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Two Different Forms of Angiotensin I-Converting Enzyme from Hog Kidney

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Angiotensin I-converting enzyme of hog kidney exists in two different forms, peak A and peak B, as shown by hydroxyapatite column chromatography. These enzymes were found to possess identical molecular weights by filtration through Sephadex G-200. The sialic acid and neutral sugar contents of peak A were roughly twice those of peak B. The peak A preparation gave a single protein component on SDS-gel electrophoresis, while peak B showed three components. After neuraminidase treatment, however, both enzyme preparations showed a single protein band with an estimated molecular weight of 90000 as determined by SDS-gel electrophoresis. It is suggested that the different mobilities of the two forms on electrophoresis depend upon the relative amounts of sialic acid in their molecules, and that these enzymes consist of two polypeptide chains.

Keywords——hog renal angiotensin I-converting enzyme; two different forms of angiotensin I-converting enzyme; molecular weight determination of glycoprotein; sialic acid contents; neuraminidase treatment

Although the angiotensin I-converting enzyme (kininase II, peptidyldipeptide hydrolase)[1] has been purified from several sources and some of its physical properties determined,[3,4] there is still much controversy with respect to its molecular weight, isozymes and structure.

We purified this enzyme from hog kidney by hydroxyapatite column chromatography as described by Oshima *et al.*[5] with some modifications, and found that there were two different forms of the enzyme (sialic acid contents: 12.59 and 7.32 μg/mg protein). In this report, we compare some properties of the two different forms of the enzyme.

SDS-polyacrylamide gel electrophoresis is not directly applicable to molecular weight determinations of glycoproteins. Glycoproteins containing more than 10% carbohydrate behave anomalously during SDS-polyacrylamide gel electrophoresis compared with standard proteins. Segrest and Jackson[6] have developed a largely empirical technique for the estimation of the molecular weights of glycoproteins by SDS-gel electrophoresis. The procedure involves determination of apparent molecular weights over a range of polyacrylamide gel concentrations (usually 4—8%). In this study, we estimated the molecular weight of angiotensin I-converting enzyme using this technique. The value obtained was markedly different from the molecular weight as determined by Sephadex G-200 gel filtration.

Experimental

Materials——Hog kidney was freshly obtained from a slaughterhouse. Angiotensin I and Bz-Gly-His-Leu were obtained from the Protein Research Foundation, Osaka, Japan. Bz-Gly-Gly-Gly and hippuric acid were synthesized in this laboratory. DEAE-Sephadex A-50, Sephadex G-200 and Dextran blue 2000 were from Pharmacia Fine Chemicals AB, Uppsala, Sweden. DEAE-cellulose was obtained from Serva Entwicklungs, Heidelberg, West Germany. Hydroxyapatite was purchased from Clarkson Chem. Co.,

1) Enzymes: angiotensin I-converting enzyme (EC 3.4.15.1); neuraminidase (EC 3.2.1.18). Abbreviations: benzoyl, Bz; sodium dodecyl sulfate, SDS.
2) Location: *Nanakuma, Nishi-ku, Fukuoka 814, Japan.*
3) E.G. Erdös, *Circ. Res.*, **36**, 247 (1975).
4) R.L. Soffer, *Ann. Rev. Biochem.*, **45**, 73 (1976).
5) G. Oshima, K. Nagasawa, and J. Kato, *J. Biochem.*, **80**, 477 (1976).
6) J.P. Segrest and R.L. Jackson, *Methods Enzymol.*, **28**, 54 (1972).

Williamport, PA, U.S.A. Neuraminidase from *Cl. perfringens*, type V[1] (specific activity, 0.6 U/mg at pH 5.1 and 37°) was a product of Boehringer Mannheim GmbH, West Germany.

