Isolation from a Salivary Gland of Granules Containing Renin and Kallikrein

By Tzu S. Chiang, Ph.D., Ervin G. Erdös, M.D., Isoo Miwa, M.D., Larry L. Togue, B.S., and Jacqueline J. Coalson, Ph.D.

ABSTRACT

Granules of the submaxillary gland of the white mouse contain both kallikrein and renin. The granules were separated and concentrated in sequential centrifugation procedures. They were more stable at room temperature and in hypertonic sucrose solutions than in the cold or in isotonic solution. The amylase, acid phosphatase, renin, kallikrein, and benzoyl-L-arginine ethyl ester esterase contents of the granules were determined. Kallikrein and renin showed a similar distribution pattern after fractionation. Granular renin released the equivalent of 9.6 μg angiotensin II amide/mg enzyme protein/min from swine serum angiotensinogen. Granular kallikrein liberated from human kininogen the equivalent of 8.4 μg bradykinin/mg enzyme protein/min.

Electron micrographs of the isolated granules showed various forms; some were spherical and symmetrical, and others were amorphous.

ADDITIONAL KEY WORDS submaxillary gland bradykinin angiotensin subcellular fractions electron microscopy differential centrifugation insoluble enzyme polymers kallidin

Granules originating from the rat submaxillary gland contain large amounts of kallikrein (1). The appearance of these particles in electron micrographs is similar to that of pancreatic zymogen granules (2). Although the presence of renin in granules has been gaining acceptance, a definite relationship between granules and renin activity has not yet been established (3, 4). In addition to kallikrein, renin was found in the submaxillary glands of the white mouse (5-8). Renin activity is concentrated in the striated ducts which have granulated cells. These findings led us to study the subcellular distribution of renin and its relationship to kallikrein in the submaxillary gland of the white mouse.

In the present communication we deal with

Methods

White male Swiss Webster mice (A. Sutter Co.) were killed by a sudden blow on the head without using anesthesia. The mice were over 90 days old and weighed about 50 g. Fresh, minced submaxillary glands were homogenized in batches of six pairs in a Dounce hand homogenizer. In the first series of experiments, the tissues were placed in a medium of 0.25M sucrose solution buffered with 0.005M Tris, pH 7.4, in an ice bath. A 10% (w/v) homogenate was prepared and filtered through four layers of gauze. The filtered homogenate was then fractionated using isotonic sucrose solutions and a refrigerated centrifuge as previously described (1).

In the second series of experiments, all procedures were done at room temperature in a 0.88M sucrose solution buffered with 0.005M Tris, pH 7.4. The unbroken cells and tissues were removed by centrifugation at 120 g for 5 minutes using a Sorvall RC-2 centrifuge. The step was repeated once. The supernatant was centrifuged again at 800 g for 20 minutes, and the precipitate was washed with sucrose solution and resuspended at 800 g. The washed precipitate obtained at 800 g contained granules and will be referred to as extract I. The supernatant was centrifuged again at 2,000 g for 20 minutes or in some experiments at 10,800 g for 1 hour and

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subsequently at 144,000 g for 1 hour in a Spinco L-2 65 ultracentrifuge. The latter operation was repeated once after washing the sediment.

Extract 1 was further fractionated by discontinuous sucrose density gradient centrifugation according to Siekevitz and Palade (2). A Spinco L-2 65 ultracentrifuge was used with the swinging bucket rotor SW 25.1 for 2 hours at 4,000 g. Four ml of extract I in 0.88M sucrose was layered on top of sucrose solutions of increasing molarity as shown in Figure 2. The granules were on the boundary between the 1.60M and 1.84M solutions. This active granular fraction will be designated as extract II.

Continuous sucrose density gradient centrifugation was done with the Spinco equipment mentioned above for 2 hours at 4,000 g. Prior to centrifugation, 2.3 ml of extract II mixed with 2.3 ml of 0.88M sucrose was layered on top of a solution with an increase in molarity from 1.60M to 1.84M. The gradients were sampled with a gravity siphoning device. Fractions of 1 ml were collected and assayed for benzoyl-L-arginine ethyl ester (BAEe) esterase and renin activity. The optical densities were measured in a Cary UV spectrophotometer at 280 nm.

