BIOCHEMICAL STUDIES ON RENIN AND A HIGH MOLECULAR WEIGHT RENIN

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CHAPTER I. INTRODUCTION

Renin (EC 3.4.99.19) is a carboxyl protease that plays a central role in blood pressure regulation and sodium-potassium homeostasis. In 1898, Tigerstedt and Bergman first recognized the existence of the enzyme in a renal extract (1), and subsequently it has been studied by many investigators.

Renal renin is synthesized and stored in the juxta-glomerular cells of kidney and is secreted into the blood stream in response to various physiological stimuli from the kidney. Renin catalyzes the first step of the renin angiotensin system (Fig. 1). The enzyme released into the circulation splits the decapeptide, angiotensin I, through cleavage of the dipeptide leucyl-leucine from the N-terminus of angio-tensinogen which is synthesized in the liver and secreted from the tissue. Angiotensin I, which has no physiological action of its own, is converted into the

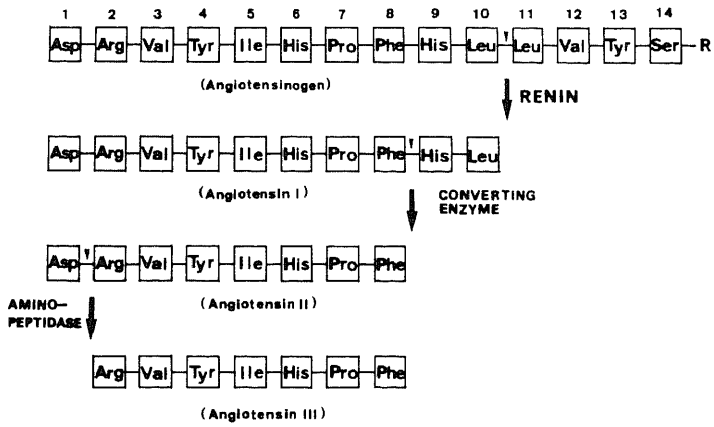


Fig. 1 RENIN-ANGIOTENSIN SYSTEM

octapeptide, angiotensin II, by a carboxydipeptidase called converting enzyme or kininase II, which removes the dipeptide histidyl-leucine from the C-terminus of angiotensin I. Angiotensin II elevates blood pressure directly through its myotropic action which causes vasoconstriction of vascular smooth muscle cells. Angiotensin II is one of the most potent blood pressor substances. On the other hand, the kallikrein-kinin system comprises a vasodepression system to counteract renin-angiotensin system effects on a vasopression. Recently, it has been reported that kallikrein might catalyze the production of renin from prorenin, a precursor of renin (2). These results suggest that the vasopression system, and renin activity in particular, may be controlled by the vasodepression system.

In 1953, Haas et al.(3) partially purified renin from hog kidney laboriously using fractional precipitation methods. However, pure renin was difficult to obtain, because the concentration of the enzyme in the tissue was extremely low, and the partially purified renin was labile for the contamination of other proteases. Recently, several special techniques have been devised and applied for purification of the enzyme. In 1975, Murakami and Inagami purified renin completely from hog kidney by a combination of affinity chromatography on a pepstatin-aminohexyl-agarose

column and conventional methods (4). They characterized the enzyme as follows: 1) the molecular weight determined by a sedimentation equilibrium method was 36,400; 2) isoelectric point was 5.2; and 3) it was a glycoprotein containing glucosamine (4). The affinity column was a useful tool for purification of renin. Subsequently, the enzyme has been isolated from hog (4,5), rat (6), dog (7) and human (8,9) kidneys by the combination method. However, these renins were not available in sufficient quantities for full characterization, such as determination of the active site and the amino acid sequence.

Extrarenal renin has been also studied in the submaxillary gland, placenta, amniotic fluid and brain (10,11). Submaxillary gland renin has long been researched in detail. Werle et al.found a very large amount of renin-like substance in the submaxillary gland of adult albino male mice in 1957 (12). However, the physiological function of the enzyme is not yet known. The submaxillary gland of albino adult mouse contained more renin in male than in female (13,14). The administration of testosterone to a female mouse increased the renin content of the tissue, while castration of the male mouse decreased its content (14). The submaxillary gland renin was released in significant amounts into the blood stream in the course of

aggregation or previous contact with other mice (15). A remarkable increase in renin activity of the submaxillary gland with age was reported in male mice, but not in female mice (16). These results indicate that renin biosynthesis is effected by androgen in the tissue of the albino mouse.

A mouse has three pairs of salivary glands: the parotid, submaxillary and sublingual glands (Fig. 2). The submaxillary glands lie beneath the lower jaw, and have an oval shape. The tissue includes digestive enzymes (amylase, protease), androgenic hormones (nerve growth factor, epidermal growth factor, etc.), homeostative enzyme(renin, kallikrein, etc.). Figure 3 shows the structure of the mouse submaxillary gland. The tissue consists of the acinus and three ducts: the intercalated, striated and interlobular duct. Amylase and kallikrein are synthesized in the acinus cell and secreted into the ducts. Renin is synthesized in the

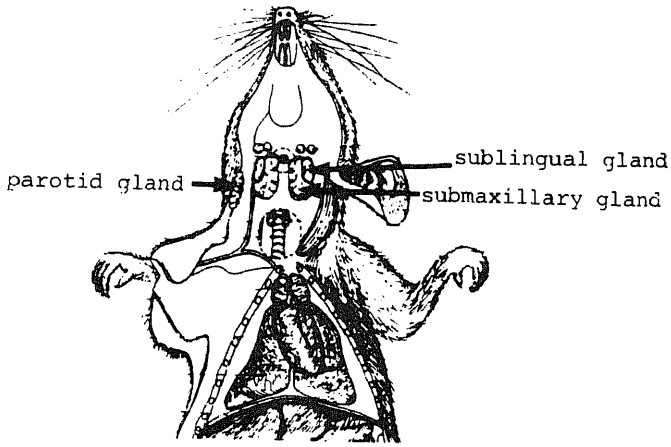


Fig. 2 LOCATION OF THE MOUSE SALIVARY GLAND

convoluted granular cell which is the differential part of the striated duct, and was released into the ducts with saliva (external secretion system) and/or into the circulation through the venous capillary tube (the internal secretion system).

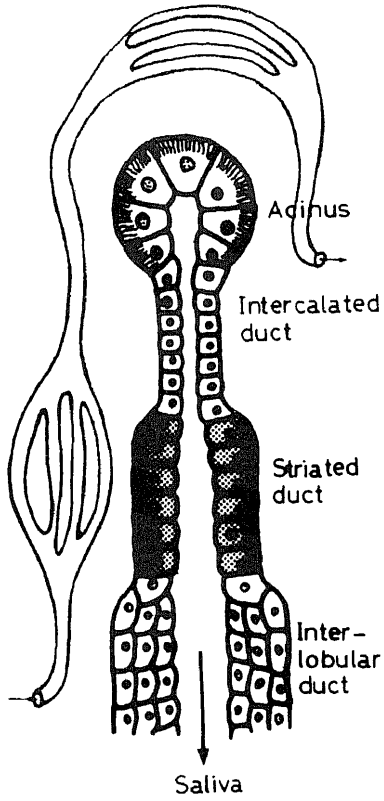


Fig. 3 STRUCTURE OF THE MOUSE SUBMAXILLARY GLAND

In 1972, Cohen et al. purified renin from the submaxillary gland of an albino male mouse by the five steps of the conventional protein fractionation technique. The final purified renins were classified into renin-A, -B, -C, -D and -E. All of their molecular weights were similar(36,000-37,000), although the isoelectric points were slightly different (5.4-5.7). Renin A was a main component; renin A and C produced a characteristic prolonged pressor response similar to that produced by renal renin, when each of them was injected into nephrectomized rats (13).

The availability of pure mouse submaxillary gland renin made it possible to study the renin molecule in detail. For example, (i) direct radioimmunoassay of renin was established, so renin contents could be determined in various mouse organs (14); (ii) the active site of the enzyme was revealed to be similar to that of pepsin (17); and (iii) submaxillary gland renin was demonstrated to be synthesized as 50,000 dalton pre-prorenin in cell-free translation system (18).

In recent years, various forms of renin have been observed in the kidney and plasma (2). In 1974, Boyd demonstrated a high molecular weight renin (Mr=60,000) in hog renal extracts (19), which was regarded as a protein-bound form composed of normal renin (Mr=40,000) and a binding protein. The next year, Leckie and McConnell also showed a high molecular weight renin in the rabbit kidney (Mr=50,000), and moreover suggested that a renin-binding protein was an intrinsic renin inhibitor (20). Since both studies, a high molecular weight renin or its binding protein has been studied in the kidney and some organs by many investigators (2).

The following questions have been posed by various investigators regarding a high molecular weight renin or a renin-binding protein. Does a renin-binding protein play an important role in the cell or after release into the blood stream? Are renin and its binding protein synthesized sequentially or are genes of these proteins cross-linked? The author tried to use two different kinds of animals useful for examination of such problems. One was a stroke-prone spontaneously hypertensive rat (SHRSP). The rat released a large amount of renin into the blood from the kidney at a malignant hypertensive stage (21). Another was an albino male mouse (JCL-ICR). The mouse synthesizes an abnormally large amount of renin in the submaxillary gland, although its physiological role is not yet known. In the present thesis, the author attempted to answer the above questions by studying renin and a high molecular weight renin biochemically in the blood and kidney of SHRSP and in the submaxillary gland of JCL-ICR mouse.

CHAPTER II. A HIGH MOLECULAR WEIGHT RENIN IN STROKE-PRONE SPONTANEOUSLY HYPERTENSIVE RAT

1. SUMMARY

The major form of renin in the plasma of the malignant phase of the stroke-prone spontaneously hypertensive rat(SHRSP) was a high molecular weight renin(Mr=56,000 ± 2,000, major isoelectric point(pI)=5.7 ± 0.08) instead of normal circulating renin of low molecular weight form (Mr=42,000±2,000).

A high molecular weight renin(Mr=52,000 ± 3,600 and pI=5.6 ± 0.07) was also detected in kidney extracts of SHRSP and control Wistar Kyoto strain of rats during all stages of their lives. However, there was no difference in the molecular weight and in the pI value of high molecular weight renin in the kidney extract between Wistar Kyoto rats and SHRSP. Molecular properties of high molecular weight renin in the kidney extracts were very similar to those of high molecular weight renin in the plasma of the malignant phase of SHRSP.

Besides high molecular weight renin, a low molecular weight renin(Mr=35,600 ± 2,900 and major pI value = 5.15 ± 0.05) was also observed in kidney extracts of Wistar Kyoto rats and SHRSP during all stages of their lives, and its

molecular properties were similar to those in their plasma. Therefore, a clear molecular shift of renin was observed in the plasma of the malignant phase of SHRSP, but not in their kidney extracts.

2. INTRODUCTION

Renin(EC 3.4.99.19) is an acid protease which plays a key role in blood pressure regulation and in certain types of hypertension by catalyzing the first step of the renin-angiotensin-aldosterone cascade.

In 1963, Okamoto and Aoki (23) developed a colony of the spontaneously hypertensive rats(SHR) by selective inbreeding of Wistar Kyoto rats. The plasma renin level of SHR has been shown to be comparable to, or slightly lower than that of the normotensive Wistar Kyoto rats during all stages of their lives. Therefore, the role of plasma renin in the SHR has been considered to be minimal. On the other hand, in the stroke-prone substrain of SHR(stroke-prone spontaneously hypertensive rats:SHRSP) developed by Okamoto et al. (21), hypertension manifests itself in two discrete stage (24,25). The initial stage of the SHRSP is similar to that of SHR with a normal or subnormal plasma renin level which is followed by a second stage with a markedly increased plasma renin level. The second stage eventually leads to brain hemorrhage

with a further rise in blood pressure. This second phase which can be considered as a model of malignant hypertension seem to be due, at least partly, to a failure in the regulation of renin secretion.

However, these results and concepts developed thus far are based on the assumption that renin is a simple fixed entity. In recent years, as the purification studies on renin have advanced and its molecular properties have been elucidated, its has become evident that multiple forms of renin (19,20,26-28) exist in the kidney and in blood circulation. Furthermore, it is also becoming clear that one form can be converted into another under certain conditions. These observations suggested that plasma renin activity alone may not be the unique index to assess the angiotensin generating capability under a variety of genetic and hypertensive conditions. The author assessed the pattern of molecular weight distribution of renins in the plasma of SHRSP with the hope to obtain a correlation of the molecular weight distribution with hypertensive or genetic conditions.

In this chapter, the author compared in detail the molecular properties of plasma and kidney renin in SHRSP with those of renin in control Wistar Kyoto rats.

3. MATERIALS AND METHODS

Plasma collection SHRSP and Wistar Kyoto rats of the original strains and strains bred from those distributed by NIH were used. Wistar Kyoto rats were used as control rats for SHRSP throughout the experiment. More than 85% of SHRSP in the breeding colony developed brain hemorrhage. All indwelling polyethylene catheter was implanted in the abdominal aorta through the femoral artery with an opening in the back of the neck. Without anesthesia and under minimum stress, a small volume of blood which was equivalent to 0.5% of the body weight (usually less than 1 ml) was collected in a tube coated with EDTA and then plasma was separated to be used fresh or frozen and stored for subsequent use.

Kidney extract Two grams of fresh rat kidney were collected from animals, after stunning or decapitation, and homogenized in 8 ml 0.01M pyrophosphate buffer(pH 6.5) containing 0.1M NaCl; the homogenate was centrifuged at 100,000 x g for 60 min, then the supernatant was used as kidney extract.

Lyophilized kidney extract Fresh rat kidneys were lyophilized and powdered. The kidney powder of 0.4g were homogenized in 8 ml of the above buffer containing 30%

methylcellosolve, 1 mM $CuSO_4$, 1 mM $ZnSO_4$ and 10 mM N-ethylmaleimide. Cu^{2+} and Zn^{2+} were added for the purpose of inhibiting cathepsins in the kidneys. The homogenate was centrifuged and the supernatant was used as lyophilized kidney extract. The largest proportion of high molecular weight renin was detected in this extract.

Renin activity Renin activity was determined by the radioimmunoassay of angiotensin I generated after incubation with partially purified rat angiotensinogen at 37°C in 0.1 M phosphate buffer (pH 6.5) containing 5 mg rat renin substrate, 5.4 mM diisopropylfluorophosphate and 5 mM EDTA. The rat renin substrate was prepared according to the method of Boucher et al. (30).

Molecular weight The molecular weight of renin was estimated by gel filtration of 1 ml sample on a calibrated column (1.5 x 90 cm) of Ultrogel AcA 44 (LKB) or Sephadex G-100 (Pharmacia). The column was eluted at a flow rate of 5 ml/h with 0.01M pyrophosphate, pH 6.5, containing 0.1 M NaCl. The column was calibrated using the following molecular weight standards: bovine serum albumin (Mr= 67,000), egg albumin (Mr=45,000) and equine skeletal muscle myoglobin (Mr=16,900). The recovery of renin activity during gel filtration was more than 85%.

Isoelectric point(pI) The pI value of renin was determined by the method of gel isoelectric focusing (31). After isoelectric focusing for 3 h at 200 V and 4°C, each gel was cut into fragments 2.5 mm in length; renin was extracted by soaking each fragment in distilled-deionized water for 10 h; and renin activity after extraction was determined by the method described above. The pH range covered was 4-6 (pH 4-6 Ampholine, LKB). Concentrations of polyacrylamide and ampholine in the gel were 5% and 2%, respectively. The recovery of renin activity during isoelectric focusing was about 30-50%.

All the procedures described above were carried out at 4°C unless otherwise stated. All reagents used in this study were of analytical reagent grade.

4. RESULTS

a) Age related differences of blood pressure, renin activity, and molecular weight in the plasma of SHRSP.

As shown in Table I, normotensive Wistar Kyoto rats had normal renin activity and low molecular weight renin as a major form of renin in their plasma through all stage of

Table I

MOLECULAR WEIGHT OF PLASMA RENIN IN WISTAR KYOTO RATS AND STROKE-PRONE SPONTANEOUSLY HYPERTENSIVE RATS

Values after a ± sign in blood pressure and plasma renin activity are standard deviations (S.D.).

	Number of rats (N)	Age (days)	Sex	Blood pressure (mmHg)	Plasma renin angiotensin I (ng per h per ml)	M_r
Wistar Kyoto rats	5	80—90	F & M	122 ± 2.0	14.8 ± 8.0	low *
	8	120	F & M	145 ± 7.8	16.3 ± 8.5	low
	4	200	F & M	139 ± 12.0	14.0 ± 5.3	low
Stroke-prone	4	60	F	168 ± 16.3	19.6 ± 4.0	low
spontaneously	8	120	F & M	180 ± 10.0	22.5 ± 5.0	low
hypertensive	6	160	F	235 ± 9.5	41.7 ± 15.1	high **
rats	9	170	M	247 ± 7.0	35.1 ± 19.3	high
	2	250	F & M	270 ± 6.0	85.2 ± 8.2	high
	10	350	F	253 ± 3.2	69.5 ± 4.1	high

* Low molecular weight renin, M_r = 42 000 ± 2000 S.D. (N = 22).