Methods——Converting enzyme activity was determined using Bz–Gly–His–Leu or Bz–Gly–Gly–Gly as a substrate by the method of Nagamatsu *et al.*[7] Protein was determined by the method of Lowry *et al.*,[8] using bovine serum albumin as a standard. The content of sialic acid in the converting enzyme was analyzed by the thiobarbituric acid assay of Warren,[9] and neutral sugar was determined by the method of Dubois *et al.*, using glucose as a standard.[10]

Standard disc gel electrophoresis was performed on 7% acrylamide gel at pH 9.4 with a current of 2 mA per tube for 2 hr.[11] For the determination of the molecular weight of the enzyme after incubation with 1% SDS and 1% 2-mercaptoethanol for 1 hr at 60°, disc gel electrophoresis was performed in 4—8% polyacrylamide gels containing SDS as described by Lanzillo and Fanburg.[12] Proteins were stained for sugar moieties using the Schiff reagent. Noncarbohydrate proteins were detected with Coomassie brilliant blue R-250.[13]

For the removal of sialic acid moieties, angiotensin I-converting enzyme (0.5 mg) was incubated at 37° with neuraminidase (0.1 mg) in 1 ml of 0.05 M Tris–HCl buffer, pH 7.4, containing 0.1 M NaCl.

Purification procedures for angiotensin I-converting enzyme from hog kidney are summarized in Table I. Hog kidney was washed with saline to remove blood. The cortex was removed from the kidney and chopped into small pieces. The chopped cortex (1500 g) was suspended in 3 l of 0.25 M sucrose in 0.05 M Tris–HCl buffer, pH 7.4. The suspension was homogenized for 4 min with a Waring blender. The homogenate was centrifuged at $5000 \times g$ for 30 min, and the supernatant was then centrifuged at $50000 \times g$ for 2 hr. The precipitate was suspended in 1.5 l of 0.25% deoxycholate in 0.05M Tris–HCl buffer, pH 7.4, using a glass homogenizer. After standing overnight in the cold, the suspension was centrifuged at $50000 \times g$ for 2 hr. The supernatant (1400 ml) was mixed with DEAE-cellulose (150 g dry wt.), and suspended in 3 l of 5 mM Tris–HCl buffer, pH 7.4. The mixture was stirred overnight at 4° and filtered using a glass funnel. After washing with the same buffer, enzyme adsorbed on the cellulose was eluted with 2 l of 0.3 M NaCl in 5 mM Tris–HCl buffer, pH 7.4. $Ca_3(PO_4)_2$ (500 g) was added to the eluate and the pH was adjusted to 6.8 with 1 N HCl. The mixture was stirred for 1 hr then centrifuged at $5000 \times g$ for 10 min at 4°. Enzyme adsorbed on the gel was extracted with 2 l of 0.1 M sodium phosphate, pH 6.8. After centrifugation to remove the gel, the supernatant was concentrated to 200 ml by filtration with an Amicon XM-100 membrane under nitrogen pressure. The concentrate was dialyzed overnight against 5 l of 5 mM Tris-HCl buffer, pH 7.4, containing 0.1 mM $CoCl_2$. The dialyzed solution was then applied to a column of DEAE-Sephadex A-50 (2.5×40 cm)

Table I. Purification of Angiotensin I-Converting Enzyme from 1500 g of Hog Kidney Cortex

Procedure	Volume (ml)	Units/ml	Total units	Spec. act. (units/mg)	Yield (%)
Deoxycholate extract of microsomal fraction	1400	3.40	4760.0	0.053	100
DEAE-cellulose, elution with 0.3 M NaCl	2000	1.47	2940.0	0.21	61.8
$Ca_3(PO_4)_2$, elution with 0.1 M sodium phosphate, pH 6.8	2000	1.14	2280.0	1.11	47.9
Dialysate after Amicon membrane concentration	210	9.54	2003.4	1.26	42.1
DEAE-Sephadex A-50 column chromatography	60	17.1	1026.0	2.6	21.6
Hydroxyapatite column chromatography peak A	9.2	52.92	486.9	3.8	
peak B	7.5	12.83	96.2	3.7	12.3
Sephadex G-200 gel filtration peak A	14.0	27.07	378.9	12.5	
peak B	12.5	5.13	64.1	12.0	9.3

Substrate: Bz–Gly–His–Leu.