Amylase was determined according to Searcy et al. (9). The substrate was soluble starch, and the reaction was run at 37°C. Protein content was measured according to Lowry et al. (10).

Acid phosphatase activity of the fractions was assayed with p-nitrophenylphosphate following Sigma technical bulletin no. 104. The enzymatic hydrolysis of 5 × 10⁻⁴M BAEe was followed at room temperature in a Cary UV recording spectrophotometer at 253 nm (1). The hydrolysis of 1 × 10⁻⁴M benzoyl-L-arginine p-nitroanilide (BAPA), as the hydrochloride, was measured at 405 nm in a 0.1M Tris buffer, pH 7.9 (1).

Renin substrate was prepared from swine serum according to Green and Bumpus (11). After repeated precipitation with ammonium sulfate, 2 μg or more of angiotensin can be released by renin from 10 mg lyophilized powder.

Angiotensin activity was expressed in equivalents of [α-L-Asp⁻¹-(NH₃)-Val²⁻]-angiotensin II (angiotensin II amide, Hypertensin, Ciba). The preparations used were standardized against Angiotensin Research Standard A supplied by the Medical Research Council. One microgram of angiotensin II amide was equivalent to 0.66 ± 0.02 SE units of the standard as tested on the blood pressure of Holtzman rats weighing about 200 g.

The angiotensinase content of the renin extracts was inactivated by keeping them at pH 3.9 for 20 minutes at 25°C (12). Renin and renin substrate were incubated at pH 6.5 in 0.05M phosphate buffer containing 0.9% NaCl. The equivalent of about 1 μg of renin protein/ml was added to 5 mg of purified swine renin substrate/ml. After 10 minutes of incubation at 37°C, the mixture was placed in a boiling water bath for 4 minutes, cooled in an ice bath, and centrifuged for 20 minutes at 3,000 g. The supernatant containing the liberated angiotensin was injected in the cannulated jugular vein of the pithed rat initially anesthetized with ethyl ether. Cardiot blood pressure was recorded on a Grass Polygraph with a Statham transducer. The figures in the text show the mean value ± SE or the mean value and the range.

Angiotensin activity was also determined on the isolated rat uterus and the guinea pig ilium (4).

The biological activity of kallikrein¹ in vitro was established by assaying on the isolated rat uterus the amount of kinin released by the enzyme (13). The substrate was human plasma heated at 56°C for 2 hours. The pH of the reaction was kept at 7.4. In contrast to rat kallikrein, mouse kallikrein is not oxytocic on the isolated rat uterus, thus no Trasylool was added to the incubation mixture prior to injecting it into the bath (1). Trasylool¹ is a proteolytic inhibitor that blocks the effects of rat kallikrein on the rat uterus. The kinin values were expressed in synthetic bradykinin² equivalents.

The in-vivo effects of kallikrein and renin as well were registered on the systemic arterial blood pressure of mongrel dogs of both sexes. The dogs were anesthetized with sodium pentobarbital, 30 mg/kg iv. Injections were given through the cannulated femoral vein, and systemic arterial blood pressure was measured through the carotid artery.

Insoluble trypsin polymer was made in this laboratory by coupling trypsin (Worthington 2X crystallized) to the copolymer of maleic anhydride and ethylene and crosslinking it with hexamethylene diene diamine (14, 15). Insoluble polymer of swine pancreatic carboxypeptidase B was prepared by the same technique. EMA 21 and 31³ were used in these two preparations. The enzyme was purified in this laboratory from acetone-dried swine pancreatic powder (16). The insoluble enzymes were stable when stored in the refrigerator in a buffer solution. Angiotensin was incubated with the trypsin polymer at pH 7.4 and with the soluble trypsin at pH 8; plasma kinin was incubated with the trypsin polymer at pH 9. Angiotensin and plasma kinin were brought in contact with the

¹Donated by Bayer, A. G.
²Donated by Schering, A. G., and Sandoz, Inc.
³Donated by the Monsanto Chemical Co.
TABLE 1