** High molecular weight renin, M_r = 56 000 ± 2500 S.D. (N = 27).

life. The molecular weight of low molecular weight renin was estimated to be 42,000 ± 2,000 S.D. (number of rats (N) = 22). In contrast to this, SHRSP was found to undergo at least two phase of the molecular weight. In the first phase up to the age of 120 days and with less than 200 mmHg blood pressure, low moelecular weight renin identical to that of Wistar Kyoto rats was a major form of renin in SHRSP (Fig. 4B). However, as the plasma renin concentration and blood pressure were further elevated, the major form of renin in almost all SHRSP began to change from low to high molecular weight renin (Table I and Fig. 4A).

b) A high molecular weight renin in the plasma of SHRSP

b-1) Molecular weight The molecular weight was estimated to be 56,000 ± 2,000 (S.D.,N=27) by gel filtration. In some animals, the transition was not complete and test results showed the presence of high and low molecular weight renins. A typical elution profile is presented in Fig. 4A. Low molecular weight renin in the SHRSP plasma was identical to that of Wistar Kyoto rats by gel filtration (Fig. 4B).

b-2) Isoelectric point To investigate whether any electric charge difference exists between high and low molecular weight renins in the plasma of SHRSP, the isoelectric points (pI) of both renins were determined. A major pI value of

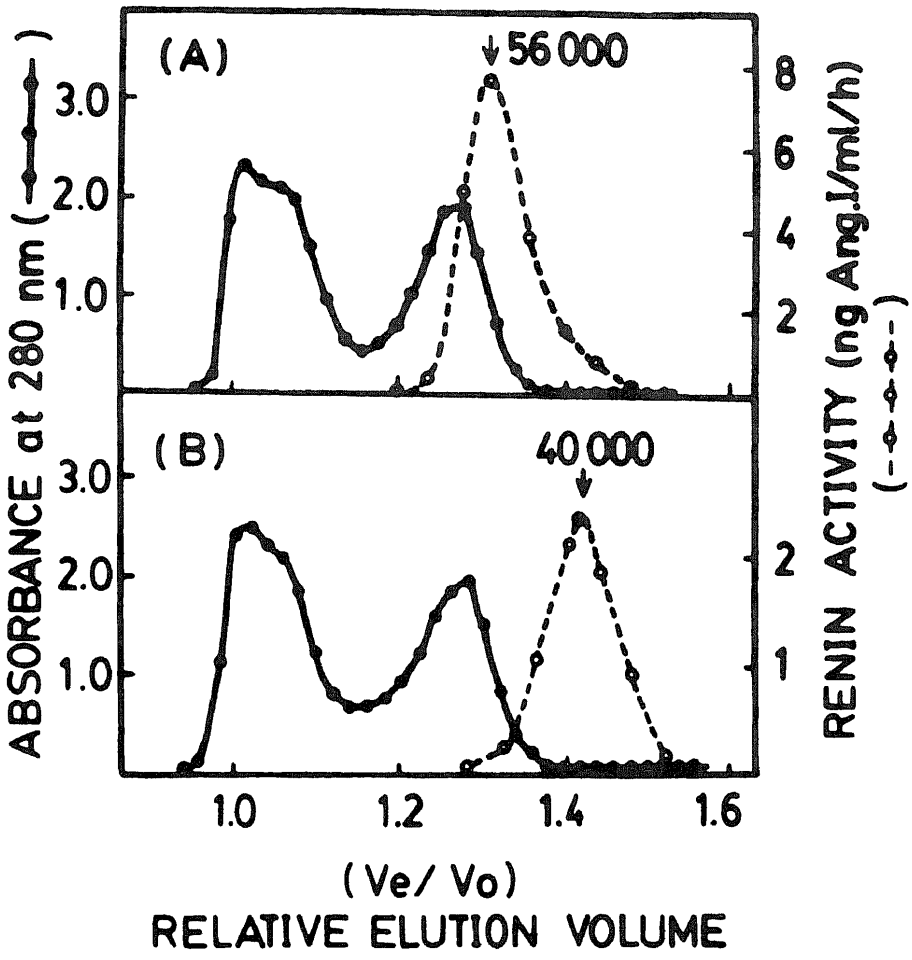


Fig. 4. Gel filtration of stroke-prone spontaneously hypertensive rats. A, malignant(250 days) stage; B, young(60 days) stage. 1 ml of plasma was applied on a Sephadex G-100 column(1.5 x 90 cm). Ang. I, angiotensin I.

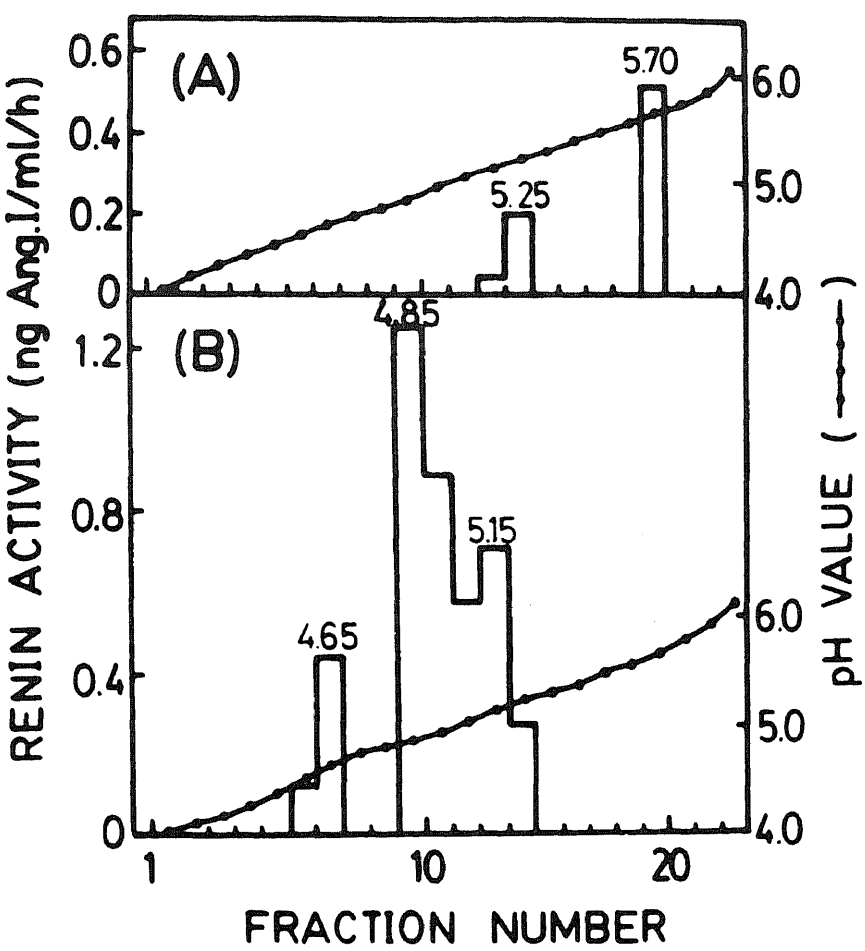


Fig. 5. Isoelectric focusing of high(A) and low(B) molecular weight renins in plasma of stroke-prone spontaneously hypertensive rats. Approx. 50 µl plasma at malignant(A) and young(B) stages were added to an Ampholyte solution(pH 4-6).

high molecular weight renin was 5.7 ± 0.08 (N=8) which was clearly different from 4.85 (Fig. 5).

c) A high molecular weight renin in the kidney extract of SHRSP

c-1) Molecular weight Since the kidney is the source of the plasma renin, the author investigated the molecular weight properties of renin in the kidneys of two strains of rats, Wistar Kyoto rats and SHRSP, at different stage of hypertension. Almost all of the renins in the kidney extracts of both strains of rats in young and adult of malignant stages were low molecular weight forms, although the malignant SHRSP (180 days) secreted high molecular weight renin into their blood. Since Inagami et al. had shown that inclusion of a thiol blocking reagent in the extraction buffer preserved high molecular weight renin of porcine or rat kidney (32), the author extracted renin from the lyophilized kidney powder with a buffer containing 5 mM N-ethylmaleimide and other protease inhibitors and found that both strains of rats in young and adult or malignant stages had high molecular weight renin (Mr= 52,300 ± 3,600 S.D., N=17) and low molecular weight renin (Mr= 35,600 ± 2,900 S.D., N= 19) in their kidney extracts (Fig. 6). Additionally, such a high molecular weight renin was not observed when renin was extracted without the reagent (Fig. 7). No conspicuous

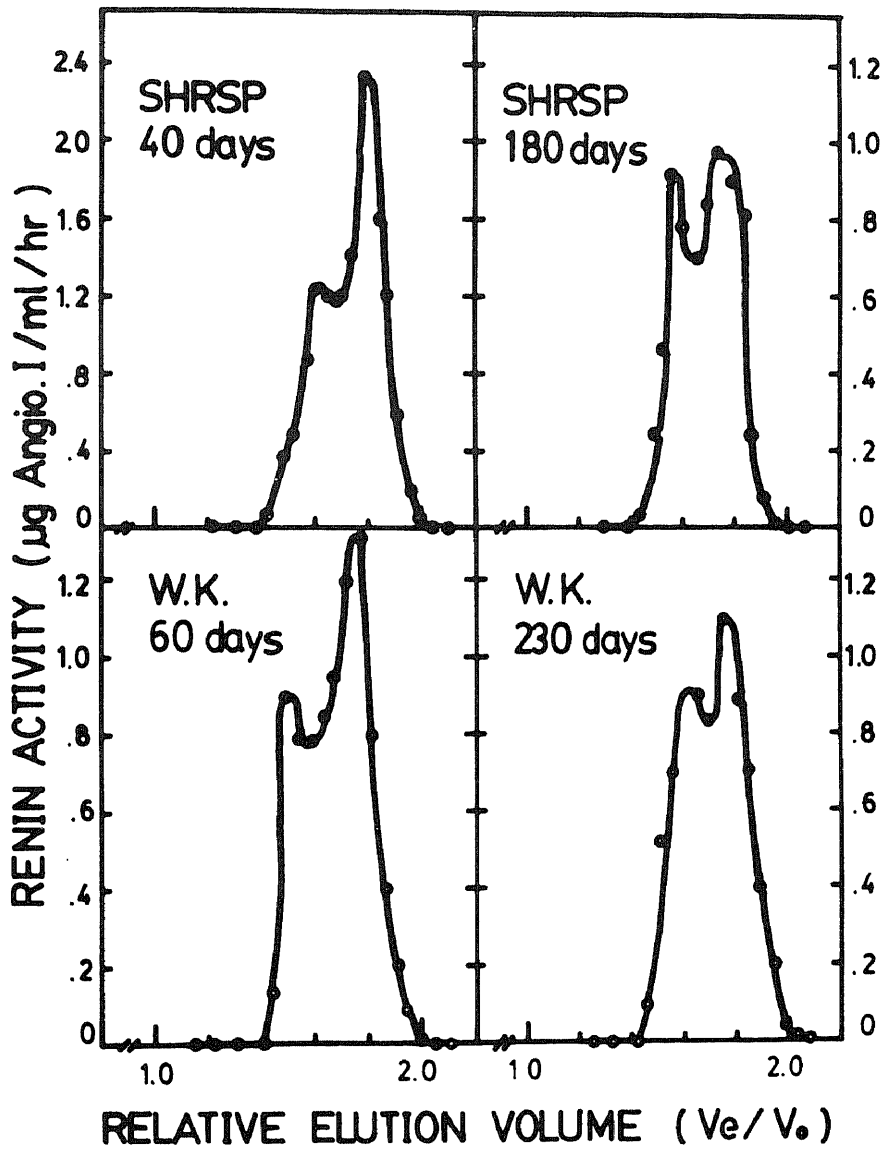


Fig. 6. Gel filtration of lyophilized kidney extract prepared with protease inhibitors from stroke-prone spontaneously hypertensive rats and Wistar Kyoto rats at young and adult or malignant stages. 1 ml of lyophilized renal extracts prepared from the two strains of rats with a mixture of protease inhibitors were applied on a Ultrogel AcA 44 column(1.5 x 90 cm).

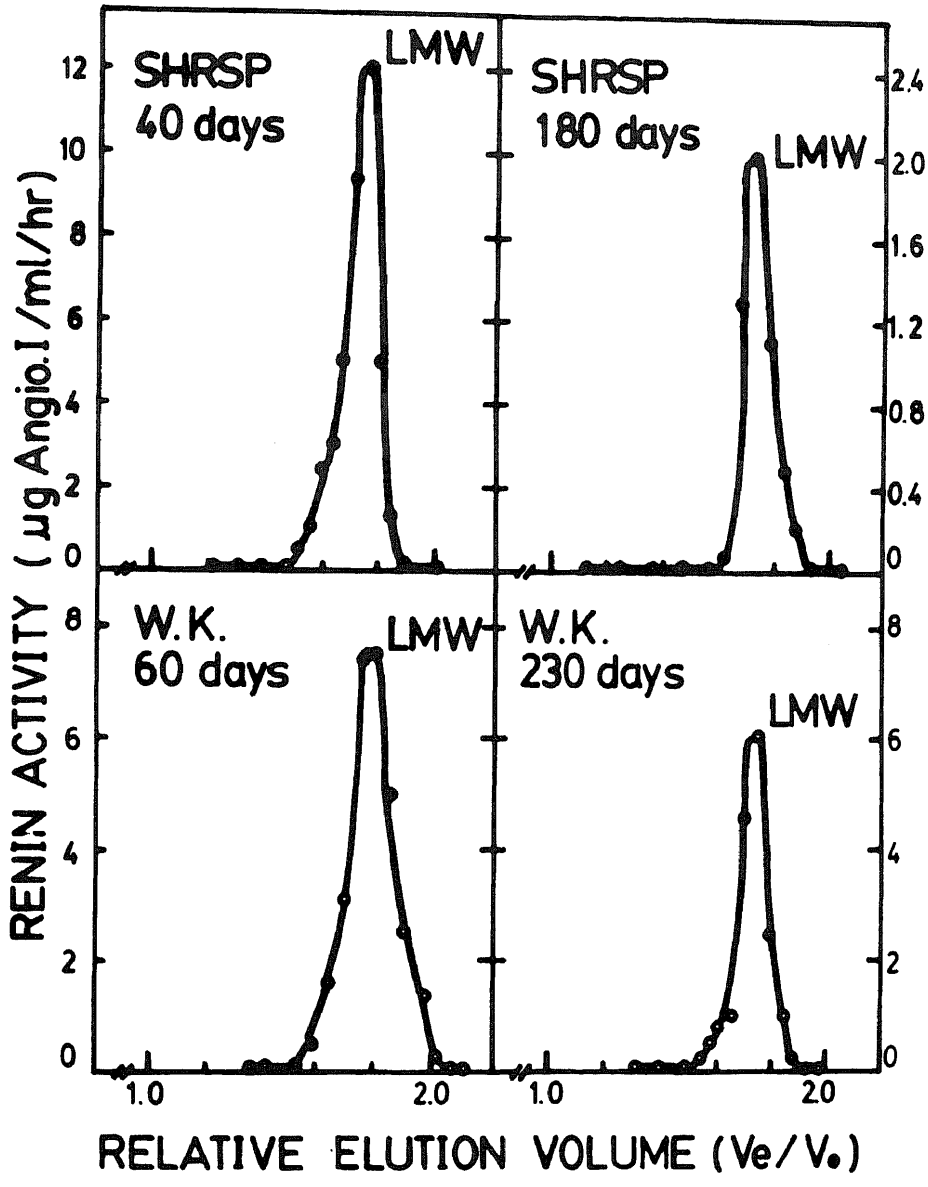


Fig. 7. Gel filtration of renal extract prepared without protease inhibitor from stroke-prone spontaneously hypertensive rats and Wistar Kyoto rats at young and adult or malignant stages. 1 ml of extracts obtained in the absence of protease inhibitor was applied on a Ultrogel AcA 44 column(1.5 x 90 cm).

difference in the molecular weight distribution of kidney renin was detected between the two strains of rats in all stages of their lives, although low molecular weight renin was the major form in young rats. Thus, the clear molecular shift of renin found in plasma of SHRSP was not observed in their kidney extracts.

c-2) Isoelectric points The major pI value of high molecualr weight renin in the kidney of the two strains of rats was 5.60 ± 0.07 (N=9) which was identical with that of high molecular weight renin in plasma. On the other hand, the major pI value of low molecular weight renin in the kidney extracts of the two strains of rats was 5.15 ± 0.05 (N=5) and two minor pI values of the two molecular weight renins were 5.38 ± 0.03 (N=5) and 4.96 ± 0.07 (N=5). There was also no clear difference in the pI profiles of low molecular weight renin between Wistar Kyoto rats and SHRSP.

d) Reversible conversion between high molecular weight renin and normal renin

The author checked a possibility that the high molecular weight renin presented in Fig. 6 and 8-A may be induced during preparation procedure, since most of renin

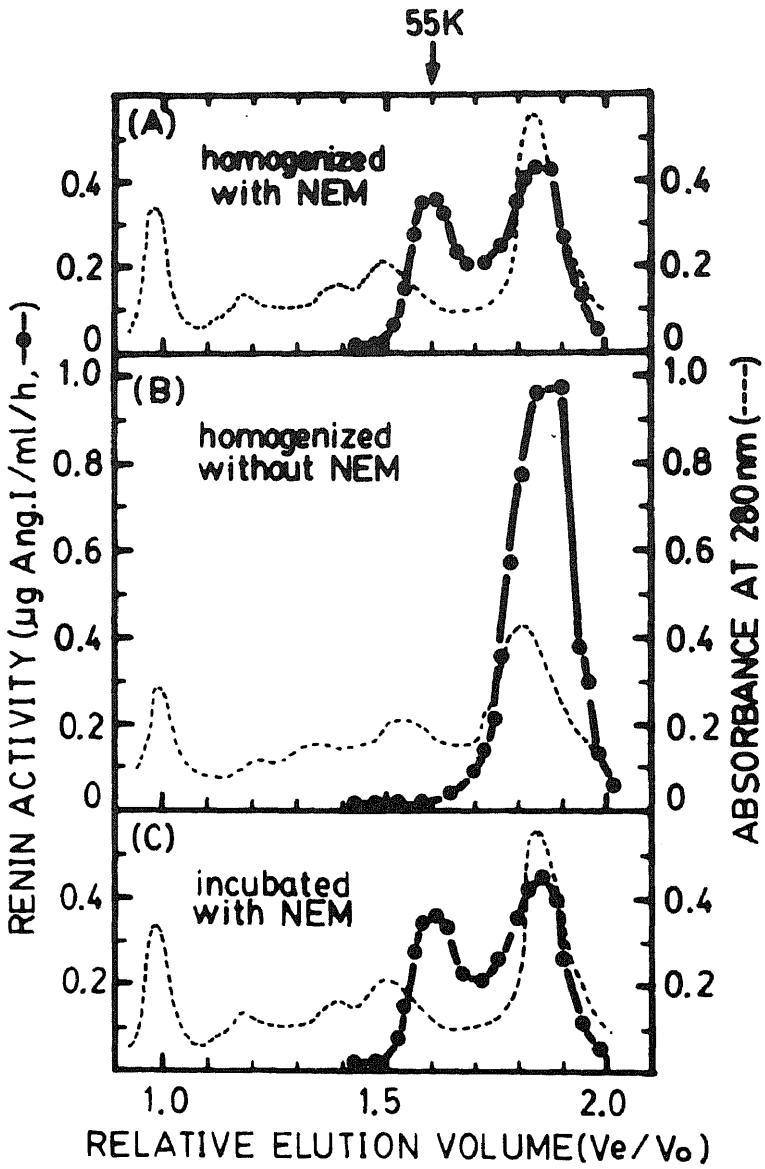


Fig. 8. Induction of high molecular weight renin by incubation of renal extract with N-ethylmaleimide.
3 ml of renal extracts of spontaneously hypertensive rats, (A):prepared with protease inhibitors; (B): obtained without inhibitors; (C): incubated with N-ethylmaleimide at 4° C for 10 min after the extract was prepared in the absence of inhibitors, was applied on a Ultrogel AcA 44 column(2.5 x 200 cm).

was eluted at 40,000 dalton region when the extract was prepared without thiol blocking reagent, as shown in Fig.7 and 8-B. A thiol blocker, N-ethylmaleimide, was added into the extract after it was prepared without the blocker, and then was applied on a column. As shown in Fig. 8-C, a high molecular weight renin besides normal renin was eluted. The elution volume was in agreement with that of the high molecular weight renin observed when the extract was prepared with thiol blocker. These results indicate that both high molecular weight renins are identical in the molecular weight.