7) A. Nagamatsu, S. Soeda, and J. Inokuchi, *Yakugaku Zasshi*, **98**, 1296 (1978).
8) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
9) L. Warren, *J. Biol. Chem.*, **234**, 1971 (1959).
10) M. Dubois, K.A. Gilles, J.K. Kamilton, P.A. Rebers, and F. Smith, *Anal. Chem.*, **28**, 350 (1956).
11) B.J. Davis, *Ann. N.Y. Acad. Sci.*, **121**, 404 (1974).
12) J.J. Lanzillo and B.L. Fanburg, *Biochim. Biophys. Acta*, **439**, 125 (1976).
13) H.R. Murer and R.C. Allen, *Z. Klin. Chem. Klin. Biochem.*, **10**, 220 (1972).

equilibrated with the same buffer. The enzyme was eluted with a linearly increasing gradient of NaCl from zero to 0.3 M in the same buffer. The enzyme fractions were collected and concentrated to about 50 ml using an Amicon PM-10 membrane. The concentrate was directly applied to a hydroxyapatite column (2.5×10.5 cm) equilibrated with 0.2 mM sodium phosphate, pH 6.8, and the enzyme was eluted as two peaks by increasing the buffer concentration to 100 mM. The two active fractions were separately applied to a Sephadex G-200 column (2.5×90 cm), and eluted with 0.05 M Tris-HCl buffer, pH 7.4, containing 0.1 M NaCl and 0.1 mM $CoCl_2$.

Results

On hydroxyapatite column chromatography, the enzyme was eluted as two peaks by increasing the buffer concentration to 100 mM. The first peak of the enzyme was eluted at a concentration of 18 mM sodium phosphate (peak A) and the second peak at a concentration of 50 mM (peak B), as indicated in Fig. 1. The peak A fraction showed only one peak on gel filtration and the elution profile of the peak B fraction was similar to that of the peak A fraction, as indicated in Fig. 2.