Percent of Renin and BAEe Esterase Released from the Granules of Extract I at 1°C and at 25°C

<table>
<thead>
<tr>
<th>Procedure</th>
<th>At 1°C% Renin released</th>
<th>At 1°C% Esterase released</th>
<th>At 25°C% Renin released</th>
<th>At 25°C% Esterase released</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standing for 40 min</td>
<td>37</td>
<td>50</td>
<td>6</td>
<td>100</td>
</tr>
<tr>
<td>Standing + Triton X-100 (0.1%)</td>
<td>100</td>
<td>100</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>Standing + 3 vol. H₂O</td>
<td>52</td>
<td>80</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

![Enzyme content of the various fractions obtained in the centrifuge. The glands were homogenized at room temperature in 0.88M sucrose solution. The granules were in sediment B, extract I.](image)

A, 120 g. sediment; B, 800 g. sediment; C, 2,000 g. sediment; D, 10,800 g. sediment; E, 144,000 g. sediment; F, supernatant.

Fractionation

In the first series of experiments, submaxillary glands of the mouse were homogenized in 0.25M sucrose at 1°C (1). The distribution of renin and BAEe esterase among the various fractions was very similar. The sediment collected at 120 g had 13% of the renin and 19% of the esterase activities. The corresponding figures for the final supernatant were 70% and 63%. There were relatively small amounts of enzymes in sediments obtained at 10,800 g and 144,000 g. The washed sediments obtained at 480 g contained granules and had 14% of renin and 13% of esterase activities of the crude homogenate. In the rat the esterase action of this fraction was shown to be due to kallikrein (1). Therefore ester hydrolysis was used as a convenient rapid in-vitro chemical technique for detecting kallikrein, but the final identification of kallikrein in the granules was done by bio-assay.

The stability of packed granules of extract
I in 0.88m sucrose was studied at 1°C and at 25°C. Table 1 shows the effects of cold, Triton X-100 and dilution of the hypertonic solution on the release of enzymes from the granules. When the granules were centrifuged briefly (2,000 g for 10 minutes) after standing on ice for 40 minutes, 37% of renin and 50% of esterase activities were recovered in the supernatant. Thus similar, but not identical, percentages of bound esterase and renin were liberated from the granules in the cold. The corresponding figures at room temperature were only 3% and 6%. Triton X-100 or water released both esterase and renin, although dilution of the hypertonic solution with water liberated more esterase than renin. Like granules from other glands that are rich in amylase or kallikrein (1, 17), granules of mouse submaxillary gland are more stable when kept at room temperature instead of on ice.

Figure 1 shows the percent distribution of renin (EC 3.4.4.15), kallikrein (EC 3.4.4.21), esterase, amylase (EC 3.2.1.1), and acid phosphatase (EC 3.1.3.2) in the fractions. One gram of homogenized gland hydrolyzed soluble starch at the rate of 1.3 mg of reducing sugar released per minute. Kallikrein and renin activities are shown below in the section on Bioassay. The esterase activity of the crude homogenate was quite high; 17,000 µmole of BAEe were hydrolyzed per minute by 1 g of tissue. The rate of cleavage of the ester by 1 mg extract I protein was 189 µmole/min. As in the rat (1), the amidase activity of the gland was very low, less than 0.1% of the rate of hydrolysis of BAEe. Two µmole BAPA/mg protein extract I/hour were cleaved. The only fraction which had significant amounts of amylase, kallikrein, esterase, and renin was the sediment that settled at 800 g (B in Fig. 1). In contrast to this finding, a significant amount of acid

![FIGURE 2](http://circres.ahajournals.org/)

**Figure 2**

Differential centrifugation of the granules in a discontinuous density gradient. Extract I in 4 ml 0.88m sucrose solution was layered on top of sucrose solutions of increasing molarity. The molarity and volume from top to bottom were: 1.02M, 4.7 ml; 1.60M, 4.7 ml; 1.84M, 3.3 ml. Centrifugation was done at 4,000 g for 2 hours. The granules were recovered in extract II. The figures for kallikrein bio-assay and protein content indicate the percent of extract I recovered in fraction no. 8.
phosphatase was in the sediment obtained at 2,000 g. The specific activity of acid phosphatase was twice as high in the sediment obtained at 2,000 g as in that at 800 g. The latter cleaved 4.4 µmole p-nitrophenylphosphate/mg protein/min.