5. DISCUSSION

SHRSP is a model for studies on the pathogenesis of strokes. It has been known that the plasma renin level in SHRSP is markedly elevated with increase of age and blood pressure. In the preliminary (29) and present investigation, the author found that an abnormal plasma renin appeared in the malignant phase of SHRSP rats with high blood pressure and high plasma renin activity. The abnormal renin with an average molecular weight of 56,000 and pI value of 5.70 was a high molecular weight renin clearly different from normal renin in the plasma of control Wistar Kyoto rats or young SHRSP with respect to its molecular weight and electrical

charge. Thus, the adult SHRSP in the malignant phase have not only very high plasma renin level, but also a large amount of high molecular weight renin in their plasma.

The molecular weight or pI value of high molecular weight renin in the plasma during the malignant phase of SHRSP was very similar or identical with those of high molecular weight renin which was found in the kidneys of all stages of Wistar Kyoto rats and SHRSP.

Inagami et al. (32) showed that a high molecular weight renin of kidney extract was detected only in the presence of thiol blocker and had suggested that high molecular weight renin was the native form stored in kidneys and that it was converted by an enzyme or agent requiring a thiol group to low molecular weight renin when it was secreted into blood.

In the present study, high molecular weight renin was observed in the extract of kidneys of SHRSP prepared in the presence of thiol blocker. On the other hand, the kidney extract prepared from the normotensive Wistar Kyoto rats or SHRSP without using thiol blocker did not contain high molecular weight renin. Moreover, the high molecular weight renin was induced by a thiol blocker (Fig. 8). Thus, in the absence of thiol blocker, high molecular weight renin seemed to be easily converted to low molecular weight renin in the kidney extracts but not in the plasma of the malignant phase of SHRSP.

Murakami and Suzuki (33) described that the conversion between high and low molecular weight renins is reversible in kidney extracts of hog. Leckie and McConnell (20) and Boyd (19) had reported renin-binding protein or renin inhibitor in rabbit and porcine kidneys which combined with low molecular weight renin to form high molecular weight renin. Therefore, the author do not have a definite answer to the question of whether high molecular weight renin is the true native form in the kidney or not.

It is interesting that a purified high molecular weight renin from porcine kidney (26) or a crude high molecular weight renin in human kidney extract also has the same pI value of 5.60 to 5.70 and similar molecular weight (approx. 60,000) as those found in Wistar Kyoto rats and SHRSP. Day et al. (27) also reported the existence of a "big" renin which has a greater molecular weight (63,000) than normal renin in plasma or kidney of patients with renal disorders. "Big" renin in human plasma and kidney is similar to high molecular weight renin in plasma of SHRSP. Further studies on high molecular weight renin and "big" renin are needed to determine their interrelationship. Besides high molecular weight renin, low molecular weight renins which had a molecular weight of 36,000 and a major pI value of 5.15 and two minor pI values of 4.96 and 5.38 were also detected in kidney extracts of Wistar Kyoto rats and SHRSP and their

molecular properties were similar to those in plasma. The molecular weight and the pI value of low molecular weight renin in rat kidney extract were nearly identical with those of pure rat renin (6). The pI values of low molecular weight renins found in the present investigation were similar to those of rat renin in the crude preparation obtained by Lauritzen et al. (34).

The author could not detect substantial differences in the molecular properties of high and low molecular weight renins in kidney extracts between the two strains of rats, SHRSP and Wistar Kyoto rats, in all stages of their lives.

These results may be explained by two following possibilities: first, renins in kidneys of both strains of rats are the same in their molecular properties and secretion of renin from kidney into blood is abnormal in the malignant phase of SHRSP; or second, in kidneys, renins of Wistar Kyoto rats are different from those of SHRSP, but the difference disappears during homogenization of kidney in which renin is localized in the granule of juxtaglomerular cells of the kidney.

The important question of why and how high molecular weight renin appears in the malignant phase of SHRSP remains to be solved.

The author have three working hypothesis on how the high molecular weight renin appears in the plasma of the

malignant phase of SHRSP. First, the high molecular weight renin is stored in the granule of juxtaglomerular cells in the kidney and secreted into blood directly. Second, low molecular weight renin is stored in the granules and secreted into blood after transformation into high molecular weight renin in kidney. Third, low molecular weight renin is stored in the granules, secreted into the blood and then transformed immediately into high molecular weight renin.

It is, thus, very important to determine the molecular form of renin in the granules in the kidney of the two strains of rats. Recently, Funakawa et al. (35,36) indicated that renin was stored in the granules of dog kidneys as the low molecular weight form and could combine with a renin-binding protein in the soluble fraction of renal cortex tissue to form high molecular weight renin. Yamamoto and Ikemoto suggested that a renin-binding protein of the dog kidney was contained not in the glomeruli but in the cortical tubules (84). More recently, Ueno et al. (66) and Takahashi et al. (80,81) demonstrated that a renin-binding protein of the hog kidney had renin-inhibitory activity, and the protein bound renin reversibly. Therefore, the author thought that the last working hypothesis was most reasonable among the three hypotheses described above, if all these results could be applied to the cases of SHRSP and Wistar Kyoto rats.

CHAPTER III. PURIFICATION OF 40,000-RENIN FROM THE SUBMAXILLARY GLAND OF ALBINO MALE MICE

1. SUMMARY

A pressor enzyme, renin, was purified about 60-fold by chromatography on an affinity column including pepstatin-aminohexyl-agarose with a high yield of 83% from the homogenate of adult mouse submaxillary glands. The renin obtained by the one-step purification was electrophoretically homogeneous on SDS-polyacrylamide gel and as active as an absolutely pure renin. The renin purified by the affinity column could be separated into five active components by chromatography on CM-cellulose. Each of those renins gave a symmetrical elution profile on the CM-cellulose column and a discrete protein band on polyacrylamide gel electrophoresis at pH 8.6. Administration of nanogram quantities of each of the two major renin fractions to nephrectomized rats caused a sustained rise of blood pressure and decrease in sensitivity of the animal to angiotensin II. This rapid and large-scale purification method using pepstatin-aminohexyl-agarose eliminates all four fractionation steps reported previously for the isolation of mouse submaxillary gland renin. Furthermore, the

antibodies were raised against fraction-A or -D renin.

2. INTRODUCTION

Recently, renin has been isolated from hog (4,5,37), rats (6), dog (7), and human (8,9) kidneys. In this isolation, an affinity column including pepstatin-amino-hexyl-agarose played a central role, because the concentration of renin in the kidneys is very low and its isolation is almost impossible without this affinity column. However, these renins were not available in sufficient quantities for full characterization, such as determination of the active site and the amino acid sequence. Furthermore, renins have also been observed to exist in active high molecular weight forms (2,38,39) and a completely inactive renin precursor (40-42) was found in kidney and plasma, although our present knowledge of these new forms of renin is far from complete.

On the other hand, a very large amount of renin-like substance was found in the submaxillary gland of adult albino male mouse (12). In 1972, this submaxillary gland renin was isolated by five steps of conventional protein fractionation techniques (13), because at that time, no suitable affinity column was available. Although Poulsen et al. (18) reported that a large precursor of mouse submaxillary gland renin was synthesized in vitro from its

purified mRNA, nothing is known about a high molecular weight renin or renin precursor in the homogenate of mouse submaxillary glands.

It was thus desirable to develop a simple isolation method to obtain pure preparations of renin, high molecular weight renin and renin precursor from mouse submaxillary gland for their identification and characterization.

In this chapter, the author described the development of a rapid and large-scale purification method for mouse submaxillary gland renin and elicitation of antirenin antibodies.

3. MATERIALS AND METHODS

Renin assay Renin activity was determined by the rate of formation of angiotensin I from renin substrate which had been obtained from hog plasma as described below. After incubation with 10 mg of hog angiotensinogen preparation at 37°C for 1 h in 0.1M phosphate buffer, pH 6.5, containing 5 mM EDTA and 5.4 mM diisopropylfluorophosphate, angiotensin I was determined by the radioimmunoassy of Haber et al. (76).

Hog renin substrate Two liters of fresh blood obtained from normal hog in 0.3% EDTA was centrifuged at 3,400 x g for 30 min. The pH of the supernatant was adjusted to 5.3

and solid ammonium sulfate was added to a concentration of 30.4 g per 100 ml. After standing for 1 h, the resulting precipitate was separated by centrifugation, suspended in 200 ml of distilled water and dialyzed for 12 h against 0.01M acetate buffer pH 5.5. The dialyzed sample was centrifuged at 9,000 x g for 20 min. The supernatant was applied to a pepstatin-aminohexyl-agarose column (4) (1.5 x 4.5 cm) equilibrated with 0.01M acetate, pH 5.5, to removed renin and eluted with the same buffer at a flow rate of 60 ml/h. Renin substrate fraction containing almost no renin activity was eluted in the flowthrough fractions. The renin substrate in the elution was again salted out by the addition of solid ammonium sulfate as described above. The resulting precipitate was dissolved in 200 ml of distilled water and dialyzed for 12 h against distilled water. The dialyzed sample was centrifuged and the supernatant was used as hog angiotensinogen preparation after lyophilization. Ten mg of the hog renin substrate was added to each renin assay tube to give maximum amount of angiotensin I during incubation.

Molecular weight The molecular weight of renin was estimated by electrophoresis on polyacrylamide gel with sodium dodecylsulphate (SDS). The gel (0.5 x 8 cm) contained 10% polyacrylamide and 0.1% SDS with or without 0.25% dithiothreitol (DTT).

Protein concentration Protein concentration was determined spectrophoretically using an extinction of E = 10.5 at 280 nm (13).

Polyacrylamide gel electrophoresis Polyacrylamide gel electrophoresis was run in a 0.5 x 8 cm gel containing 7.5% polyacrylamide at pH 8.6 with a constant current of 2 mA per gel according to Davis (43).

Effect of submaxillary gland renin on blood pressure Rats were bilaterally nephrectomized 16 h prior to the experiments. Under sodium pentobarbital anesthesia, a catheter was inserted into the femoral vein for injection of submaxillary gland renin and standard solution of angiotensin II. Another catheter was inserted into the carotide artery and connected to a manometer for pressure recorder(Erma Optical Works, Ltd., Tokyo).

Antirenin antibodies Pure renin, fraction-A or -D (0.1 mg), was mixed with Freund's complete adjuvant and then it was injected intradermally at multiple sites in the back of a rabbit(Japan White) at weekly interval. High titer antibodies were obtained after 5 weeks.

4. RESULTS

a) Purification Renin in the homogenate of male mouse submaxillary gland was completely adsorbed at pH 5.5 on an affinity column including pepstatin-aminohexyl-agarose, prepared according to the method of Murakami and Inagami (4), and eluted with 0.1M Tris-HCl buffer containing 0.5M NaCl, pH 7.4 (Fig. 9), although hog kidney renin was eluted with 0.1M acetic acid of pH 3.2(4). Renin was purified about 60-fold on this affinity column with yield of 83% (Table II). This renin fraction was further purified on a CM-cellulose column by elution with a shallow NaCl gradient (Fig. 10). Each of the fractions showed a symmetrical elution profile on the CM-cellulose column, and the protein peak coincided with the renin activity (Fig. 10).

b) Purity The purified renin by the affinity chromatography was electrophoretically homogeneous on SDS-polyacrylamide gel with or without a reducing reagent, DTT (Fig. 11), and was fully active. The further separated renins by CM-cellulose chromatography gave a discrete protein band individually on polyacrylamide gel electrophoresis at pH 8.6, indicating electrophoretic homogeneity, as shown in Fig. 12.

Table II Purification of renins from mouse submaxillarey gland.

Preparation	Total protein(mg)	Total activity(unit*)	Specific activity (unit/mg)	Yield (%)	Purification
Crude extract	678.4	13,700	20.2	100	1
Affinity column	9.6	11,400	1,190	83.2	58.9
CM-cellulose	5.9	5,280	895	38.5	44.3
fraction-A	2.1	2,500	1,190	18.2	58.9
fraction-B	0.6	400	667	2.9	33.0
fraction-C	2.0	1,700	850	12.4	42.1
fraction-D	0.6	300	500	2.2	24.8
fraction-E	0.6	383	638	2.8	31.6

*1 unit: 1 μg angiotensin I/h.

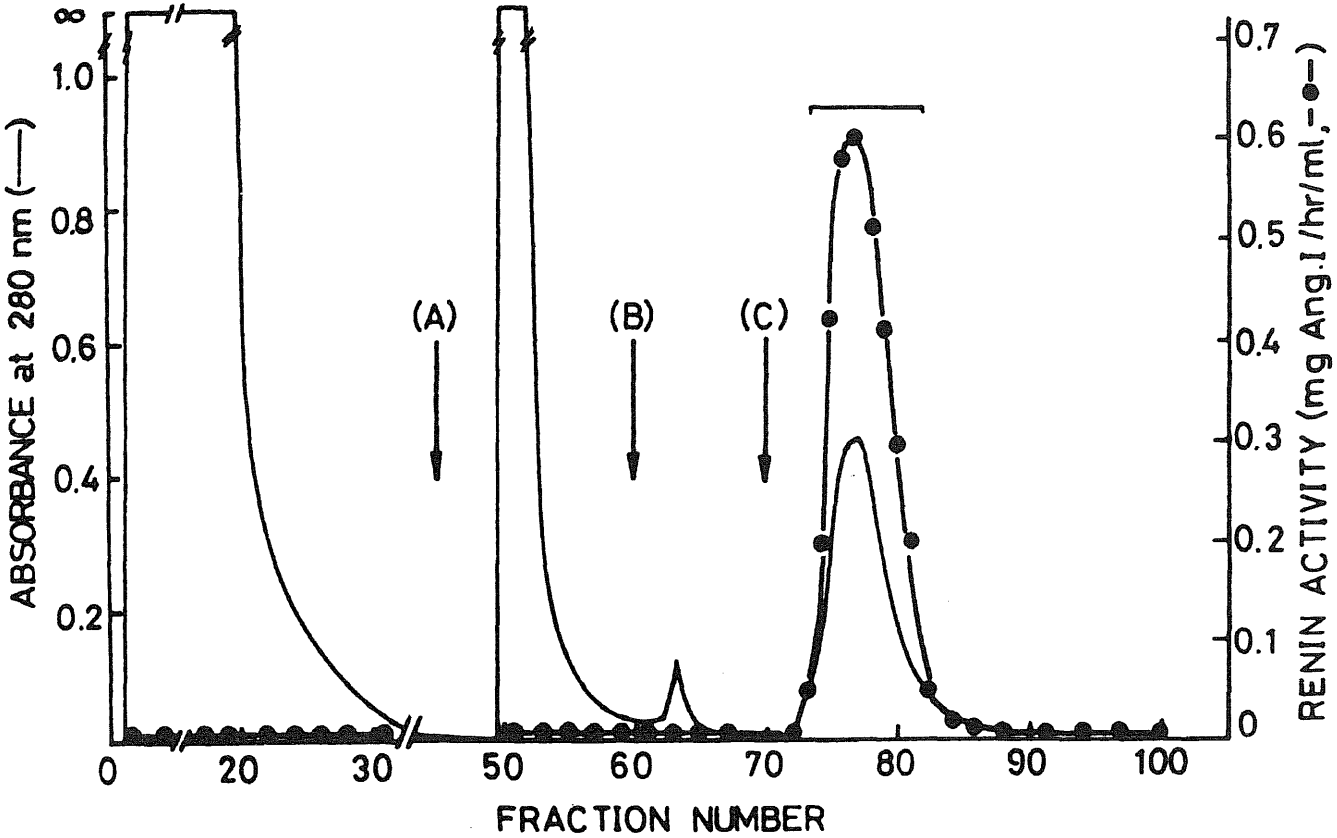


Fig. 9. Affinity chromatography of renin in the homogenate of male mouse submaxillary glands on a pepstatin-aminohexyl-agarose column. Submaxillary glands were excised from 21 adult male albino mice(8-week-old, JCL-ICR) under ether anesthesia and immediately frozen and stored in liquid nitrogen. The frozen submaxillary gland (4.1g) were homogenized in 40 ml of 0.02M acetate buffer, pH 5.5. The homogenate was centrifuged at 100,000 x g for 60 min. The supernatant was applied to a pepstatin-aminohexyl-agarose column (4) (0.5 x 20 cm) which had been preequilibrated with the above buffer. Components were eluted with 3 different buffers: A, 0.02M acetate buffer, pH 5.5 containing 0.5M NaCl; B, 0.1M Tris-HCl buffer, pH 7.4; C, 0.1M Tris-HCl, pH 7.4 containing 0.5M NaCl. The flow rate was 10 ml/h and fractions of approximately 3.5 ml were collected.