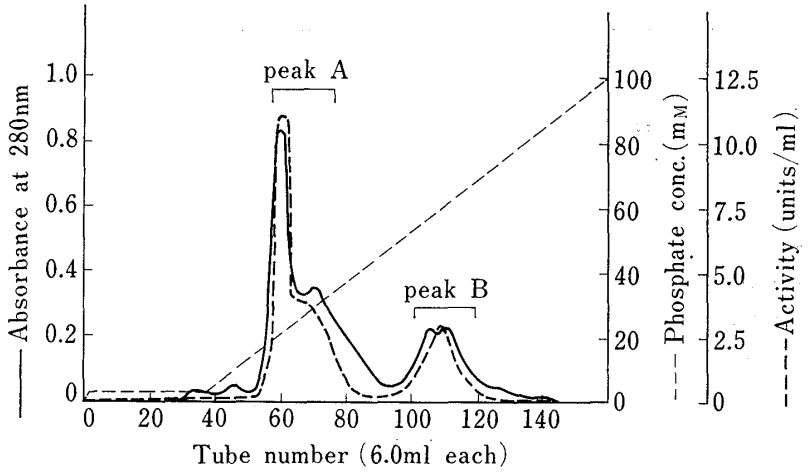

Fig. 1. Hydroxyapatite Column Chromatography

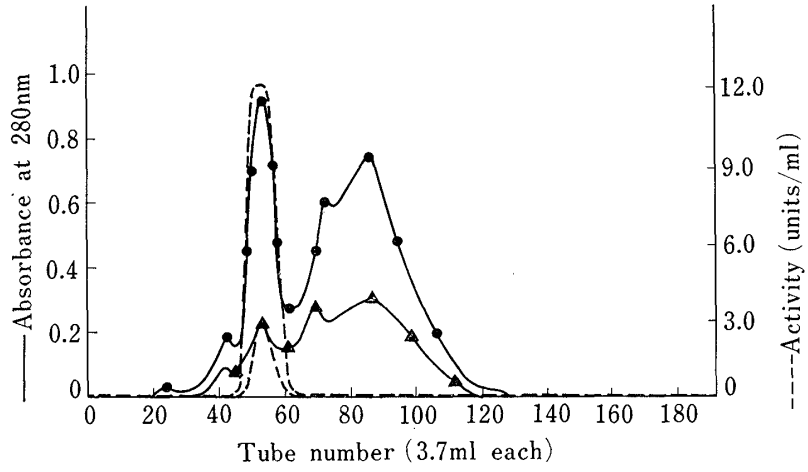

Fig. 2. Sephadex G-200 Column Chromatography
—●—: peak A. —▲—: peak B.

The molecular weights of peak A and peak B were both estimated to be 300000 by gel filtration on Sephadex G-200 (2.5×95 cm). The specific activities of peak A and peak B were identical and were 12±1 units per mg protein and 30±1 units per mg protein with

Bz–Gly–His–Leu and Bz–Gly–Gly–Gly, respectively. The ratio of the total enzyme activities of peak A and peak B was about 6: 1.

The analytical data for sugars are presented in Table II, compared with those of another group.[14] Peak A contained 12.59 μg of sialic acid and 73.5 μg of neutral sugar per mg of protein. On the other hand, peak B contained 7.32 μg of sialic acid and 40.5 μg of neutral sugar.

Table II. Carbohydrate Contents of Angiotensin I-Converting Enzyme from Hog Kidney, Rabbit Lung and Rabbit Serum

Carbohydrate	Hog kidney		Rabbit lung[14]	Rabbit serum[14]
	Peak A	Peak B		
	μg/mg protein			
Sialic acid	12.59	7.32	35.4	110.0
Neutral sugar	73.5	40.5	162.0	126.3
Amino sugar	not tested		92.6	85.7

The enzymic assays of peak A and peak B were performed with 5 mM Bz–Gly–His–Leu and Bz–Gly–Gly–Gly in 0.1 M potassium phosphate buffer, pH 7.4, both in the presence of Cl ions (0.3 M NaCl) and in the absence of Cl ions. The enzymic activities of peak A and peak B were dependent on Cl ions. The optimum pH for the activity of peak A was 8.3 for Bz–Gly–His–Leu and that of peak B was 8.6 for the same substrate.

The purified enzyme preparation of peak A showed two protein bands on gel eletrophoresis under the standard conditions, and a single major component on gel eletrophoresis in the presence of SDS, while the purified enzyme preparation of peak B showed a single protein band under the standard conditions and three major components in the presence of SDS, as indicated in Fig. 3. Further studies were carried out to determine whether the

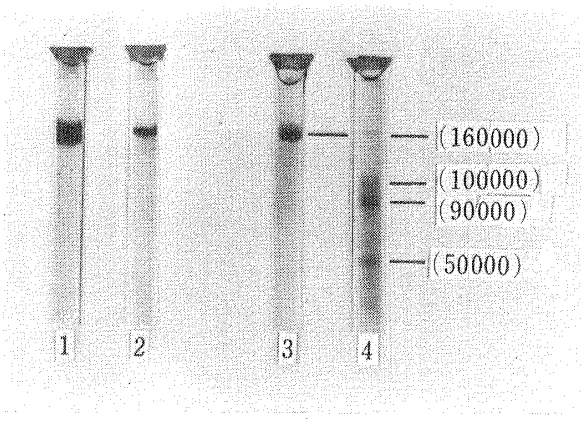

Fig. 3. Gel Electrophoresis under Standard and denaturing Conditions

1, standard gel electrophoresis of peak A (20 μg); 2, standard gel electrophoresis of peak B (20 μg); 3, SDS-gel electrophoresis of peak A (20 μg); 4, SDS-gel electrophoresis of peak B (37 μg). For the determination of molecular weight, 10 μg per gel of each of the following marker proteins was used; RNA-polymerase β′ (mol. wt. 165000), RNA-polymerase β (mol. wt. 155000), β-galactosidase (mol. wt. 130000), phosphorylase a (mol. wt. 100000), bovine serum albumin (mol wt. 68000), RNA-polymerase α (mol. wt. 39000).