The washed sediment obtained at 144,000 g (E of Fig. 1), so called microsomal fraction, had 1% or less of the activities. Most of the renin and esterase were in the final supernatant.

Extract I was further fractionated by layering it on top of a discontinuous sucrose density gradient and centrifuging it for 2 hours (Fig. 2). The granular fraction found in a layer between 1.60 and 1.84M sucrose solutions was named extract II (Fig. 2). About 70% of the esterase and renin were recovered in the various sucrose gradient fractions, half of this value was concentrated in extract II. Extract II also contained about half of the total protein content and kallikrein activity (see Bioassay) and hydrolyzed 266 µmole of BAEe/mg protein/min.

Additional similarities in the distribution of renin and the esterase (kallikrein)-containing particles were seen in continuous density gradient centrifugation (Fig. 3). Extract II was layered on top of a sucrose solution of linearly increasing density from 1.60M to 1.84M sucrose and centrifuged. This third subsequent centrifugation also did not separate the activities, the distribution curves of esterase and renin being quite similar.

Inhibition of the BAEe esterase after 30 minutes of incubation with selected inhibitors is shown in Table 2. The inhibitors (Nutritional Biochemical Corp.), soybean and ovomucoid trypsin inhibitor, were ineffective. Trasylol (1, 18) and diisopropyl fluorophosphate (DFP) blocked the activity of the enzyme. Thus the inhibition pattern of this enzyme also suggests the identity of the esterase with kallikrein.
TABLE 2

<table>
<thead>
<tr>
<th>Source of enzyme</th>
<th>Hydrolysis of BAEe (μmol/mg protein/min)</th>
<th>Soybean trypsin inhibitor (100 μg/ml)</th>
<th>Ovomucoid trypsin inhibitor (100 μg/ml)</th>
<th>Trasylol (50 units/ml)</th>
<th>DFP (5 × 10⁻⁴ M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract I</td>
<td>189</td>
<td>0</td>
<td>0</td>
<td>79</td>
<td>99</td>
</tr>
<tr>
<td>Extract II</td>
<td>266</td>
<td>3</td>
<td>7</td>
<td>81</td>
<td></td>
</tr>
</tbody>
</table>

**BIOASSAY**

Renin in the extracts was identified by its direct effect on the blood pressure and by the release of angiotensin from angiotensinogen. Injecting renin intravenously into the pithed rat or intact dog raised the mean systemic arterial blood pressure. Owing to tachyphylaxis, subsequent injections of renin into the same animals were not effective. Renin incubated at pH 6.4 with angiotensinogen prepared from swine serum (12) liberated a substance that contracted isolated rat uterus and guinea pig ileum and raised the blood pressure of the pithed rat. Under the conditions of the incubation, 1 g tissue equivalent of crude homogenate liberated 450 ± 28 μg of angiotensin II amide/min or 3.3 ± 0.2 μg of angiotensin II amide/mg protein of crude homogenate/min (n = 14). The corresponding values in μg of angiotensin II amide released per mg protein per minute of extracts I and II were 5.6 ± 0.63 μg (n = 10) and 9.6 ± 1.2 μg (n = 6).

Figure 4 shows the rate of liberation of angiotensin by renin. The effect of increasing the substrate concentrations on the amount of angiotensin set free is also demonstrated here. The pressor substance was completely inactivated after incubation with 100 μg/ml of trypsin in 60 minutes; 71% of the activity disappeared after incubation with insoluble trypsin polymer (200 μg/ml). As expected, the insoluble polymer of carboxypeptidase B was ineffective against angiotensin. Extract of swine kidney cortex also inactivated the peptide (19). The hypertensive activity was not influenced by boiling the solution for 4 minutes or by pretreating the animal with phenoxybenzamine, 0.2 to 0.4 mg/kg iv. Contractions of the isolated guinea pig ileum induced by angiotensin were blocked by 1 μg/ml atropine (8). Renin was not inhibited by DFP (2 × 10⁻⁴ M) or Trasylol (250 units/ml). Submaxillary renin did not release angiotensin from human plasma heated for 2 hours at 56°C, although such plasma was the source of kininogen in the kallikrein experiments. It is likely that submaxillary renin liberated angiotensin I which was subsequently converted to the more potent angiotensin II.