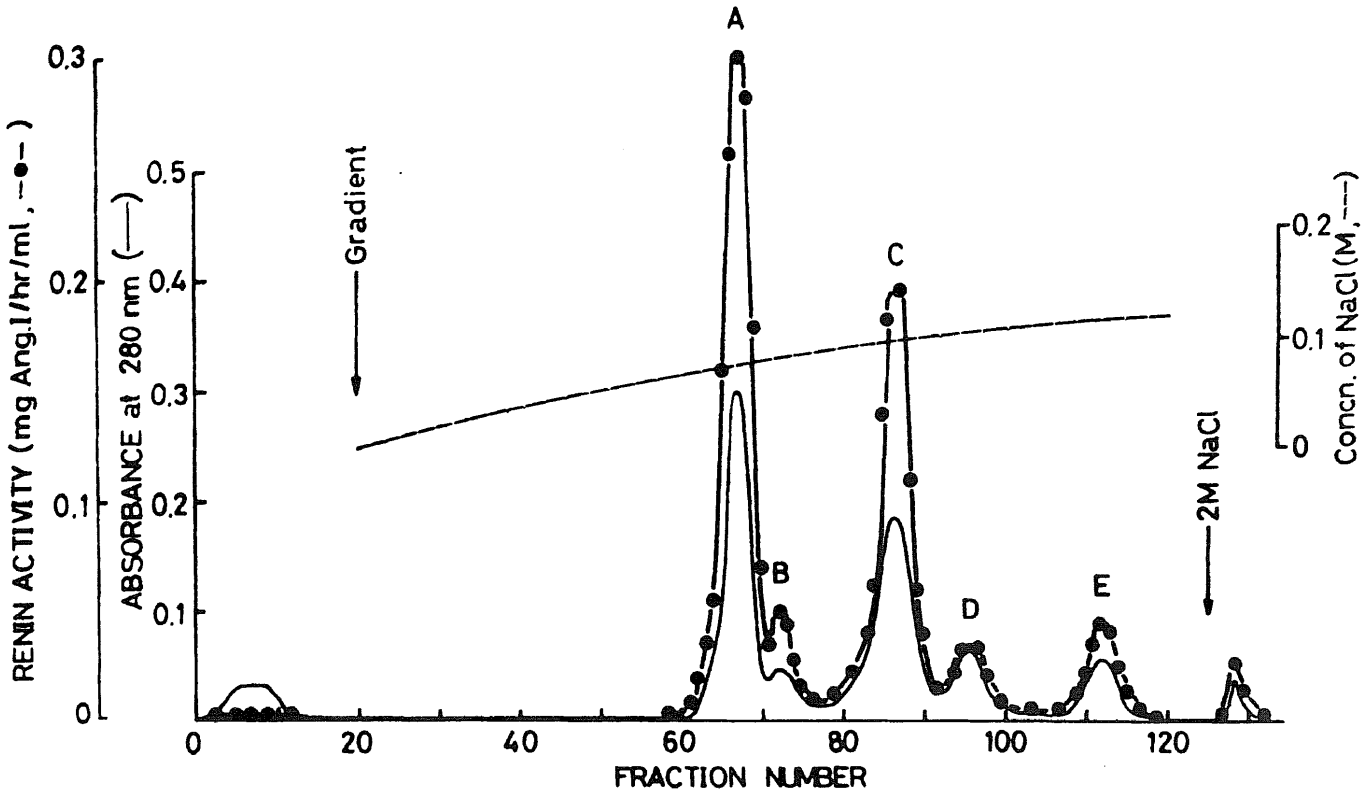


Fig. 10. Chromatography of renin fractions from the affinity column on CM-cellulose. The renin fractions shown by the bracket in Fig. 9 were pooled and equilibrated with 0.02M acetate buffer, pH 5.05, then applied to a CM-cellulose column (0.6 x 25 cm, Whatman CM 52) previously equilibrated with the same buffer. Renins were eluted with a convex gradient of 0-0.125M NaCl in the buffer, generated from a 200 ml constant volume mixing chamber. The flow rate was 6 ml/h and 2 ml fractions were collected.

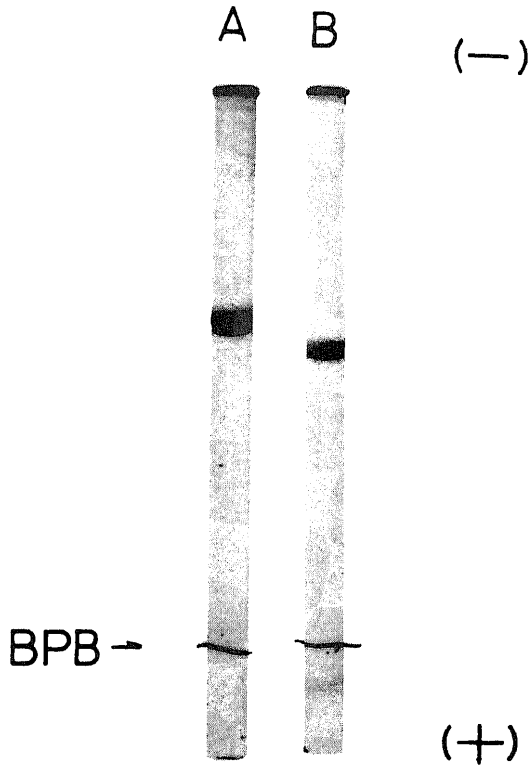


Fig. 11. SDS-polyacrylamide gel electrophoresis of the renin purified on the affinity column (Fig. 9). Electrophoresis of a 5 μg sample was carried out as described in MATERIALS AND METHODS in this chapter. A: without DTT, B: with DTT.

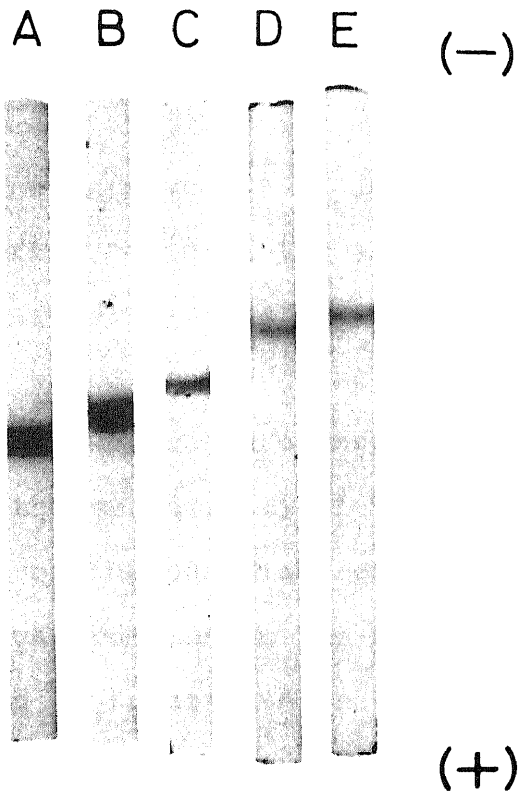


Fig. 12. Polyacrylamide gel electrophoresis of the renin purified on the CM-cellulose column (Fig. 10). Electrophoresis was carried out as described in MATERIALS AND METHODS in this chapter (7-10 μg of samples). A,B,C,D, and E present fraction-A,-B,-C,-D, and -E, respectively.

c) Molecular weight The molecular weight of the purified submaxillary gland renin was determined by SDS-polyacrylamide gel electrophoresis to be 40,000 without DTT and to be 33,000 (fraction-A,-B,-C and -E) and 38,000 (fraction-D) in the presence of DTT, as shown in Fig. 13.

d) Bioassay The intravenous injection of a small dose (18 ng) of the purified renin by the affinity chromatography in nephrectomized rats elicited a clear and prolonged (over 30 min) increase in blood pressure, as shown in Fig. 14. Along with the renin-like pressure response, there was a marked decrease in the sensitivity of rats to angiotensin II following injection of the purified renin. Moreover, the effect of each active fraction on the blood pressure was examined. When 20 ng of fraction-A or -C was administrated to nephrectomized rats, there was a sustained rise in the blood pressure which was almost identical to that in Fig. 3. However, there was no rise in the blood pressure when large quantities (0.8 to 1 μg) of each of the minor fractions (B, D,and E) were injected.

e) Elicitation of antirenin antibodies Double diffusion of pure submaxillary gland renin (fraction A) and rabbit antirenin antibodies on an Ouchterlony plate produced a

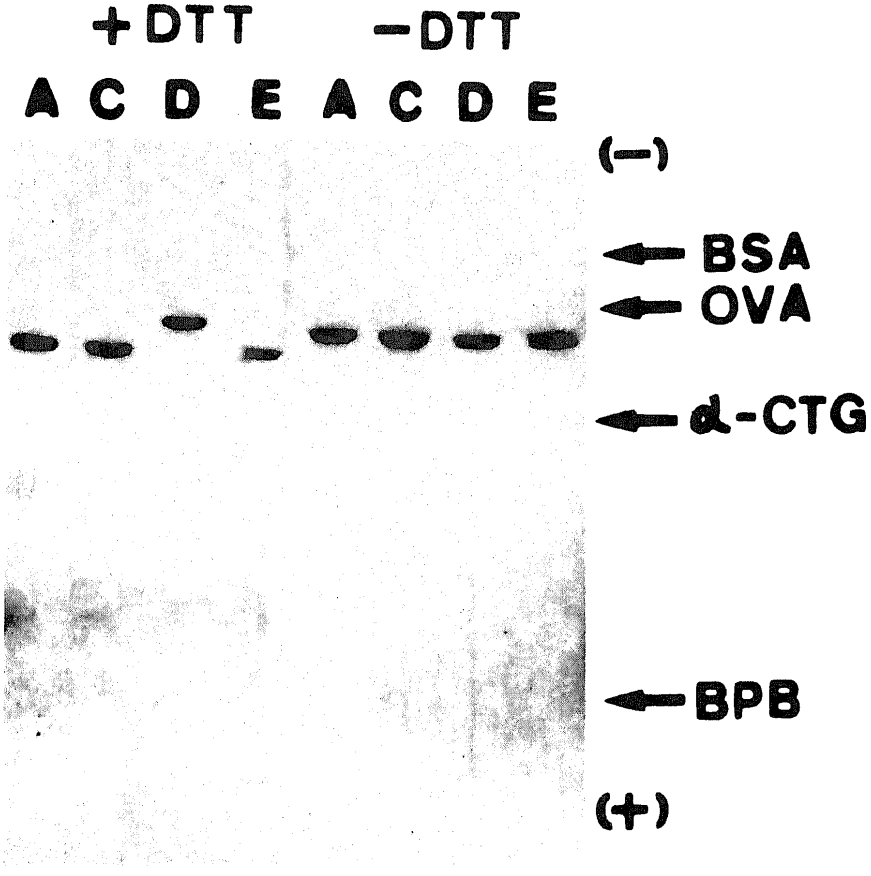


Fig. 13. Slab SDS-polyacrylamide gel electrophoresis of the renin purified on the CM-cellulose column (Fig. 10). Electrophoresis of a 15-25 μg sample was run in a 10 x 15 x 0.1 cm slab gel containing 15% polyacrylamide at pH 8.6 with a constant current of 25 mA according to Laemmli (51). Proteins were stained in 0.1% Coomassie Brilliant Blue R 250. A,C,D,and E present fraction-A,-C,-D, and -E renin, respectively. BSA; bovine serum albumin, OVA; ovalbumin, α-CTG; α-chymotrypsinogen A, BPB; bromophenol blue.

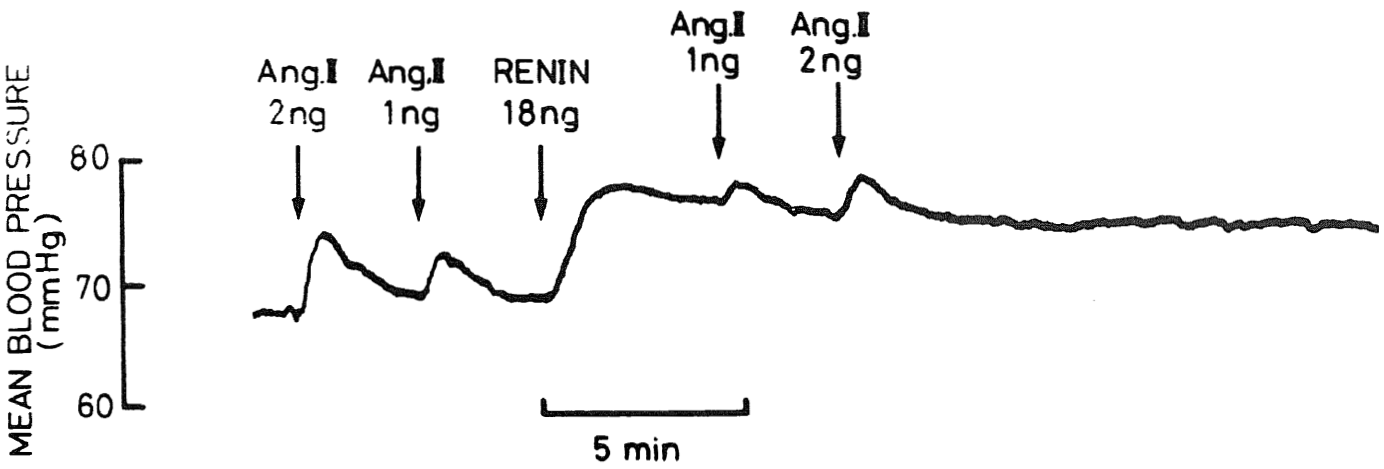
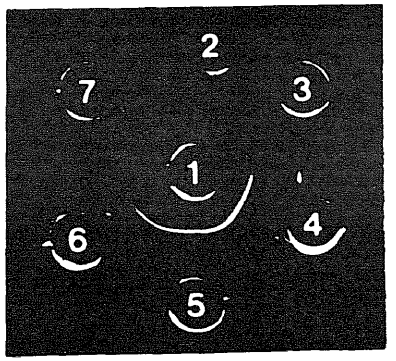
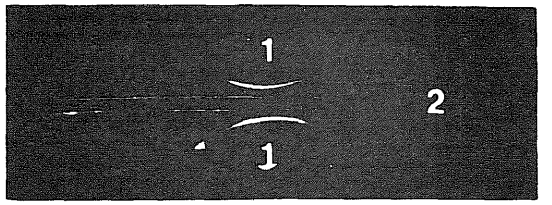


Fig. 14. Typical response of the blood pressure of rats to injection of renin. After the intravenous injection of 2.0 or 1.0 ng of standard angiotensin II, 18 ng of submaxillary gland renin purified on the affinity column (Fig. 9) was injected intravenously. The same dose of angiotensin II as before was again injected during the sustained rise of the blood pressure caused by renin infusion.



(A)



(+) (—)

(B)

Fig. 15. Immunodiffusion and immunoelectrophoresis patterns of fraction A renin and anti-fraction A renin antibodies. The left figure(A) shows the immunodiffusion pattern of renin and antirenin antibodies by Ouchterlony's method (52). Pure fraction A renin (2.8 µg) was placed in the No. 1 well. Antisera were diluted 2-,4-,8-, 16-, 32- and 64-fold with saline and then 15 µl of each of the diluted antisera was left set for 16 h. The right figure (B) presents the immuno-electrophoresis pattern by Scheidegger's method (53). Pure fraction A renin (2.8 µg) was placed in the well. After electrophoresis, the antiserum (100 µl) was placed in the trough and diffusion was allowed. The electrophoresis was run at pH 8.6, veronal buffer (μ = 0.025) with a constant current of 5 mA per cm at 4°C

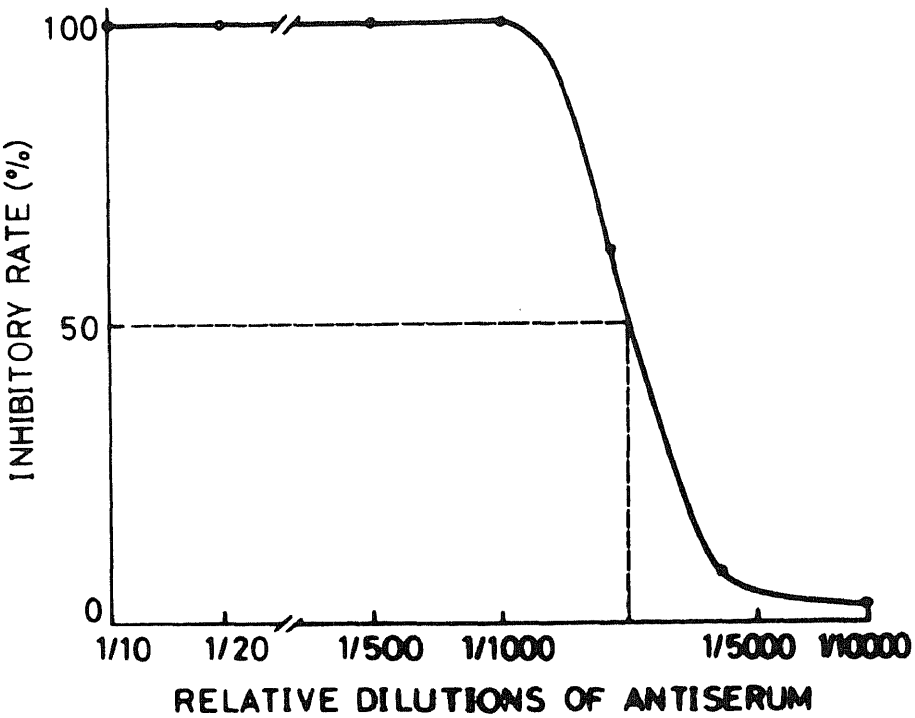


Fig. 16. Neutralization of the renin activity by the anti-fraction A renin antibodies. Pure fraction A renin, which could generate 800 pg angiotensin I per ml per h from the angiotensinogen, was incubated with relatively diluted antiserum (100 µl) for 16 h at 4°C. Then the renin activity of the mixture was determined under the same conditions described in MATERIALS AND METHODS in this chapter.

