RENIN INHIBITOR IN THE SUBMAXILLARY GLAND OF MALE MOUSE

HITOSHI MIYAZAKI, FUMIAKI SUZUKI*, SHIGEHISA HIROSE, NAOTO UENO, YUKIO NAKAMURA* and KAZUO MURAKAMI**

Institute of Applied Biochemistry, The University of Tsukuba, Ibaraki 305, and *Department of Agricultural Chemistry, Gifu University, Naka, Kagamigahara 504, Japan

ABSTRACT

A renin inhibitor was found in the mouse submaxillary gland. The inhibitor has a molecular weight of 64,000 as estimated by gel filtration on Ultrogel AcA 44. Its isoelectric point was at 5.0-5.1. The inhibitor was completely inactivated by heating or acidification. This suggests that the inhibitor is a protein.

KEY WORDS renin / renin inhibitor / mouse submaxillary gland

The renin-angiotensin system is connected with secretion of kidney renin into blood and renin plays an important role in the regulation of the blood pressure and in homeostasis of fluid and electrolyte. Renin is also located in other organs. For example, renin was found in the submaxillary glands of male mice (24). Renin concentration in the submaxillary gland reaches the level 400-fold greater than in the kidney (13). The submaxillary gland renin was isolated, characterized (3, 12, 22) and used as a model enzyme of renal renin (14), because it was available in large quantities and was similar to renal enzyme. However, little is known about renin inhibitor in the submaxillary gland although some preliminary search was made for proteinous renin inhibitor or renin-binding protein in the kidney (2, 5, 11, 17, 18, 20). This paper describes a proteinous renin inhibitor found in the submaxillary gland.

MATERIALS AND METHODS

Submaxillary Gland Extract

One and a half grams of frozen submaxillary glands of albino male mice (JCL-ICR, 8 week-old) were homogenized for 1 min using a Polytron PT 10/35 homogenizer (Kinematica GmbH)

in four volumes of 0.01 M pyrophosphate buffer, pH 6.5, containing 0.5 M NaCl and 1 mM diisopropylfluorophosphate (DFP). The homogenate was centrifuged at 100,000 *g* for 60 min, and the supernatant was used as submaxillary gland extract.

Renin Assay

Renin activity was determined by the rate of formation of angiotensin I from hog renin substrate which had been obtained from hog plasma (17). Samples (100 μ l) were incubated with 10 mg of hog angiotensinogen preparation at 37°C for 30 min in 0.1 M phosphate buffer, pH 6.5, containing 5 μ M captopril (SQ 14225; a potent competitive inhibitor of angiotensin I converting enzyme, K_i =1.7 nM; reference 4) and 7 mM phenylmethyl sulfonyl fluoride (PMSF) in a total volume of 0.3 ml, and then angiotensin I was determined by the radioimmunoassay of Haber *et al.* (7).

Angiotensinase Assay

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

** To whom reprint requests should be made

Pure Renin

Pure renin was isolated from the mouse submaxillary gland using a pepstatin-aminohexyl-agarose column by a published procedure (22). It was diluted to a concentration of 0.1 mg/ml with 0.1 M Tris-HCl buffer, pH 7.4, containing 0.1% BSA, and used as a pure renin preparation which was capable of generating 50 μ g angiotensin I/ml/hr. Renin concentration was determined using an extinction coefficient, $E_{1\text{cm}, 280\text{nm}}^{1\%} = 10.5$ (3).

Renin-Inhibitory Activity

Samples (60 μ l) were incubated with 40 μ l (4 μ g) of pure renin at 37°C for 30 min. Aliquots (100 μ l) were then assayed for renin to determine their inhibitory activity; after angiotensin I generation, the reaction mixtures were diluted 100 times and subjected to radioimmunoassay of angiotensin I.

Molecular Weight

The apparent molecular weight of renin inhibitor was estimated by gel filtration on a column (1.5 $\times$ 90 cm) of Ultrogel AcA 44. The following molecular weight standards were added to a sample: bovine serum albumin ($M_r=68,000$), egg albumin ($M_r=45,000$), and α -chymotrypsinogen A ($M_r=26,000$). The column was eluted, collecting 1 ml fractions at a flow rate of 8 ml/hr, with 0.01 M pyrophosphate, pH 6.5, containing 0.1 M NaCl. All the above procedures were carried out at 4°C unless otherwise stated.

Isoelectric Focusing

Isoelectric focusing in density gradient (23) was achieved in a 110 ml glass column. After focusing, 1 ml fractions were collected from the bottom of the column.