The kallikrein in the glandular preparations cleaved a kinin from the kininogen. The kinin contracted the isolated rat uterus and guinea pig ileum. The kinin activity was not decreased by incubating with trypsin polymer or by adding atropine to the bath housing the guinea pig ileum. Incubation with 0.05 ml of carboxypeptidase B polymer suspension immediately inactivated the kinin. Injection of the plasma kinin to pithed rat had no effect on the blood pressure. One milligram of tissue of the homogenized gland released the equivalent of 54 ± 68 μg of bradykinin in 1 hour (n = 3) from human plasma, whereas 332 (321 to 352) μg bradykinin was liberated by 1 mg of extract I protein and 504 ± 148 μg by extract II.

Injection of the glandular kallikrein of the crude homogenate or extracts I or II caused transient hypotension in the dog. The effect was not tachyphylactic and did not decrease upon repeated administration. One gram of homogenized gland contained 220 ± 13 Frey Units (FU) of kallikrein (n = 5); 3.4 (3.5 to 3.2) FU of kallikrein were found in 1 mg of extract I protein and 6.3 (4 to 8.2) FU in extract II (n = 3).

The hypotensive effects of kallikrein were not inhibited by Trasylol (500 units/ml) or soybean trypsin inhibitor (100 mg/ml) after
Liberation of angiotensin as a function of the purified swine renin substrate concentration (A) and as a function of incubation time (B). The source of renin was a granular fraction of the submaxillary gland of the mouse (extract I). The incubation time for A was 10 minutes; 10 mg swine serum substrate were used for B.

30 minutes of preincubation. The release of kinin by the kallikrein preparations was prevented by $2 \times 10^{-8}$ M DFP but not by 100 $\mu$g/ml soybean trypsin inhibitor or 500 units/ml Trasylol.

**MICROSCOPY**

The granules were investigated by phase-contrast and electron microscopy. As in the rat (1), mouse extract I contained vesicles that were observed under a phase-contrast microscope at 500x magnification. The particles were dissolved when a drop of Triton X-100 was added to the slide. Electron micrographs of the granules of extract II showed a fairly homogenous layer where the intensity of the electron density in granules was consistent (Fig. 5). The size and shape of the granules varied. Some were spherical bodies, ranging up to $2\mu$ in diameter, which resembled the kallikrein granules isolated from the rat gland (1). Others were more ellipsoid or amorphous.

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Electron photomicrography of granules from the submaxillary gland of the white mouse that contain kallikrein and renin. 11,000× magnification. Granules were separated in discontinuous density gradient centrifugation (extract II).

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Discussion

It is now generally accepted that renin is located in the juxtaglomerular complex of the kidney, but the exact component within the complex that contains renin is not settled. A good positive correlation exists, however, between the index of granulation and renin content (4).

Our experiments showed the simultaneous presence of renin and kallikrein in a granular layer obtained from the homogenized submaxillary gland of the white mouse. The distribution of the two enzymes in the granules was alike in four different centrifugation procedures and three subsequent ones. Renin activity was assayed by measuring the angiotensin released by the enzyme from swine angiotensinogen on the arterial blood pressure of the pithed rat. The rise in blood pressure caused by the administration of liberated angiotensin was identical with the response to the synthetic peptide.

The BAEe esterase activity of the homogenized gland was taken as an indicator of the presence of kallikrein. Kallikrein in the granules, however, was identified by determining the amount of kinin released by the enzyme from human plasma kininogen on the isolated rat uterus. The hypotensive action of kallikrein was measured on the systemic arterial blood pressure of the dog.