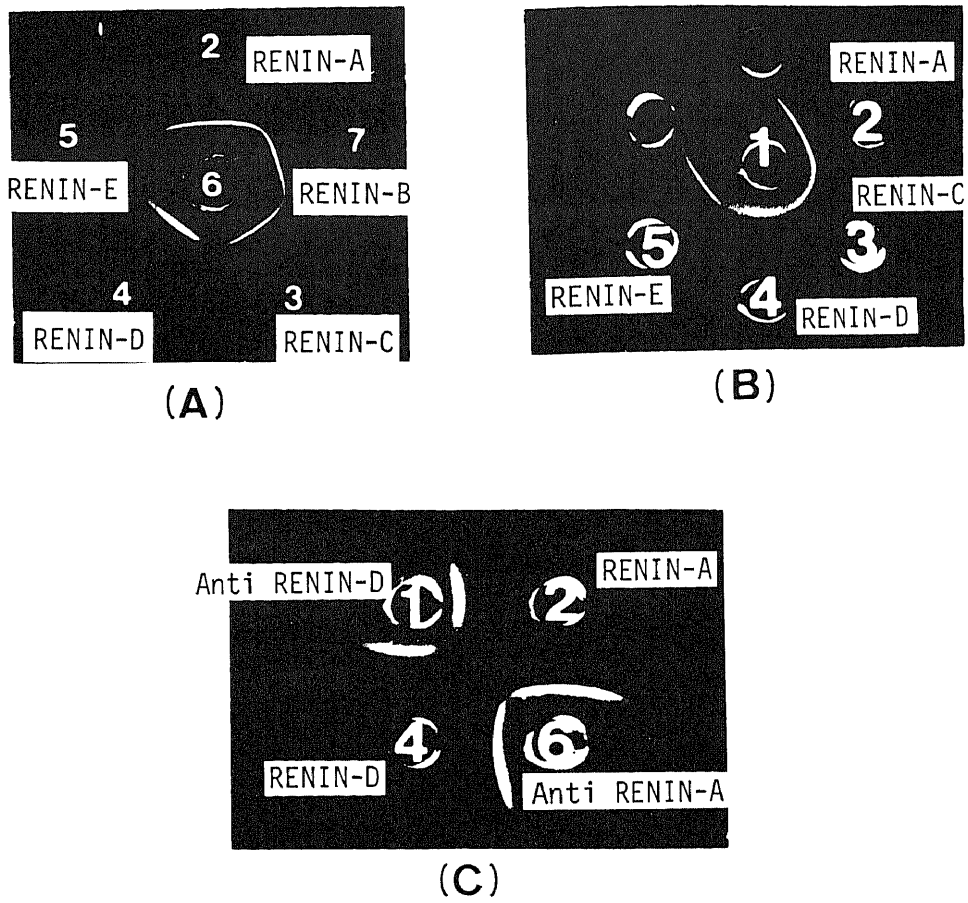


Fig. 17. Immunological cross-reactivity among fraction-A, -B,-C,-D, and -E renin. Fig.(A): immunodiffusion pattern of anti-fraction-A renin antibodies(6), fraction-A(2), -B(7), -C(3), -D(4), and -E(5) renin. Fig.(B): immunodiffusion pattern of anti-fraction-D renin antibodies (1), fraction-A(2), -C(3), -D(4) , and -E(5) renin. Fig.(C): immuno-diffusion pattern of anti-fraction A renin(6), anti-fraction D renin(1) antibodies, fraction-A(2), and fraction-D(4) renin. Other experimental conditions were similar to that described in the legend for Fig. 15.

single precipitate band until the original antisera solution was diluted 2 times with saline (Fig. 15A). Similarly, a clear precipitation line of submaxillary gland renin and antisera was also observed in an immunoelectrophoresis (Fig. 15B). The antisera used in this experiment neutralized 50% of submaxillary gland renin at 1 : 2,000 dilution, as shown in Fig. 16. Double diffusions of fraction-A , -B, -C, -D, -E and the antirenin antibodies of fraction-A or -D was observed to produce a single precipitate band, as shown in Fig.17A and B. Moreover, Fig. 17C shows that anti-fraction A renin antibodies and anti-fraction D antibodies cross-react with fraction-D renin and fraction-A renin, respectively.

5. DISCUSSION

In this study, the author established a rapid and large scale isolation of submaxillary gland renin using an affinity column and a CM-cellulose column. As much as 9.6 mg of the purified renin was obtained from 4.1g of male mouse submaxillary gland by the method. This high yield is marked contrast to the yield in the case of kidneys. For example, 90 kg of hog kidney was needed to obtain the same amount (9.6 mg) of pure hog kidney renin (4).

The elution profile of the fractions on CM-cellulose in

Fig.10 was similar to that of renin-A, -B, -C, -D, and -E on the final CM-cellulose column reported by Cohen et al. (13). However, the yield of fraction-C in the present method is much higher than that of renin-C in Cohen's method. Moreover, it is noteworthy that (i) the present method eliminates all four consecutive fractionation steps in Cohen's method (13) to obtain pure submaxillary gland renin, (ii) one step of affinity column chromatography starting from the homogenate of submaxillary gland is sufficient to obtain a preparation which is as absolutely pure renin, and (iii) the combined yield of renin activity in the present method is much higher than that in Cohen's method.

In 1973, Murakami et al. (45) initiated a preliminary study on the isolation of renins from hog kidney and mouse submaxillary gland, using pepstatin-aminoethyl polyacrylamide gel. However, this gel (45) had a very low affinity for renin, probably due to the short spacer or to the low content of pepstatin in the gel. Pepstatin-aminohexyl-agarose gel (4) is superior to pepstatin-aminoethyl polyacrylamide for isolation of renin from kidneys (4) or from submaxillary glands.

The final CM-cellulose column was effective in separating the five active fractions, but did not increase their specific activity. Fraction-A and -C, the major renin components in the submaxillary gland are more active than

the minor fraction-B, -D, -E, although the five renins were identical immunologically. Since, large amounts of proteases are present in mouse submaxillary glands (44), fraction-B, -D and -E may be degradation products formed from fraction-A and -C during their isolation. To check this possibility, renins in male mouse submaxillary gland were isolated by the procedure described above in the presence of a mixture of proteinase inhibitors (10 mM EDTA, 5 mM tetrathionate, and 1 mM diisopropylfluorophosphate). However, almost no change was seen in the elution profile on the CM-cellulose column. More recently, Catanzaro et al. (78) demonstrated by cell-free translation and pulse-chase experiments that mouse submaxillary gland renin was synthesized as a preprorenin (Mr=45,000) which was converted to a prorenin (Mr=43,000) and then to renin (Mr=38,000) by rapid processing, which was cut slowly to give a two-chain form (Mr=33,000$\pm$5,000). In this study, only fraction-D renin was determined to have a molecular weight of 38,000 by SDS-PAGE with DTT, although the molecular weight of other renins was 33,000. Moreover, five renins were immunologically identical (Fig. 17). These results indicate that fraction-D renin, which was converted from prorenin, was modified slowly to be fraction-A, -B, -C and -E renins by actions of some proteases.

CHAPTER IV. MOLECULAR WEIGHTS OF RENIN AND THEIR RENIN ACTIVITIES IN THE SUBMAXILLARY GLANDS OF 1- TO 8-WEEK-OLD MICE

1. SUMMARY

Renin activity in the extract from the submaxillary gland of 1- to 8-week-old albino male mice was found to increase with increase of age and to reach a maximum in 6-week-old mice in which the renin activity was 7.5×10^5 times as high as that of 1-week-old mice. The author found three unfamiliar renins of different molecular weights (Mr=48,000, Mr=37,000, Mr=31,0000 in addition to a normal renin (Mr=41,000) in the submaxillary gland of 1- to 3-week-old mice. An unfamiliar renin (31,000) increased notably in the submaxillary gland of 6-week-old mice, but it almost disappeared in the gland of 7-week-old mice in which a normal renin (Mr=41,000) increased and became a major form of renin. The renin activity in all three of the unfamiliar renins was completely inhibited by antibodies raised against pure normal renin, suggesting that all the unfamiliar renins found have a precursor common to normal renin.

2. INTRODUCTION

A very large amount of renin-like substance has been found in the submaxillary gland of adult male mice (12,14, 16,54-57), although its physiological role is not yet known. This enzyme was purified and characterized, and its enzymatic and molecular properties (13) and its pressor activity (59,60) were found to be very similar to renal renin(E.C. 3.4.99.19). Since mouse submaxillary gland renin could be purified in a large quantity, it was used as a model of renal renin to delineate the detailed features of the active site of renin (17).

In recent years, high molecular weight renin (Mr= 50,000-63,000) and inactive renin or prorenin have been observed in kidney or blood circulation (2,32,38,39,61). However, neither high molecular weight renin nor inactive renin were detected in the mouse submaxillary gland and the only form of renin demonstrable in the submaxillary gland was an active 40,000 dalton form (13,62). In this chapter, the author showed mouse submaxillary gland renins of different molecular weights observed in 1- to 7-week-old mice and the age-related difference in their renin activity.

3. MATERIALS AND METHODS

Preparation of crude extract The submaxillary glands at each week were excised from mice of the JCL-ICR under ether anesthesia, immediately homogenized with 10 volumes of 0.01 M pyrophosphate buffer pH 6.5, containing 0.1 M NaCl with or without 5 mM sodium tetrathionate, 10 mM N-ethyl-maleimide, 10 mM EDTA and 1 mM diisopropylfluorophosphate, and centrifuged at 100,000 x g for 60 min. The supernatant of the homogenate was used as the crude extract from the submaxillary gland.

Molecular weight estimation The crude extract (0.5 ml) from the submaxillary glands of each week old mice was applied to a calibrated column (1.5 x 90 cm) of Ultrogel AcA 44. Renin was eluted at a flow rate of 6 ml/h with 0.01M pyrophosphate buffer, pH 6.5, containing 0.1 M NaCl. Bovine serum albumin (Mr=67,000), egg albumin (Mr=45,000), and α-chymotrypsinogen (25,000), were used as the standard proteins in the estimation of the molecular weight.

Renin assay and elicitation of antirenin antibodies were described in MATERIALS AND METHODS in CHAPTER II.

4. RESULTS

a) Age-related difference in the renin activity Table III shows the renin activity in the submaxillary gland of 1- to 8-week-old albino male mice. Renin activity of the submaxillary gland increased notably with increase of age and reached a maximum in 6-week-old mice in which the activity was 1.5×10^4 μg angiotensin I/g(wet weight)/h and was 7.5×10^5 times as high as that of 1-week-old mice.

b) Age-related difference in molecular size of renin

Using gel filtration, the author examined the molecular weight of the submaxillary gland renin from each stage of 1- to 7-week-old mice. Fig.18 shows a gel filtration profile of renin activity in crude extract from the submaxillary gland of 2-week-old mice. The author found three unfamiliar renins of different molecular weight in the submaxillary gland of 2-week-old mice in addition to normal renin with a molecular weight of 41,000. They were tentatively designated renin-48 (Mr=48,000), renin-37 (Mr=37,000) and renin-31 (Mr=31,000). All three of these unfamiliar renins were new types of renin in the submaxillary gland. The normal and unfamiliar renins observed in Fig.18 were also detected in the extract from the submaxillary gland in 1-week-old and 3-week-old mice.

Table III *Age-Related Differences in the Renin Activity and the Weight of Submaxillary Glands of Albino Male Mice, the JCL-ICR Strain*

Age (week)	Renin Activity (μg Angiotensin I/g*/hr)	(fold)	Submaxillary gland weight** (mg)	Body weight (g±SD)	Number of mice (n)
1	2.0×10^{-2}	(1)	16.7	4.9±0.05	30
2	5.6×10^{-2}	(2.8)	28.7	6.8±0.08	15
3	2.9	(1.5×10^{2})	47.5	13.8±0.95	8
4	8.4×10^{2}	(4.2×10^{4})	76.0	22.2±0.61	8
5	1.6×10^{3}	(8.0×10^{4})	127	28.7±1.20	6
6	1.5×10^{4}	(7.5×10^{5})	164	32.4±1.00	6
7	5.8×10^{3}	(2.9×10^{5})	180	34.0±1.60	6
8	2.2×10^{3}	(1.1×10^{5})	200	34.4±1.30	6

*one gram of submaxillary glands in the wet weight
**the wet weight of a pair of the submaxillary glands

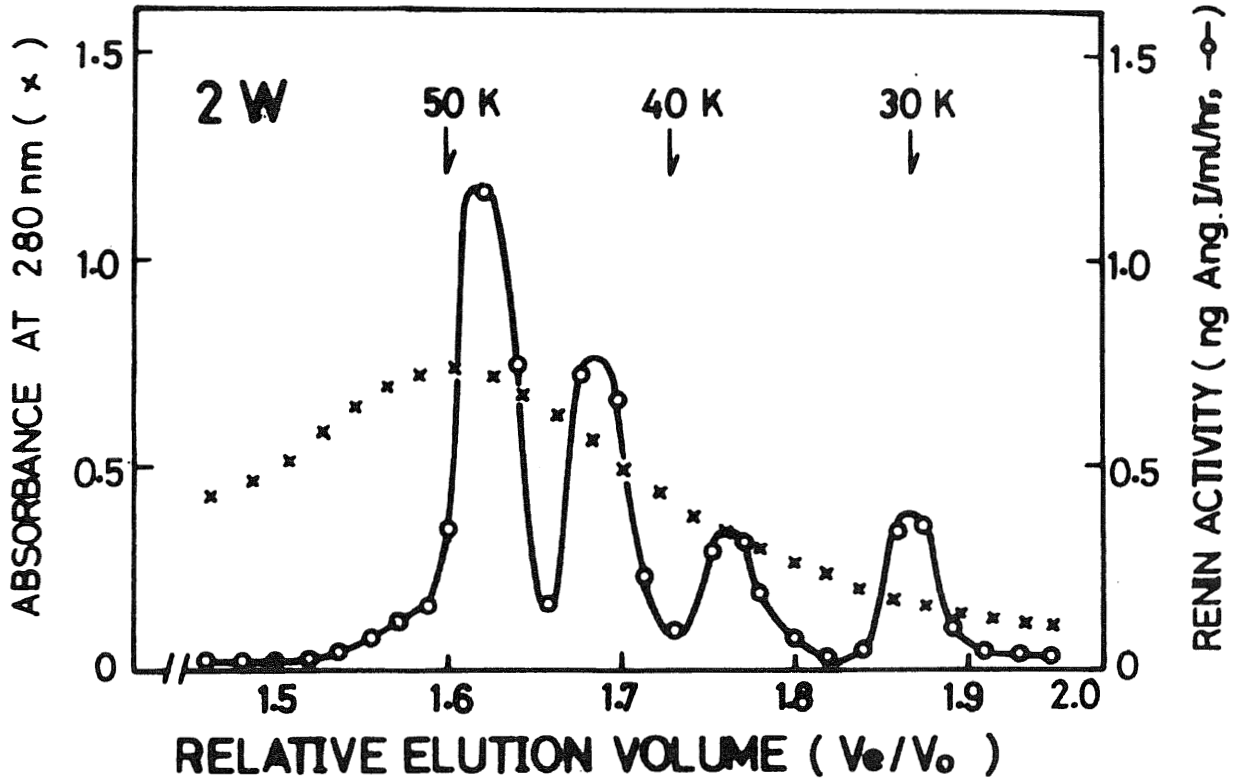


Fig. 18. Gel filtration profile of renin activity in the crude extract prepared from submaxillary glands of 2-week-old mice.

Figure 19 shows a gel filtration profile of renin activity in the crude extract from the submaxillary gland in 4-, 5-, 6- and 7-week-old mice. Together with a large increase of renin activity, a new type of renin, renin-31, increase notably in the submaxillary gland of 6-week-old mice, but it had almost disappeared in the gland of 7-week-old mice in which normal renin increase and became a major form of renin. The molecular shift from renin-31 in 6-week-old mice to normal renin in 7-week-old mice was accompanied by a decrease of renin activity in the submaxillary glands.

Renin-31, -37 may be degradation products from the high molecular weight renin (Renin-41, -48) because submaxillary gland of male mice contains a large amount of proteases (44). To minimize this possibility, the author extracted renin from the submaxillary gland of 2-week-old male mice in the presence of protease inhibitors as described in MATERIALS AND METHODS. The elution profile of the extract was similar to that of the extract in the absence of inhibitor, as shown in Fig. 20.

c) Immunological identification of renin-like substances

The author examined the immunological properties of renin-like substances (Renin-48, -37, -31) appearing in the submaxillary glands of 2-week-old male mice, which was prepared in the presence of protease inhibitors. As shown in Fig.20,

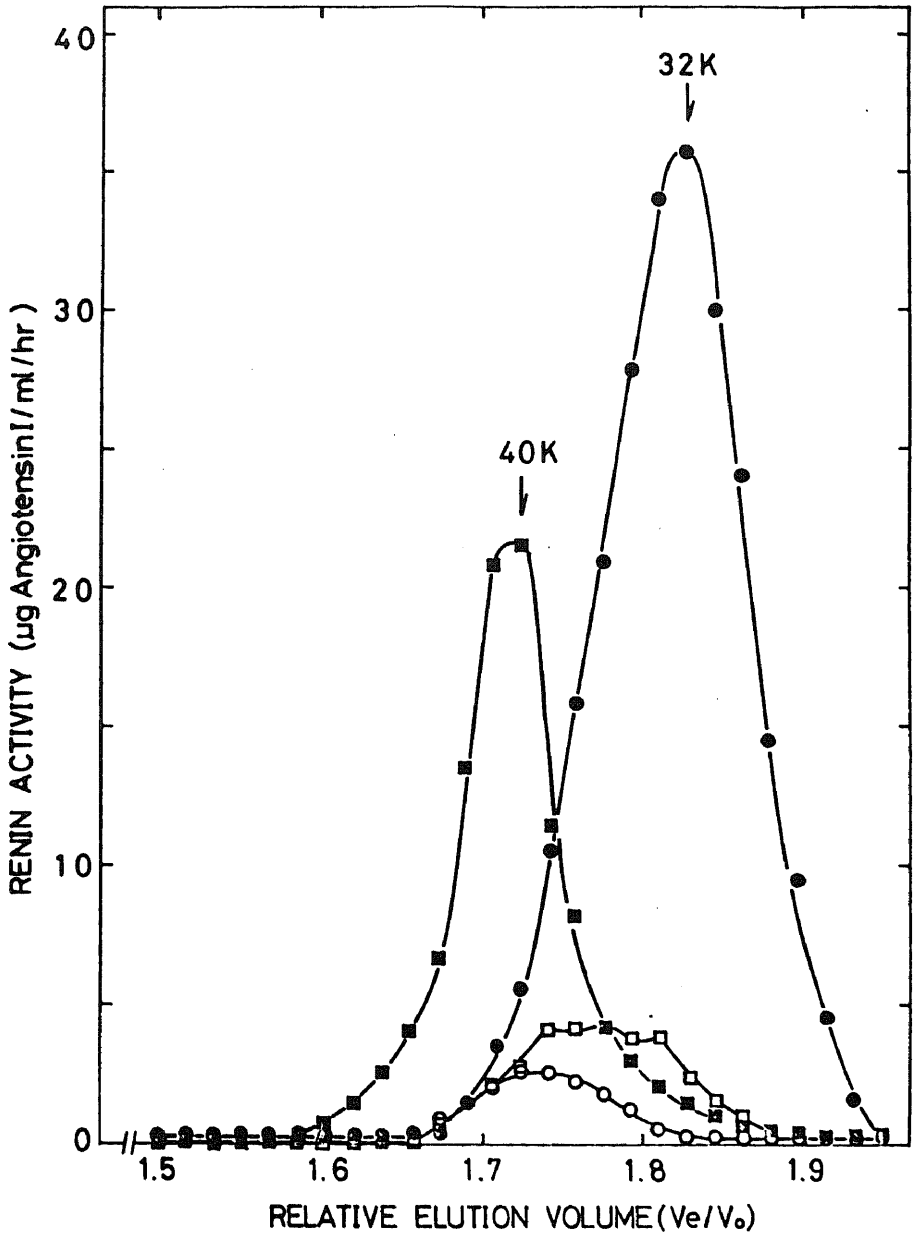


Fig. 19. Gel filtration profiles of renin activity in the crude extract from the submaxillary gland of 4- to 7-week-old mice. The elution priofiles of the extracts from 4-, 5-, 6- and 7-week-old mice were expressed as -O-, -□-, -●-, and -■-, respectively.