Chemicals

Pepstatin-aminohexyl-agarose was prepared by the method of Murakami and Inagami (16). Bovine serum albumin was obtained from Seikagaku Kogyo. Ovalbumin, α -chymotrypsinogen and phenylmethylsulfonylfluoride (PMSF) were from Sigma. Diisopropylfluorophosphate (DFP) was from Fluka AG Chemicals. Pepstatin A and Ultrogel AcA 44 were obtained from Protein Research Foundation and LKB, respec-

tively. Captopril (SQ 14225) was kindly supplied from Sankyo Company.

RESULTS

Evidence for the Existence of a Renin Inhibitor in the Male Mouse Submaxillary Gland

For the detection of renin inhibitor in the submaxillary gland, it was necessary to separate renin from renin inhibitor. Most of the renin activity present in the male mouse submaxillary gland was removed by absorption on the pepstatin-aminohexyl-agarose (Fig. 1). Strong renin-inhibitory activity was found in the remainder which was not absorbed by the affinity column (Fig. 1). This renin-inhibitory activity was completely lost after an acidification to pH 3.0, although renin was stable after the same treatment. The inhibitory activity was also completely lost after boiling for 3 min. For the definite demonstration of the presence of the renin inhibitor, however, the following possibility must be ruled out: the apparent renin-inhibitory activity may be due to decomposition of angiotensin I which was produced during renin reaction by angiotensinase in the submaxillary gland or due to degradation of renin added for the determination of renin-inhibitory activity. Angiotensinase activity was not detected in fractions with renin-inhibitory activity. The inactivated renin (renin-inhibitor complex) could be re-activated by acidification. This result excludes the other possibility that the inactivation is due to the degradation of renin by a non-specific action of proteases. It was thus concluded that there was an acid-labile renin inhibitor in the submaxillary gland.

Estimation of the Molecular Weight and the Isoelectric Point of Renin Inhibitor

The molecular weight of the inhibitor in the submaxillary gland extract was estimated to be 64,000 by a gel filtration on Ultrogel AcA 44 (Fig. 2), and its isoelectric point was determined to be 5.0–5.1 by isoelectric focusing method (Fig. 3).

DISCUSSION

Endogenous or synthetic inhibitors of renin have been studied by many investigators. Lipid inhibitors were isolated from the plasma (9, 10), kidney (15, 19, 21) and bile (8). The antibody against renin and the synthetic analogues of

Fig. 1 Separation of renin inhibitor from renin in the submaxillary gland extract by affinity chromatography on a pepstatin-aminohexyl-agarose column. The extract (4 ml) prepared as described in Materials and Methods was applied to a pepstatin-aminohexyl-agarose column (0.6 × 20 cm) equilibrated with 0.01 M pyrophosphate, pH 6.5 containing 0.5 M NaCl. Then the column was eluted with the same buffer and 0.5 M Tris-HCl buffer, pH 7.5. Fractions of 1 ml were collected at a flow rate of 10 ml/hr and assayed for renin activities and renin-inhibitory activities. The inhibitory activities with and without acidification are represented by —○— and —●—, respectively. Protein concentration and renin activity are shown by ---●--- and —▲—, respectively.

Fig. 2 Gel filtration profile of renin inhibitor on an Ultrogel AcA 44 column. Three milliliters of the renin inhibitor fractions (tube Nos. 4-9, Fig. 1) were applied to an Ultrogel AcA 44 column. After gel filtration, each fraction was assayed for renin-inhibitory activity. The other experimental conditions are described in Materials and Methods.

renin substrate, which were more specific to renin, have also been examined (6). In this paper, we have demonstrated the presence of a proteinous inhibitor of renin and partially characterized it. Since a common biological mechanism controlling protease activity involves the formation of enzyme-inhibitor complexes, such an inhibitor of renin has long been searched. The key to our successful demonstration was the use of i) affinity gel for the removal of renin from the inhibitor preparation, ii) the male mouse

submaxillary gland which is the richest source of renin and possibly of a renin inhibitor, and iii) little dilutions of renin-inhibitor complexes when examining the inhibitory activity since the complexes tend to dissociate upon dilution.

The molecular weight and the isoelectric point of renin inhibitor found in this study is very similar to those of renin inhibitor in the kidney (our unpublished data). The submaxillary gland of albino male mouse contains a large amount of biologically active polypeptides and

Fig. 3 Isoelectric focusing of renin inhibitor. Three milliliters of the renin inhibitor fractions (tube Nos. 4-9, Fig. 1), containing β -lactoglobulins A and B (2 mg) as internal standard, were used as a sample. The separation was achieved in a 110 ml column using 1% ampholine at 1,000 V for 21 hr at 4°C, and each fraction from pH 2.9 to 8.7 was assayed for renin-inhibitory activity. The other experimental conditions are described in Materials and Methods.

proteinases (1) such as nerve growth factor, epidermal growth factor, arginine esterase and renin of unknown physiological roles. Further biochemical and physiological studies on renin inhibitor in the submaxillary gland may give a clue to understand the role of renin in the gland.

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