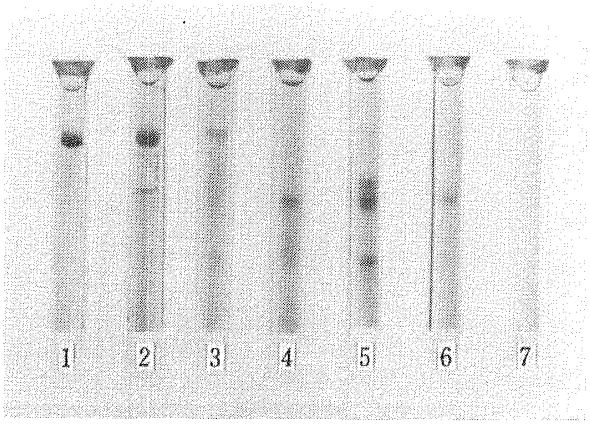

Fig. 4. SDS-gel Electrophoresis of Peak A and Peak B before and after Neuraminidase Treatment

1, peak A (20 μg) control; 2, peak A (20 μg) treated with neuraminidase for 3 hr; 3, peak A (20 μg) treated with neuraminidase for 24 hr; 4, peak A (20 μg) treated with neuraminidase for 48 hr; 5, peak B (37 μg) control; 6, peak B (18 μg) treated with neuraminidase for 48 hr; 7, neuraminidase only.

14) M. Das, J.L. Hartley, and R.L. Soffer, *J. Biol. Chem.*, **252**, 1316 (1977).

different electrophoretic bands of the purified enzyme preparations were due to variation in the sialic acid content of the proteins or not. The enzyme preparations were treated with neuraminidase to remove sialic acid residues. The treated enzymes were then subjected to SDS-gel electrophoresis. As shown in Fig. 4, after treatment with neuraminidase for 48 hr, both enzyme preparations gave a single identical protein band. The mobilities of the neuraminidase-treated enzymes were similar to that of the protein band with an apparent molecular weight of 90000 in the native enzyme preparation of peak B. This observation indicated that the native enzyme preparation of peak B was a mixture of three glycoproteins which contained different amounts of sialic acid; one contained much more sialic acid than the other two, and had the greatest mobility. Our observations also indicated that polypeptide chains which contained a substantial amount of covalently bound carbohydrate migrated at rates which were not proportional to their molecular mass. However, such polypeptide chains migrated at rates proportional to their mass when the sialic acid moieties were removed by neuraminidase treatment.

In all the experiments, incubation with neuraminidase did not affect the activity of the angiotensin I-converting enzyme. Thus, the sialic acid moiety of the enzyme is probably not involved in the catalytic activity of the enzyme. The pH optima of the neuraminidase-treated enzymes were compared with those of the native preparations (Fig. 5). While the native peak A preparation had an optimum at pH 8.3, neuraminidase-treated peak A was maximally active at pH 8.6 for the hydrolysis of Bz–Gly–His–Leu. However, the optimum pH of peak B was 8.6 and was unchanged by neuraminidase treatment.