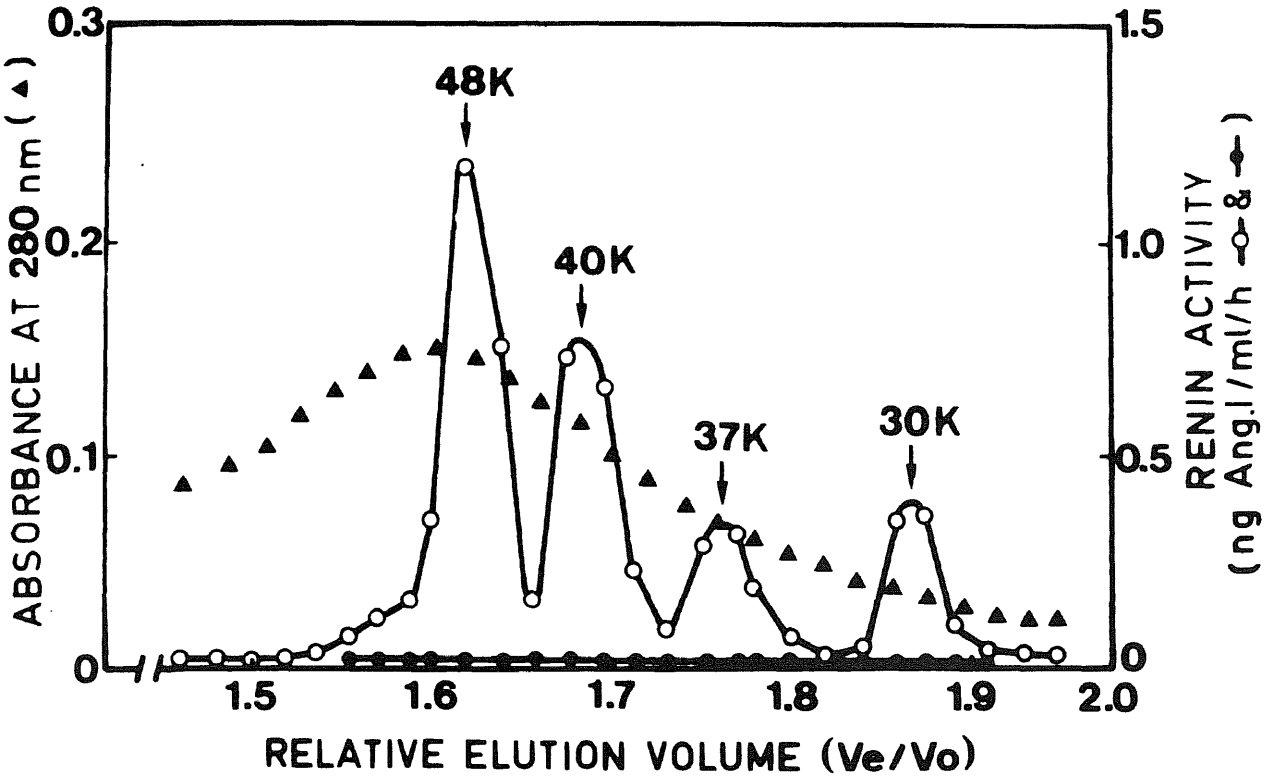


Fig. 20. Multiple elution profile of renin activity in the 2-week-old mice and its inhibition by anti-fraction A renin antibodies. The inhibition of renin activity was observed as follows; a sample(100 µl) was incubated with the anti-fraction A renin antibodies(1:100 dilution) for 16 h at 4° C and then renin activity was analyzed under the same condition described in MATERIALS AND METHODS of chapter II. Renin activity was not detectable in the diluted antibodies. Renin activity with and without the antibodies is presented as -●-, and -O-, respectively.

all the renin activities of the renin-like substances were completely inhibited by the antisera, indicating that all the four renin-like substances are immunologically identical to renin and not pseudorenin or cathepsin D. Moreover, each renin activity of renin-31, -37, -41 in 6-, 5-, and 7-week-old mice, respectively, was also completely inhibited by the antibodies. This is the first demonstration supporting a view that renin-like substances have a precursor common to renin.

5. DISCUSSION

The notable age-related difference in the renin activity is compatible with that reported by Bhoola et al. (16), although they could not detect renin activity in the mouse submaxillary gland of 1- to 4-week-old mice. This is the first presentation to determine renin activity quantitatively in the submaxillary gland from 1- to 8-week-old mice. The radioimmunoassay for the renin assay used in the present investigation enabled us to determine the very low renin activity in the submaxillary gland from 1- to 4-week-old male mice.

In this study, renins are designated according to their average molecular weight to the nearest whole 1,000 as determined by gel filtration on Ultrogel AcA 44. This nomen-

clature was also used by Levine et al. (28) and Overturf et al. (38).

The finding of most interest in this study is the presence of a high molecular weight renin in the submaxillary gland (Mr=48,000). The high molecular weihgt renin was easily demonstrable in young male mice, but difficult to find in the matured stage. Recently, Poulsen et al. (18) demonstrated that renin in the mouse submaxillary gland was synthesized as 50,000 dalton single chain polypeptide in cell-free translation systems. However, this inactive prorenin was not identical to the active renin-48 found in this study, although its molecular weight was similar. This active high molecular weight renin is not considered to be pre-prorenin or prorenin because the precursor form are inactive in the kidney (41,42) and plasma (63), although these inactive form of renin are not yet detected in the extract of submaxillary gland. The high molecular weight renin may be a complex of renin and its binding protein like that proposed in the kidney (19,20).

CHAPTER V. DEMONSTRATION AND CHARACTERIZATION OF A HIGH MOLECULAR WEIGHT RENIN IN THE SUBMAXILLARY GLAND

1. SUMMARY

A high molecular weight renin (Mr=75,000±3,000) was found in the submaxillary gland of adult male mice by a sedimentation equilibrium method. Most renin in the submaxillary gland is supposed to be the high molecular weight form. This high molecular weight renin was converted into normal renin (Mr=40,000±3,000) by acidification or dilution and reconstituted from normal renin and a renin-binding protein fraction. The high molecular weight renin was also clearly separated from normal renin by three cycles of gel filtration. A renin-binding protein was purified to be apparently homogeneous on polyacrylamide gel electrophoresis by gel filtration, pepstatin-aminohexyl-Sepharose 4B column chromatography and DEAE Sepharose CL-6B column chromatography. Its molecular weight was estimated to be 25,000 by SDS-polyacrylamide gel electrophoresis. The renin-binding protein inhibited the renin activity potently and bound renin to form 75,000 dalton renin. It was, therefore, concluded that the high molecular weight renin was composed of normal renin and a renin-binding protein.

2. INTRODUCTION

Male mice submaxillary glands synthesize large amounts of an enzyme which is very similar to renal renin in molecular and enzymatic properties (13,14). The mouse submaxillary gland (MSG) renin has been easily purified (60,91,92) and used as a substitute for renal renin: the active site of MSG renin was studied in detail (17); a mRNA of the renin was isolated from MSG and translated (18,90); a complete amino acid sequence of MSG renin and its precursor were determined from the cDNA (64,89) and the protein preparation (65); moreover, the pathway of renin biosynthesis was examined in MSG (78,88).

Since an initial investigation by Boyd (19), a high molecular weight renin, which is considered to be a complex of normal renin and a renin-binding protein, has subsequently been studied in the kidney, brain and plasma (29,39, 70,71,77,81,82). Many investigators have attempted to identify the high molecular weight renin in MSG of an adult age by various methods such as gel filtration (46), immunoprecipitation (62), and affinity column chromatography containing antirenin antibodies (91). However, no high molecular weight renin could be demonstrated to exist in MSG. The demonstrable form of renin in MSG has been considered to be only an active 40,000 dalton form. In the

present study, existence of a high molecular weight renin was elucidated in MSG extract by a new method, sedimentation equilibrium analysis with a tabletop air-driven ultracentrifuge, and the high molecular weight renin was proved to be composed of normal renin and a renin-binding protein.

3. MATERIALS AND METHODS

Preparation of the submaxillary gland extract The submaxillary gland of adult male mice (JCL-ICR, 8-week-old) were homogenized with 4 volumes buffer (0.01M pyrophosphate, pH 6.5) of the wet weight of the tissue in the presence of 1 mM diisopropylfluorophosphate and 0.1M NaCl. The homogenate was centrifuged at 100,000 x g for 60 min, and the supernatant was used as a crude extract.

Purification of Normal Renin Fraction-A, -B, -C, -D and -E renins were obtained from the submaxillary gland according to the method described in chapter II. Mixture of those renins was used as normal renin in this study.

Gel Filtration of the Extract The crude extract (50 ml) from the submaxillary glands of 8-week-old mice or other sample was applied to a calibrated column (5.0 x 80 cm) of Ultrogel AcA 44. Renin was eluted at a flow rate of 56 ml/h with 0.01M pyrophosphate buffer, pH 6.5, containing 0.1 M NaCl. Bovine serum albumin (Mr=67,000), egg albumin (Mr=

45,000), and α-chymotrypsinogen (Mr=25,000), were used as standard proteins in the estimation of the molecular weight.

Molecular Weight Determination Molecular weights of renin were determined by a new method, sedimentation equilibrium analysis with a tabletop air-driven ultracentrifuge. The centrifuge was developed to determine the molecular weight of protein in the crude preparation level (67,68). Each sample(100 μl), which contained 1 kBq ^{14}C -methylated egg albumin or ^{14}C -methylated bovine serum albumin as an internal standard, was centrifuged at 35,000 rpm for 24 h at room temperature with a Beckman Airfuge and then fractionated according to the method of Ueno et al. (66). Each fractionated sample(10 μl) was diluted with 990 μl of 0.1M phosphate, pH 6.5, containing 0.1% bovine serum albumin and analyzed for the renin activity and ^{14}C -radioactivity. The molecular weights of proteins were calculated from the plots of ln c versus r^2 . The c is the concentration of solute in the fractionated sample and r is the radial distance. The solute concentration was shown as total renin activity in the fractionated sample or ^{14}C -radioactivity. Assuming globular shape, the slope obtained from the plots was related to the molecular weight (Mr) of a protein according to the following equation: $d\ln c/dr^2 = Mr\omega^2(1-\upsilon\rho)/2RT$, where υ is the partial specific volume of the solute, ρ is the solution density, T is the absolute

temperature, R is the gas constant and ω is the angular velocity. A value of 0.73 was assumed for the partial specific volumes of renin and other internal standard proteins.

Acidification The pH of the sample was adjusted to pH 3.0 with 6N-HCl; the acidified sample was stood for 60 min at 4°C; the pH was readjusted to 6.5 with 6N-NaOH; moreover, bovine serum albumin was added into the acidified sample to attain a protein concentration of 10 mg/ml before the centrifugation to provide density stabilization.

Renin assay using a fluorogenic substrate The structure of the fluorogenic substrate, which was synthesized by Murakami et al. (83), is succinyl-Arg-Pro-Phe-His-Leu-Leu-Val-Tyr-4-methylcoumaryl-7-amide. Fluorometric renin assay was carried out as follows. This procedure measures the extent of cleavage of the Leu-Leu bond in the substrate by the renin action in terms of the quantity of 7-amino-4-methylcoumarine liberated by the subsequent digestion of Leu-Val-Tyr-4-methylcoumaryl-7-amide with leucine aminopeptidase (Sigma). The standard assay mixture contained 250 µl of 0.05M pyrophosphate buffer, pH 6.5, containing 0.1 mM zinc acetate, 25 µl of substrate (1 µmole/4ml of dimethylformamide) and 25 µl of renin preparation. The mixture was incubated for 5-15 min at 37°C and the reaction was terminated by heating it for 5 min at 100°C. After

cooling, 50 mU leucine aminopeptidase was added to the mixture. This final mixture was incubated for 90 min at 37°C and the amount of free 7-amino-4-methylcoumarine was measured using Hitachi spectrofluorometer model 204. Excitation and emission wave lengths were 380 and 440 nm, respectively. The instrument was standerdized daily so that the fluoresence intensity of 1.0 μM 7-amino-4-methyl-coumarine solution gave a full scale response on a chart recorder.

Renin-inhibitory activity The renin-inhibitory activity was assayed by the following two methods. (1) Determination by using hog plasma renin substrate: each sample (70 μl), eluted from a pepstatin aminohexyl-Sepharose 4B column, was incubated with 30 μl (12 ng) of pure submaxillary gland renin at 37°C for 30 min, and then the remaining activity of renin in the incubated mixture was determined by the radioimmunoassay of angiotensin I. (2) Determination by using a fluorogenic renin substrate: each sample (70 μl), eluted from a pepstatin-aminohexyl-Separose 4B column or DEAE Sepharose CL-6B column, was incubated with 30 μl of pure submaxillary gland renin (0.3 μg) at 37°C for 30 min, and then the remaining activity of renin in the mixture was determined according to the procedure described above. For control experiments, buffer solutions (70 μl) instead of sample solutions were incubated with the renin solution. The

initial velocity of the enzyme reaction could be measured under those experimental conditions.

Angiotensinase activity Angiotensinase activity was determined by the decomposition rate of angiotensin I. Samples (100 μl) were incubated with 40 μM angiotensin I under the condition identical with that for renin assay, and the remaining angiotensin I content was determined by the radioimmunoassay.

Elicitation of antirenin antibodies and determination of renin activity by radioimmunoassay of angiotensin I were carried out by the methods described in chapter II.

4. RESULTS

a) Demonstration of a high molecular weight renin in the mouse submaxillary gland. A crude extract of male mice submaxillary gland was prepared as described in "Materials and Methods", and the molecular weight of mice submaxillary gland renin was estimated by the following two methods.

a-1) Sedimentation Equilibrium Analysis Figure 21 shows linear relationships between the logarithm of renin activity and the square of the distances from the center of a rotor in a tabletop centrifuge. The molecular weight of renin was calculated to be 75,000±3,000 (n=4, Fig. 21A). The

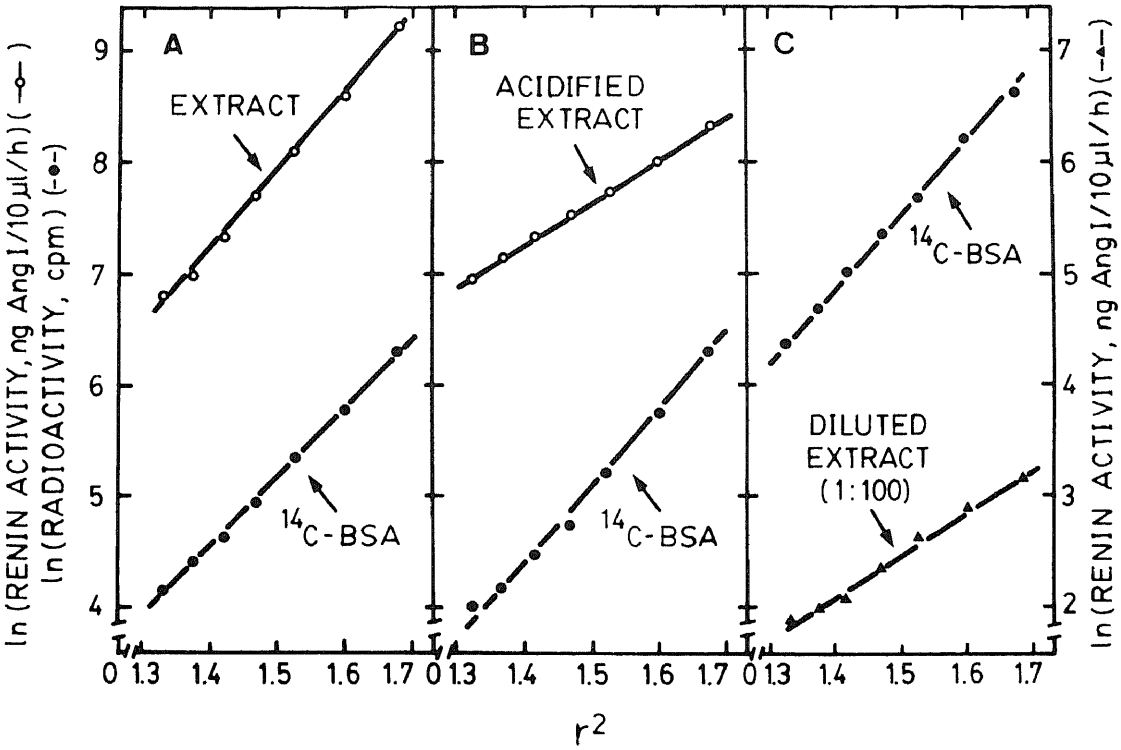


Fig. 21 Sedimentation equilibrium profiles of renin in the submaxillary gland extract. Sedimentation equilibrium profiles of renin are presented in Fig. A, B and C upon the submaxillary gland extract, the acidified extract and the diluted extract, respectively. The extract was acidified at pH 3.0 and 4°C for 15 min, and then the pH of the extract was readjusted at 6.5 with 0.1M NaOH. The acidified and diluted extracts were centrifuged after their protein concentrations were increased to 10 mg/ml by addition of bovine serum albumin to provide density stabilization. Abbreviations: ^{14}C-BSA, ^{14}C-methylated bovine serum albumin.

molecular weight of renin was decreased to 40,000±3,000 after acidification of the extract at pH 3.0 and 4°C for 15 min or after dilution of the extract (1:100) (Fig. 21B and C). However, the molecular weight of renin was calculated to be 75,000 even after the extract was concentrated to one-half of its original volume by ultrafiltration. That of pure normal renin (0.6 mg/ml) was also determined to be 40,000 ± 3,000 (n=4). These results indicate that major renin in the mouse submaxillary gland is a high molecular weight form (Mr=75,000) and this form was not detected by the methods conventionally used for molecular weight estimation. To confirm the above results, the molecular weight of mouse submaxillary gland renin was analyzed by a conventional method, gel filtration.

a-2) Gel Filtration As anticipated, most renin activity in the submaxillary gland was eluted in the normal 40,000 dalton region by gel filtration (fraction II in Fig. 22A). This result is consisted with those obtained by many investigators (46,62,91). However, a minor renin fraction appeared in a high molecular weight region (Fraction I in Fig. 22A). This fraction was concentrated and was applied again to the same column. A small portion of renin activity, which was 3-5% of total renin activity in the extract, was observed at a high molecular weight fraction (fraction III in Fig. 22B). The fraction III was also concentrated and

