyme I is a recently described nonrenin carboxyl protease induced by immunization with renin.1 It has a pH optimum of 4.7 and, like enzyme II, is highly active, producing angiotensin I at up to 2900 ng/ml serum/hr. Our results indicate that it is not identical to enzyme II for several reasons. Enzyme II was obtained only in the serum of nephrectomized or normal dogs by removing an inhibitor and then activating a precursor, proenzyme II (Tables 1 and 2). In contrast, enzyme I was induced by immunization of dogs, guinea pigs, rabbits, and rats with renin from the kidneys of various species.1 The substrate requirements of enzyme II differed from those of enzyme I: dog substrate treated for 1 hr at 38 °C and pH 2.5 was fully active as the substrate for enzyme II, but it retained only 1% of its reactivity with enzyme I. Also indicative of different substrate affinities, the rate of angiotensin I formation from tetradecapeptide renin substrate was 12 times higher than that from dog angiotensinogen for enzyme II, while it was only six times higher for enzyme I.
Nonidentity of Enzyme II with Pepsin, Cathepsin D, Plasmin, Tonin, or Cathepsin G

This inhibition by a low concentration of pepstatin also distinguished enzyme II from cathepsin D (ID50% = 1 ng/ml) and from pepsin (ID50% = 28% ng/ml).

The relative rate of angiotensin formation with dog angiotensinogen and with tetradecapeptide renin substrate was 1:12 for enzyme II, 1:200 for pepsin, and 1:6000 for cathepsin D (Table 7). Plasmin reacting at a concentration of 4 CTA units/ml with renin substrate from dog serum produced only negligible amounts of angiotensin, 8 ng/ml/hr, while enzyme II produced up to 2250 ng/ml serum/hr.

Plasmin was inhibited by each of the five inhibitors (Table 3), and by specific antibody γ-globulin prepared to human plasminogen (Table 4), but none of these acted to inhibit enzyme II. Plasmin is precipitated quantitatively with 1.0 M sodium chloride, in acid solution, while enzyme II was not precipitated. Enzyme II is not identical to tonin, because tonin produces angiotensin II, rather than angiotensin I, has maximal enzymatic activity at pH 6.8 rather than at pH 4.7, and is not inhibited by pepstatin. Cathepsin G differs from enzyme II, since cathepsin G generates angiotensin II and not angiotensin I, has its maximum enzymatic activity at pH 7.5, and is inhibited by diisopropyl fluorophosphatase, while enzyme II was not inhibited by 1 × 10^-3 M diisopropyl fluorophosphate.

Angiotensin Formation by Enzyme II and by Endogenous Renin at Neutral Reaction

The endogenous renin in normal dog serum at 38 °C and pH 7.2, produced angiotensin I at a rate of 0.7 ng/ml/hr, while only 0.10 ng/ml/hr was produced by the residual renin retained in the serum 48 hours after bilateral nephrectomy. Enzyme II, which also was obtained from the serum of three dogs 2 days after bilateral nephrectomy produced angiotensin I at 38 °C and pH 7.2 at a mean rate of 20 ng/ml serum/hr (range, 13–34 ng/ml serum/hr). This rate is 29 times higher than that of renin in normal dog serum and 200 times higher than that of renin present after nephrectomy.

Activation of Proenzyme II to Enzyme II

Optimal conditions were established for the formation of enzyme II as a prerequisite for its future isolation (Table 1). A combination of prolonged storage in the frozen state followed by incubation at elevated temperature activated proenzyme II to the angiotensin-producing enzyme II, apparently as a result of the conversion of endogenous plasminogen to plasmin and the subsequent activation by plasmin. It has been observed that after the removal of an inhibitor in serum fractions, autocatalytic conversion of plasminogen to plasmin can take place in the refrigerator. Great interindividual variation was found by Highsmith among animals in the level of saline-soluble, labile plasminogen activator from canine tissue. We also observed that the level of endogenously produced enzyme II varied considerably among animals, presumably because of variations in the concentration of the endogenous plasminogen activator.

The endogenous activating enzyme of serum appears to be plasmin, because both the enzyme and authentic plasmin converted proenzyme II to enzyme II (Table 2); were inhibited identically by low concentrations of five inhibitors (Table 3) and by antibody to human plasminogen (Table 4); and were blocked completely by the inhibitor present in the serum of humans, dogs, hogs, and cattle (Table 5).

Comparison of Present and Previous Activation Procedures

The present study treated the serum at 0 °C and pH 2.5 to remove angiotensinase and converting enzyme and as a prerequisite for the subsequent activation of proenzyme II to enzyme II. This treatment is not unusual or extreme since considerably more extreme conditions have been reported for the activation of other biologically important, peptide-producing pathways. Astrup extracted the plasminogen activator of various tissues with 2 M potassium thiocyanate and precipitated the activator at pH 1.0, all at room temperature. Hedner and Nilsson activated plasminogen to plasmin at pH 2.0 and 25 °C to remove antiplasmin substances.

Vogel et al. isolated plasma prekallikrein to kallikrein at pH 2.0 and 37 °C. Sealey et al. activated prorenin by dialysis for 24 hours at pH 3.3, while Haas et al. isolated renin from the kidneys of 12 species at pH 1.6.

None of the five procedures that activated dog proenzyme II (Tables 1 and 2) resulted in the formation of enzyme II from human, hog, or bovine serum (data not shown). Whether or not these sera contain proenzyme II has yet to be established. In addition, although an activating-enzyme inhibitor of usually high chemical stability is normally present in serum, this inhibitor could be separated only from dog serum, not from human, hog, or bovine serum (data not shown).

Our results indicate that enzyme II has the capacity to produce angiotensin I at a much higher rate than the renin normally present in serum. The new proenzyme II-enzyme II system is therefore potentially a highly active generator of angiotensin I, the precursor of angiotensin II that acts on vascular smooth muscle to produce vasoconstriction and elevated blood pressure. Strengthening this supposition is the finding that three of the five methods investigated to activate proenzyme II involved components of the plasmin fibrinolytic system, including plasminogen proactivator and activator, plasminogen, and plasmin itself (Tables 2–4). In addition to its fibrinolytic action, plasmin initiates the
factor XII pathway to generate kallikrein and kinins that act on vascular smooth muscle to produce vaso-
dilatation and systemic shock. Plasmin thus may con-
stitute a link between the two potent counterbalanced
mechanisms for blood pressure regulation, the depres-
sor kinin system and the pressor system of angiotensin
formed by enzyme II.

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