A variety of enzymes can hydrolyze BAEe, some of which were found in the submaxillary gland (20). Kallikrein is quite active in splitting this ester substrate, and this reaction provides the basis of a convenient chemical assay for this enzyme. The relationship between lowering of the blood pressure and the hydrolysis of ester bonds varies according to the source of kallikrein (13, 18). The granular fraction had a high esterase activity and a relatively low hypotensive effect. These would suggest that either the separated granules contain another esterase in addition to kallikrein, or that the ratio of esterase to kininogenase activity is very high in these animals. The amount of kallikrein found in the crude homogenized mouse gland agrees with published figures (18), but it is less than in the rat (1, 18). Of all tissues studied, the rat submaxillary gland has the highest concentration of kallikrein, 3,000 FU/g.

The esterase activity of the granular fraction was inhibited by DFP and Trasylol but not by soybean or ovomucoid trypsin inhibitors. Although Trasylol inhibited the esterase, it did not block the kininogenase action in the preparation (13). It is possible that owing to some steric effects, Trasylol inhibits only the ester hydrolysis by kallikrein of the mouse submaxillary gland. Trasylol is an effective inhibitor of rat submaxillary kallikrein (1), but it fails to inhibit glandular kallikrein of some other animals (18). The glandular preparation had a negligible amidase action, as shown with BAPA substrate.

In addition to their effects on the blood pressure, renin and kallikrein can also be distinguished by the inhibition of kallikrein but not renin by DFP, by the different pH optima of the enzymes, and by the strong tachyphylactic effect of the injected renin preparation. Their substrates, angiotensinogen and kininogen, were prepared by different procedures. Rodent glandular kallikrein releases a kinin from human kininogen (1), while animal renin is inactive on human angiotensinogen (12, 21). Injection of kinin did not effect the blood pressure of the pithed rat. Atropine blocked in part the effect of angiotensin on the isolated guinea pig ileum but not the effect of plasma kinin.

Angiotensin was inactivated by soluble and insoluble polymerized trypsin preparation, while the plasma kinin activity was abolished by incubation with insoluble polymerized carboxypeptidase B. The use of insoluble polymers of enzymes for inactivating and thereby characterizing peptides offers some advantages over the application of soluble enzymes. Since the insoluble enzyme sediments very easily from the incubation mixture, it will not interfere with the bio-assay. The polymerized enzymes can be stored easily in a refrigerator. Trypsin (14) and carboxypeptidase B were very stable when bound to polymer in contrast to their lack of stability in solution.

Of the other enzymes assayed, only the
particle-bound amylase was found in the same granular fraction as renin or kallikrein. The relative activity of amylase in the submaxillary gland is low compared to that in the parotid gland (17). The presence of acid phosphatase is frequently associated with the lysosomes. The highest specific activity in the present experiments was found in the 2,000 g sediment that contained very little of the other enzymes (C of Fig. 1).

This may be the first time that renin granules were extracted, concentrated, and remained intact enough for observations with an electron microscope. In differential centrifugation, the granules behaved like the "large renin granules" of the kidney (22). All granules looked equally dense in electron micrographs. However, more bound esterase than renin was released from the granules when kept in the cold or when distilled water was added.

The spherical granules looked like zymogen granules found in the pancreas (2) or in the submaxillary gland of the rat (1). Others were more elliptoid or amorphous and are similar to those observed in the juxtaglomerular apparatus of the kidney (23-26). It is possible that the granules lost their shape in the hypertonic sucrose solution, but hypertonic sucrose was used initially to separate zymogen particles from the pancreas (2). In addition, isotonic sucrose solution was the medium in our two control experiments, where we still harvested a heterogeneous population of granules. The question whether the same individual particles contain both hypertensive and hypotensive enzymes cannot be definitely answered. The possibilities are that either kallikrein and renin occur in the same individual granules or two types of granules contain two different types of vasoactive enzymes. The more amorphous ones may have renin, the round and symmetrical ones may contain kallikrein. The similar distribution of activities in the various centrifugation procedures speaks against this assumption. It can be stated safely, however, that the most potent endogenous hypertensive and hypotensive enzymes were found in the same granular layer originating from a salivary gland.

Acknowledgments

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References

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