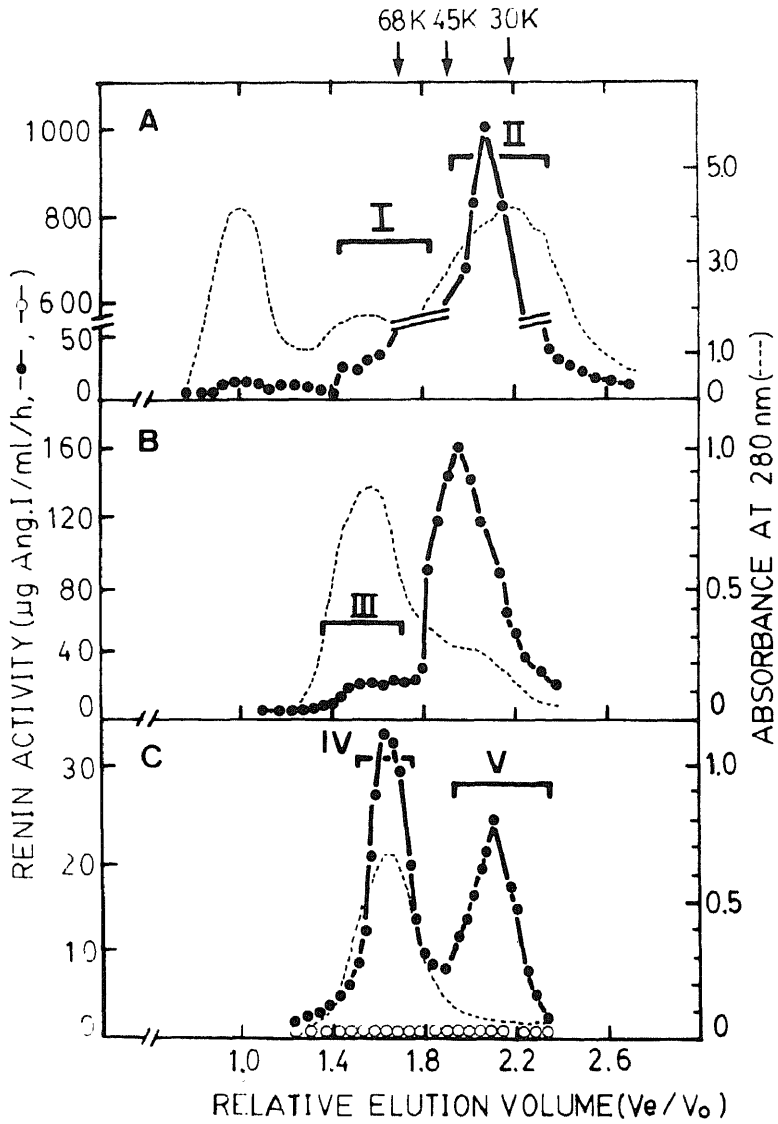


Fig. 22 Gel filtration profiles of renin in the submaxillary gland extract on an Ultrogel AcA 44 column. The renin elution profiles when the submaxillary gland extract was applied to an Ultrogel AcA 44 column (5.0 x 80 cm, Fig. A) were presented along with rechromatography of the fraction I on the same column (Fig. B) and rechromatography of the fraction III on the same column (Fig. C). Fraction I and III were rechromatographed after concentration to less than 50 ml by ultrafiltration. The renin activity was determined before (-●-) and after (-○-) incubation with the antifraction A renin antibodies (1:100 dilution) for 16 h at 4°C.

chromatographed on the same column. A high molecular weight renin was clearly observed at 75,000 dalton region by the last gel filtration (Fraction IV in Fig. 22C).

The activity of renin eluted from the column was completely neutralized by the anti-renin A antibodies, indicating that the high molecular weight renin was not pseudorenin (48,49) or γ-renin (72). The molecular weight of the high molecular weight renin was also determined to be 75,000±3,000 by a sedimentation equilibrium analysis with a Beckman Airfuge (Fig. 23).

b) Formation of a high molecular weight renin by incubation of renin with renin-binding protein fraction Renin in a normal renin fraction (fraction II in Fig. 22) was removed by pepstatin-aminohexyl-Sepharose 4B column chromatography as described in the legend to Fig. 24, and a renin-free fraction thus obtained was used as a renin-binding protein fraction. A high molecular weight renin (Mr= 75,000±3,000, n=4) was observed by the sedimentation equilibrium method (Fig. 24) after the renin-binding protein fraction was incubated with pure normal renin. The concentrations were the same as the ones in the submaxillary gland extract. The molecular weight of the normal renin was determined to be 40,000±3,000 (n=4) by the same method (Fig. 24). This value of 40,000 ± 3,000 was in good agreement with

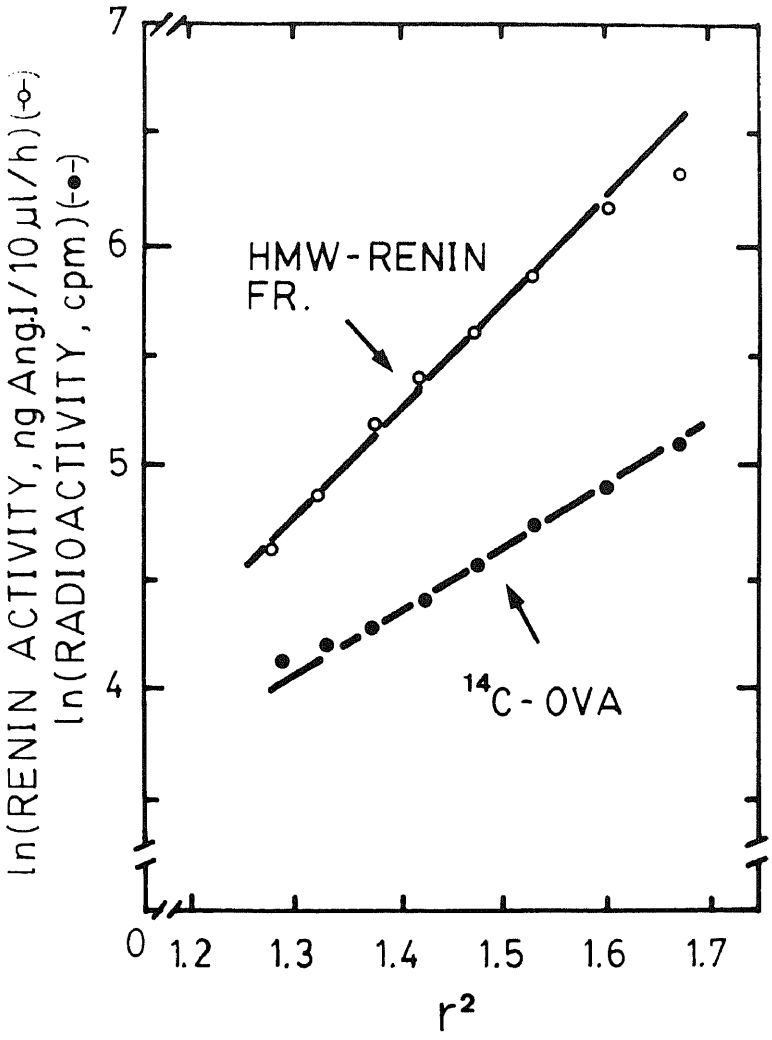


Fig. 23 Sedimentation equilibrium analysis of a high molecular weight renin fraction obtained by gel filtration. Renin contained in the fraction IV (Fig. 2) was analyzed by the sedimentation equilibrium method after the protein concentration was adjusted to 10 mg/ml with bovine serum albumin.

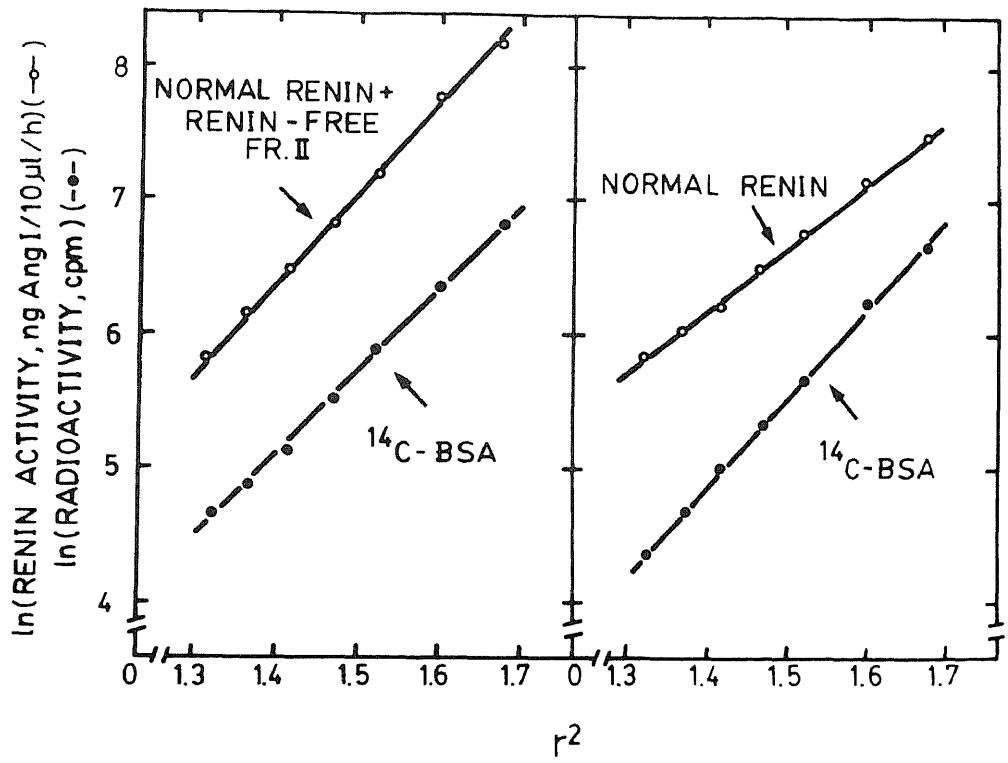


Fig. 24 Sedimentation equilibrium analysis of pure renin incubated with a renin-binding protein fraction. Pure renin of 1 ml (0.6 mg/ml) was incubated with a renin-binding protein fraction of 1 ml (20 mg/ml), and aliquots (100 µl) were analyzed by sedimentation equilibrium analysis (left figure). As a reference, pure renin (0.3 mg/ml) was also analyzed by the method (right figure). The renin-binding protein fraction was prepared as follows. The fraction II (Mr=20,000-40,000) in Fig. 2A was applied to a pepstatin-aminohexyl-Sepharose 4B column (0.5 x 20 cm) in the presence of 0.5M NaCl. The flowthrough fraction (a renin-free fraction II) was used as a renin-binding protein fraction. Abbreviations: RENIN-FREE FR. II, renin-free fraction II; ^{14}C-BSA, ^{14}C-methylated bovine serum albumin.

that determined by many investigators, using different methods (2,13,46,60,62,91,92).

c) Purification of a renin-binding protein The author thought that a renin-binding protein possibly inhibited renin, and tried to isolate a renin inhibitor from the submaxillary gland in order to obtain a pure renin-binding protein. A high molecular weight renin fraction, the fraction III in Fig. 22B, was used as a starting material for the purification. The fraction III was applied to an Ultrogel AcA 44 column and the fraction V was obtained (Fig. 22C). The fraction V, which contained normal renin and a renin-binding protein, was applied to a pepstatin-amino-hexyl-Sepharose 4B column for separating renin from other protein. Renin-inhibitory activity was eluted by 0.5M NaCl from the column, as shown in Fig. 25. The inhibitory activity could be measured using two renin substrates: hog angiotensinogen and a fluorogenic substrate.

The renin inhibitor preparation was further purified to be homogeneous on polyacrylamide gel electrophoresis by DEAE Sepharose CL-6B column chromatography (Fig. 26 and 27). The renin-inhibitory activity, which inhibited 60% of the renin activity at maximum, was eluted from the column, as shown in Fig. 26. None of the inhibitory fraction decomposed angiotensin I (40 μM), or inhibited the activity of leucine

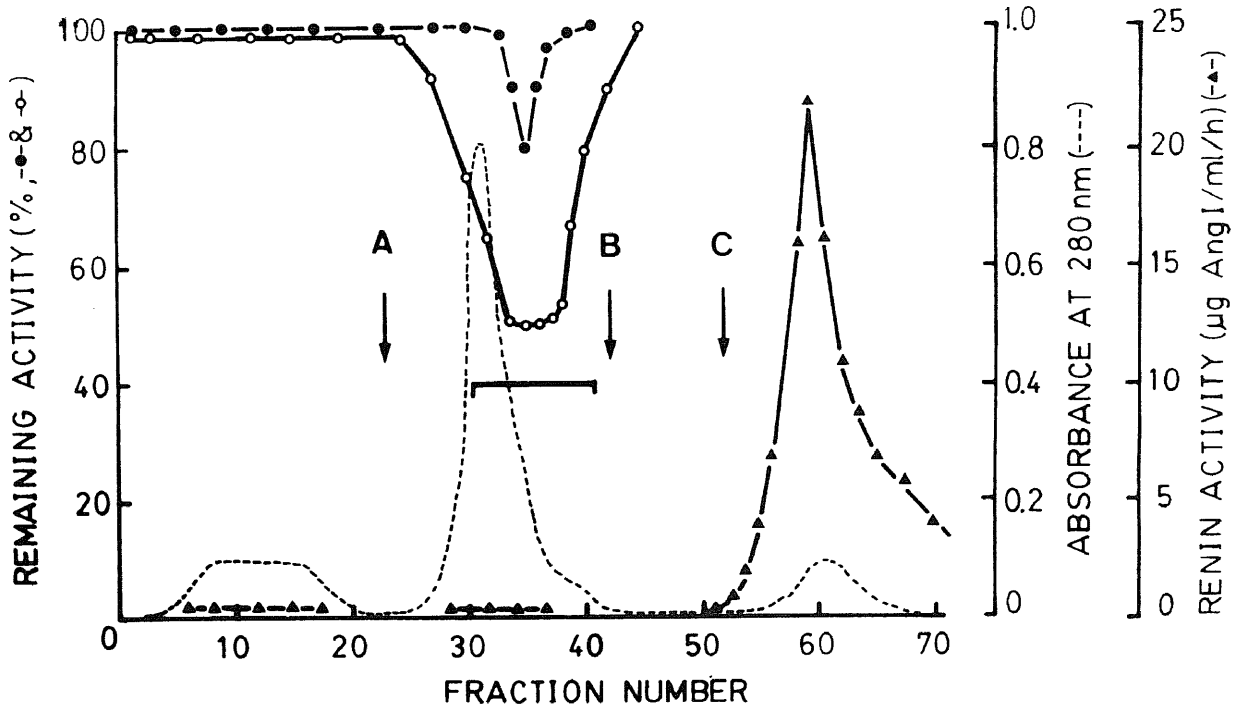


Fig. 25. Pepstatin-aminohexyl-Sepharose 4B column chromatography of a renin-binding protein preparation which was given by gel filtration of a high molecular weight renin fraction. The fraction V, which was obtained by gel filtration of fraction III (a high molecular weight renin fraction), as shown in Fig. 22, was applied to a pepstatin-aminohexyl-Sepharose 4B column (0.5 x 20 cm). Proteins were eluted by 0.01M pyrophosphate, pH 6.5, containing 0.5 M NaCl (A) and then 0.1 M Tris-HCl, pH 7.5, (B). The renin-inhibitory activity was measured using a hog renin substrate (-●-, 80 ng angiotensin I equivalent/ml) and a fluorogenic renin substrate (-○-, 20 μM). The renin activity, which was eluted by 0.5 M Tris-HCl, pH 7.5 (C), was presented as -▲-.

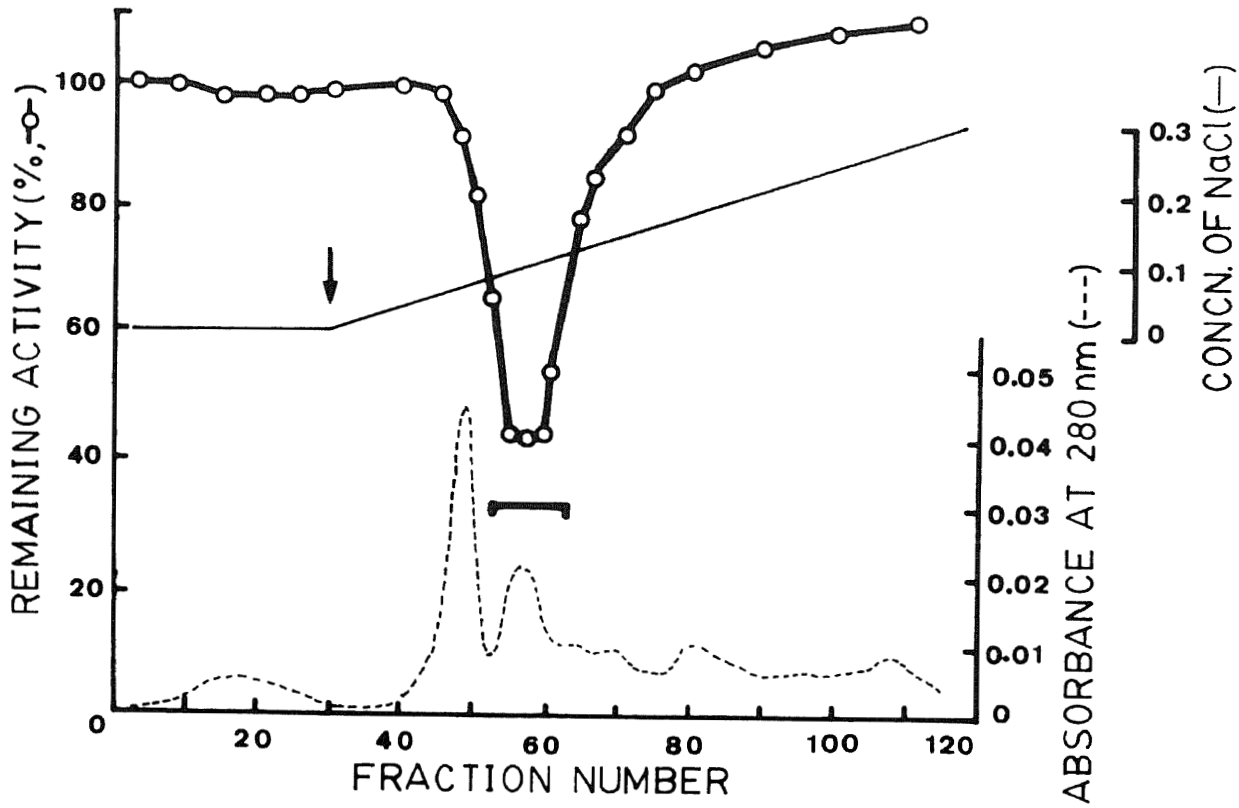


Fig. 26. DEAE Sepharose CL-6B column chromatography of a renin inhibitor preparation obtained by pepstatin-amino-hexyl-Sepharose 4B column chromatography of a renin-binding protein preparation. The inhibitory fraction of renin activity, presented by a bracket in Fig. 25, was dialyzed against 0.02 M Tris-HCl, pH 8.0, and applied to a DEAE Sepharose CL-6B column (1.0 x 18 cm) previously equilibrated with 0.02 M Tris-HCl, pH 8.0. Proteins were eluted with a linear gradient of 0-0.3M NaCl in the buffer. The renin inhibitory activity was measured using a fluorogenic renin substrate (-O-). The flow rate was 8 ml/h and 2 ml fractions were collected.