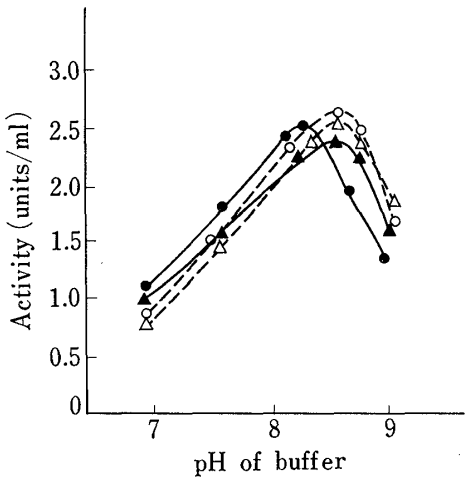

Fig. 5. pH Optima of Native and Neuraminidase-treated Enzymes

The enzyme preparations were dialyzed overnight against 1 mM potassium phosphate, pH 7.8, in the cold. The activities of the dialyzed enzymes were assayed in 0.1 M Tris-HCl containing 0.1 M NaCl, using Bz-Gly-His-Leu as a substrate.
—●—: peak A, —▲—: peak B; ---○---: peak A treated with neuraminidase for 48 hr; ---△---: peak B treated with neuraminidase for 48 hr.

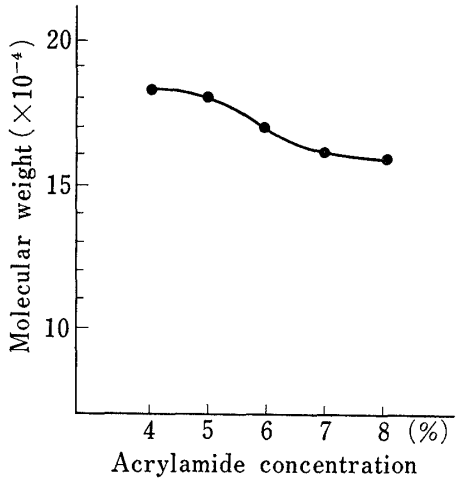

Fig. 6. Plot of Observed Molecular Weight of Angiotensin I-Converting Enzyme Peak A from Hog Kidney *vs.* Acrylamide Concentration on 4—8% SDS-Polyacrylamide Gels

Each point is the mean of 4 determinations. Calibration of gels was carried out as described in "Experimental."

Molecular weight determinations on SDS-polyacrylamide gels were carried out with the peak A preparation using the method described by Lanzillo and Fanburg.[12] The data were treated as specified by Segrest and Jackson.[4] The mobility was plotted against log molecular weight for the marker proteins in the 4—8% gels. The apparent peak A molecular weights were obtained from these curves and plotted against acrylamide concentration as shown in Fig. 6.

Discussion

The enzymic properties of the two different forms of angiotensin I-converting enzyme from hog kidney were identical with regard to Cl ion dependence, behavior on gel filtration and specific activity. These enzymic properties were similar to those of angiotensin I-converting enzyme from hog kidney reported by Oshima *et al.*[5]

Although peak B of angiotensin I-converting enzyme from hog kidney is markedly different from peak A in behavior on SDS-gel electrophoresis, several properties suggest that they may be closely related. Both peak A and peak B are sialoproteins, and the effects of neuraminidase treatment are similar. Thus there are grounds for proposing that the components of peak A and peak B may be identical in their essential structural features and may differ only in their sugar moieties. Our results also indicate that the angiotensin I-converting enzyme in hog kidney is a glycoprotein consisting of subunits that are not readily dissociable without neuraminidase treatment, and that the differences in reported molecular weights are not related to idiosyncrasies in the purification schemes. It is interesting to note that our result for hog kidney angiotensin I-converting enzyme on a 7.5% SDS-polyacrylamide gel showed an apparent molecular weight of 160000, while Oshima *et al.*[15] observed an apparent molecular weight of 195000 for the enzyme from hog kidney on a 5% SDS-polyacrylamide gel. The variation in molecular weights at different acrylamide concentrations indicates that molecular weight observations of glycoproteins on SDS-polyacrylamide gel at a single acrylamide concentration are potentially inaccurate, as reported by Lanzillo and Fanburg.[12]

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15) G. Oshima, A. Gecse, and E.G. Erdös, *Biochim. Biophys. Acta*, **350**. 26 (1974)