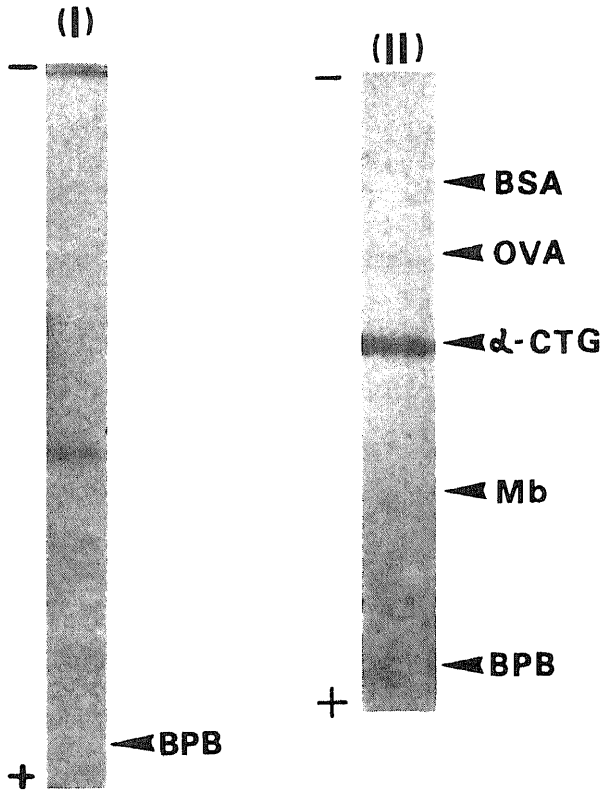


Fig. 27. Polyacrylamide slab-gel electrophoresis of purified renin inhibitor (renin-binding protein). The renin inhibitory fraction, presented by a bracket in Fig. 26, was concentrated and aliquots (25 µl) were subjected to a 7.5%-polyacrylamide gel electrophoresis (I), or to a 12.5% SDS-polyacrylamide gel electrophoresis (II). A protein was stained by Coomassie blue R-250. Abbreviations: BSA, bovine serum albumin; OVA, ovalbumin; α-CTG, α-chymotrypsinogen A; Mb, myoglobin; BPB, bromophenol blue.

aminopeptidase (50 mUnit) under the present experimental conditions. The activity of renin which was inactivated by the renin inhibitor was completely recovered by dilution with more than 10 volumes buffer. The renin inhibitor did not react with antirenin antibodies raised against submaxillary gland renin. Its molecular weight was estimated to be 25,000 by SDS-polyacrylamide gel electrophoresis (Fig. 27).

The purified renin inhibitor was checked by sedimentation equilibrium analysis whether the inhibitor was the same as the renin-binding protein, one component of 75,000 dalton renin. The molecular weight of renin was determined to be 75,000±3,000 by the analysis after the concentrated renin inhibitor was incubated with pure submaxillary gland renin at 37°C for 30 min (Fig. 28). That of pure normal renin was calculated to be 38,000±3,000 according to the same method. These results indicated that the purified renin inhibitor was identical with the renin-binding protein.

Therefore, 100 μg of a renin-binding protein was purified from 30g of the submaxillary gland (150 mice) by those chromatography (Table IV).

5. DISCUSSION

This study is the first to demonstrate a high molecular weight renin in the mouse submaxillary gland. A new tech-

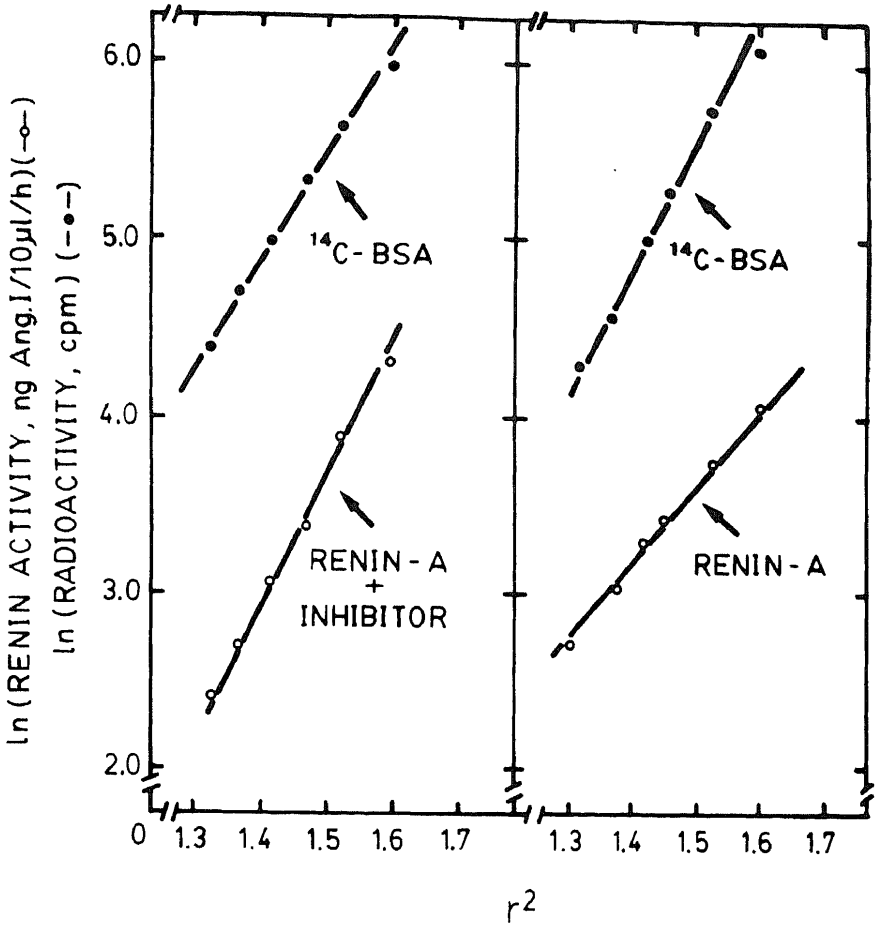


Fig. 28. Sedimentation equilibrium analysis of fraction-A renin incubated with a pure renin inhibitor. The renin inhibitory fraction, shown by a bracket in Fig. 26, was concentrated to one-tenth of its original volume and aliquots (70 µl) were incubated with 30 µl of fraction-A renin (0.3 µg) at 37°C for 30 min. Then the molecular weight of renin in the incubated mixture was determined by the sedimentation equilibrium method (left figure). For a control experiment, fraction-A renin (0.3 µg) was analyzed by the method after incubation with 70 µl of 0.02M Tris-HCl, pH 8.0, containing 0.1% of bovine serum albumin (right figure).

Table IV. Purification of a renin-binding protein

Steps	Total protein (mg)	Concn*(μg/ml) Producing 50% inhibition	Purification (fold)
1. High molecular weight renin fraction (Fr. III**)	100	-	
2. Gel filtration	8.0	-	
3. Pepstatin-AH-Sepharose 4B column	4.0	70	1.0
4. DEAE Sepharose CL-6B column	0.1	5.8	12

*The concentration of the sample protein required for 50% inhibition of purified renin (45 ng/ml).
**Fraction III was obtained by gel filtration of the submaxillary gland, as shown in Fig. 22.

nique, sedimentation equilibrium analysis with a tabletop ultracentrifuge, was used for the molecular weight determination in this study, because it has been difficult to find the high molecular weight renin in the submaxillary gland by conventional techniques. For example, the high molecular weight renin could hardly be detected in the first gel filtration but clearly detected after three cycles of the gel filtration on the same column (Fig. 22). This is probably due to the dissociation of high molecular weight renin into normal renin and renin-binding protein during gel filtration.

The present data obtained by the sedimentation equilibrium analysis also suggest that most renin in the submaxillary gland is a high molecular weight form (Fig. 21A). The high molecular weight renin was converted into normal renin by acidification or dilution (Fig. 21B and C), indicating that the high molecular weight renin under study is composed of renin and a renin-binding protein. This indication was confirmed by a reversible formation of high molecular weight renin from pure renin and a renin-binding protein fraction (Fig. 24). These results are similar to those with the high molecular weight renin from hog kidney extract which has been studied extensively (2,19,66,80). However, there is a definite difference in the size and other properties of high molecular weight renin in hog

kidneys and mouse submaxillary glands. The molecular weight of 75,000 for the high molecular weight renin in the submaxillary gland obtained in this study is lower than the 113,000 renin in hog kidney extract which was measured using the same sedimentaion equilibrium method (66). This result is consistent with the fact that a molecular weight of 20,000-40,000 for renin-binding protein in the submaxillary gland is lower than that of 56,000 in the hog kidney extract which was measured using the same gel filtration method (19,66,82). Takahashi et al. (81) purified a renin-binding protein from hog kidney extract and obtained a molecular weight of 40,000 by SDS-polyacrylamide gel electrophoresis. On the other hand, its molecular weight in the submaxillary gland was estimated to be 25,000 by the electrophoresis (Fig. 27). Thus, the molecular weight of renin-binding protein probably depends on the species or organ.

A renin inhibitor was purified to be apparently homogeneous on polyacrylamide gel electrophoresis from the submaxillary gland (Fig. 27). The protein was identical to the renin-binding protein, because the inhibitor bound renin molecule to form 75,000 dalton renin (Fig. 28) and inhibited the renin activity reversibly. It was, therefore, expected that the renin activity of the complex, renin and its binding protein, could be hardly detected. However, as shown in Fig. 24 and 28, the activity of 75,000 dalton renin as well as that of 40,000 dalton renin could be clearly

determined by radioimmunoassay of angiotnsin I. This puzzling result was probably explained by the following reasons. The activity of 75,000 dalton renin was assayed after dilution with more than 1000 volumes buffer in order to adjust the amount of angiotensin I produced by the renin action to the calibrated range (50 pg/ml - 600 pg/ml). During the dilution, the 75,000 dalton renin was altered to active 40,000 dalton renin and the activity of 40,000 dalton renin was measured instead of that of 75,000 dalton renin, because the 75,000 dalton renin was convertible into 40,000 dalton renin by dilution (Fig. 21).

Submaxillary gland renin has been demonstrated to be synthesized as a preprorenin with molecular weight of 45,000 by cell-free translation experiment using mRNA of renin by many investigators (18, 86-90). The renin-binding protein was considered to be distinct from pre-peptide and pro-peptide produced from prepro- or pro-renin, because the molecular weight of the binding protein was 10 times larger than those of the peptides. Moreover, the renin-binding protein was not a degradation product of renin, because none of immunological precipitation line was observed between renin-binding protein and antirenin antibodies on Ouchterlony plate. These results suggested that the renin-binding protein was encoded by a gene differed from that of renin.

CHAPTER VI. CONCLUDING REMARKS

Renin, which is a carboxyl protease, plays a central role in blood pressure-volume regulation and sodium-potassium homeostasis. Recently, a new type renin, a high molecular weight renin, has been demonstrated in the kidney extracts and plasma. The high molecular weight renin has been regarded as a complex of renin and its binding protein. However, its physiological role was not elucidated, because it is very difficult to purify a high molecular weight renin or a renin-binding protein. In this thesis, the author studied biochemically renin and a high molecular weight renin in some renin-rich organs of two abnormal animals, i.e. the plasma and kidney of stroke-prone spontaneously hypertensive rat (SHRSP) and the submaxillary gland of JCL-ICR mouse.

As described in chapter II, the major form of renin in the plasma of the malignant phase of SHRSP was a high molecular weight renin (Mr=56,000 $\pm$ 2,000, major isoelectric point (pI=5.7 $\pm$ 0.08) instead of normal circulating renin of low molecular form. Such a high molecular weight renin (Mr= 52,000 $\pm$ 3,600 and pI=5.6 $\pm$ 0.07) was also detected in the kidney extracts of SHRSP and control Wistar Kyoto strain of rats during all stages of their lives, when the extract was

prepared in the presence of N-ethylmaleimide. However, there was no difference in the molecular weight and in the pI value of a high molecular weight renin in the kidney extract between Wistar Kyoto rats and SHRSP. Molecular properties of a high molecular weight renin in the kidney extracts were similar to those in the plasma of the malignant phase of SHRSP.

Besides high molecular weight renin, a low molecular weight renin (Mr=35,600 $\pm$ 2,900 and major pI value = 5.15 $\pm$ 0.05) was also observed in kidney extract of Wistar Kyoto rats and SHRSP during all stages of their lives, and its molecular properties were similar to those in their plasma. Therefore, a clear molecular shift of renin was observed in the plasma of the malignant phase of SHRSP, but not in their kidney extract. During examination of these renins, the author found that a high molecular weight renin converted to normal renin reversibly: a high molecular weight renin was not eluted by gel filtration in the SHRSP-kidney extract prepared until a thiol blocker, N-ethylmaleimide, was added in the extract, suggesting that a renin binding substance existed in the extract. This observation was also confirmed in the normal rat and hog kidney extracts.

From chapter III through V, the author examined a high molecular weight renin in the submaxillary gland of JCL-ICR mouse. In chapter III, rapid and large-scale

isolation method of the submaxillary gland renin was established in order to obtain pure antigen and pure normal renin which was one component of a high molecular weight renin. Renin was purified about 60-fold by chromatography on an affinity column including pepstatin-aminohexyl-Sepharose 4B with a high yield of 83% from the homogenate of adult mouse submaxillary glands. The enzyme obtained by the one-step purification was electrophoretically homogeneous on SDS-polyacrylamide gel and as active as an absolutely pure renin. Administration of nanogram quantity of the purified enzyme to nephrectomized rats caused a sustained rise of blood pressure and decrease in sensitivity of the animal to angiotensin II. The enzyme purified by the affinity column could be separated into five active componenets by chromatography on CM-cellulose. Their renins were tentatively referred as fraction-A, -B, -C, -D and -E renin in order of elution volume. This elution pattern was similar to that observed when the enzyme was purified in the presence of various protease inhibitors. Each of those renins gave a symmetrical elution profiles on the CM-cellulose column and a discrete protein band on polyacrylamide gel electrophoresis at pH 8.6. Furthermore, antibodies against fraction-A or -D renin were raised from rabbits. This rapid and large-scale isolation method eliminates all four fractionation steps reported previously by Cohen et al. (13) for the

purification of mouse submaxillary gland renin.

The migration distance of fraction-D renin was different from that of other renins on SDS-polyacrylamide gel electrophoresis in the presence of dithiothreitol, although all renins were moved similarly in the absence of the reagent. Fraction-D renin was migrated at 38,000 dalton region and other renins were moved at 33,000 dalton region by the electrophoresis. However, all five renins were immunologically identical. These results suggested that fraction-D renin was altered to fraction-A, -B, -C and -E renins by actions of some proteases during storage in the convoluted granular cell of the submaxillary gland or during homogenization.

As described in chapter IV, the author measured the renin activity and the molecular weight of renin in the submaxillary glands from 1 to 8-week-old male mice by radioimmunoassay of angiotensin I and by gel filtration, respectively. The renin activity was found to increase notably with increasing age and to reach a maximum in 6-week-old mice in which the renin activity was 7.5×10^5 times as high as that of 1-week-old mice. Moreover, the author found three unfamiliar renins of different molecular weights (Mr=48,000, 37,000 and 31,000) in addition to a normal renin (Mr=41,000) in the tissue of 1- to 3-week-old mice. An

unfamiliar renin (31,000) increased notably at 6-week-old, but it almost disappeared at 7-week-old mice in which a normal renin increased and became a major form of renin. The renin activity in all three of the unfamiliar renins was completely inhibited by antibodies raised against pure normal renin (fraction-A and -D renin), suggesting that all the unfamiliar renins described in this chapter have a precursor common to normal renin.

As described in chapter V, a high molecular weight renin (Mr=75,000±3,000) was found in the submaxillary gland of adult male mice by a new method, sedimentation equilibrium analysis with a tabletop air-driven ultracentrifuge. Most renin in the submaxillary gland is supposed to be the high molecular weight form. This high molecular weight renin was converted into normal renin (Mr=40,000±3,000) by acidification or dilution and reconstituted from normal renin and a renin-binding protein fraction. The high molecular weight renin was also clearly separated from normal renin by three cycles of gel filtration. A renin-binding protein was purified to be apparently homogeneous on polyacrylamide gel electrophoresis by gel filtration, pepstatin-aminohexyl-Sepharose 4B column chromatography and DEAE Sepharose CL-6B column chromatography. The renin-binding protein (Mr=25,000) inhibited the renin activity

potently and bound renin to form 75,000 dalton renin. Thus, it was concluded that the high molecular weight renin was composed of normal renin and a renin-binding protein.

In this thesis, the author demonstrated high molecular weight- (HMW-) renins in the plasma and kidney of SHRSP and in the mouse submaxillary glands (MSG). Their molecular weights which were estimated by gel filtration were different as follows, although molecular weights of normal renins in those organs and the plasma were similar (Mr= 36,000-42,000). They were 52,000, 56,000, 48,000 and 75,000 in the plasma at malignant stage, in the kidney, in MSG at young age and in MSG at adult age, respectively. These differences in molecular weights are probably due to distinctions among renin-binding proteins contained in those HMW-renins. This thought is supported by the following facts. The molecular weight of HMW-renin in MSG at adult age (Mr=75,000, chapter V) was different from that in the hog kidney (Mr=113,000, ref. No=66) when their renins were analyzed by a sedimentation equilibrium method. Migration distances on SDS-polyacrylamide gel electrophoresis were different between purified renin-binding protein in MSG (Mr=25,000, chapter V) and that in the hog kidney (Mr= 40,000, ref. No=81,82). Therefore, it was concluded that the molecular weight of renin-binding protein, at least, depended on the species or organ.

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