J. Biochem. 125, 348-353 (1999)

Human Renin-Binding Protein Is the Enzyme *N*-Acetyl-D-Glucosamine 2-Epimerase

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Received September 7, 1998; accepted October 30, 1998

The existence of human renin-binding protein (RnBP) in the kidney has been shown by the isolation and characterization of a complex of porcine renin-human RnBP [S. Takahashi *et al.* (1985) *J. Biochem.* 97, 671-677]. However, the properties of the free form of human RnBP had not been understood, because of the limitation of materials. In the present study, we have expressed human RnBP in *Escherichia coli* JM 109 cells under the transcriptional control of *taq* promoter and purified it by conventional column chromatographies. The purified recombinant human RnBP (rhRnBP) exists as a dimer and inhibits porcine renin activity through formation of a complex of porcine renin with rhRnBP, the so-called high-molecular-weight renin. Moreover, the rhRnBP catalyzes the interconversion between *N*-acetyl-D-glucosamine (GlcNAc) and *N*-acetyl-D-Mannosamine (ManNAc) with the apparent K_m values of 21.3 mM for GlcNAc and 12.8 mM for ManNAc, and 0.13 mM for effector ATP. ATP is essential for the GlcNAc 2-epimerase activity of human RnBP. These results indicate that the human RnBP is a GlcNAc 2-epimerase.

Key words: *N*-acetyl-D-glucosamine (GlcNAc), *N*-acetyl-D-mannosamine, binding protein, GlcNAc 2-epimerase, renin.

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Renin [EC 3.4.23.15] is a highly specific aspartic proteinase mainly synthesized in the kidney and plays an essential role in blood pressure control. Renin-binding protein (RnBP) is a proteinaceous renin inhibitor that was first isolated from porcine kidney (*1*). RnBP strongly inhibits renin activity with the dissociation constant of 0.2 nM and forms a complex with renin, the so-called high-molecular-weight renin (*2, 3*). The purified porcine RnBP exists as a dimer and dissociates into a monomer in the presence of sulfhydryl-oxidizing and -alkylating reagents (*4*). The primary structures of porcine (*5*), rat (*6*), and human (*6*) RnBPs have been deduced from nucleotide sequences by cDNA cloning. The RnBPs have a conserved leucine-zipper motif, which is essential for the formation of RnBP homodimer and RnBP-renin heterodimer (*5-9*). Recently, human (*10*) and rat (*11*) RnBP genes were isolated from genomic DNA libraries. Both of them span about 10 kb and consist of 11 exons separated by 10 introns. The human gene was found to be located in human chromosome X by means of PCR of hybrid DNA from human and hamster somatic cells (*10*). Moreover, the human RnBP gene has been mapped in the distal Xq28 chromosomal band (*12, 13*).

Interestingly, cDNA cloning of *N*-acetyl-D-glucosamine (GlcNAc) 2-epimerase [EC 5.3.1.8] from porcine kidney showed that GlcNAc 2-epimerase was identical with porcine RnBP (*14*). The GlcNAc 2-epimerase activity has also been found in several rat tissues including kidney, liver, and salivary gland (*15*). However, the properties of human GlcNAc 2-epimerase had not been understood because of a limitation of materials.

In the present study, we report the expression, purification, and characterization of the human RnBP and demonstrate that the human RnBP is the enzyme GlcNAc 2-epimerase.

MATERIALS AND METHODS

Materials—DEAE-Sepharose CL-6B, Sephacryl S-100 HR, and Mono Q HR5/5 and Superdex 75 columns were obtained from Pharmacia Biotech; restriction enzymes, DNA ligation kit, *Escherichia coli* JM109 competent cells, and PCR amplification kit from Takara Shuzo. Porcine kidney renin and RnBP were purified by the method of Takahashi *et al.* (*1*). The purified porcine kidney renin and RnBP showed a single protein band on SDS-PAGE. Rabbit anti-porcine renin and anti-porcine RnBP antibodies were prepared by the methods of Takahashi *et al.* (*2, 3*).

Construction of Expression Plasmid—To shorten the distance between the ribosome-binding site and the start codon, a fragment of about 0.3 kb of the human RnBP cDNA, pHRB6 (*6*), was amplified with a sense primer containing an additional *Eco*RI site upstream of the start codon, 5′GGAATTCAGGACATGGAGAAA3′, and an anti-

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Abbreviations: ELISA, enzyme-linked immunosorbent assay; GlcNAc, *N*-acetyl-D-glucosamine; HMW, high molecular weight; ManNAc, *N*-acetyl-D-mannosamine; RnBP, renin-binding protein; rhRnBP, recombinant human RnBP.

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sense primer 11 bp downstream of *Sma*I site of the cDNA clone, 5′AAGGCACACTTCTTGCCAGG3′. The PCR was performed over 30 cycles of denaturation (95°C, 1 min), annealing (55°C, 1 min), and extension (72°C, 1 min). The amplified fragment was digested with *Eco*RI and *Sma*I, and the resulting fragment was subcloned into *Eco*RI-*Sma*I sites of pUK223-3 (*16*). The 1-kb *Sma*I fragment of pHRB6 was subcloned into the *Sma*I site of the above plasmid. The resulting right-oriented expression plasmid, designated as pUKHRB6 (Fig. 1), was used to transform *E. coli* JM109 cells.

GlcNAc 2-Epimerase Activity—GlcNAc 2-epimerase activity was assayed by measuring the rate of interconversion of GlcNAc and ManNAc. The reaction mixture (0.1 ml) contained 0.1 M Tris-HCl, pH 7.5, containing 10 mM $MgCl_2$, 50 mM ManNAc, 5 mM ATP, and 20 μl of enzyme solution. After incubation at 37°C for 30 min, the mixture was boiled for 5 min to terminate the reaction. The reaction products were quantified by high performance liquid chromatography (HPLC) with pulsed amperometric detection using Dionex Bio-LC gradient pump and Dionex Carbopac PA-1 column equipped with a Dionex Model PAD2 detector (*17*). One unit of enzyme activity was defined as the activity producing 1 micromole of GlcNAc per min under the assay conditions.

Preparation of Biotinylated Anti-Porcine Renin IgG—The biotinylated polyclonal anti-porcine renin IgG was prepared by the method of Leary *et al.* (*18*). The rabbit anti-porcine renin IgG (5 mg) dissolved in 3 ml of 30 mM triethanolamine, pH 7.6, containing 3 M NaCl was mixed with 20 μl of 20 mg/ml solution of biotin-*N*-hydroxy succinimide in dimethylformamide. After stirring on a rotary shaker at 25°C for 2 h, the reaction mixture was extensively dialyzed against 30 mM triethanolamine, pH 7.6, containing 3 M NaCl. The dialysate was stored at 4°C until use.

Enzyme-Linked Immunosorbent Assay (ELISA) for Porcine Renin—Microtiter wells were coated with 0.2 ml of 0.1 mg/ml rabbit anti-porcine renin IgG in 0.1 M sodium bicarbonate buffer, pH 8.5, by incubation overnight at 4°C. The coated microtiter wells were washed five times with 20 mM sodium phosphate buffer, pH 7.4, 0.15 M NaCl, and 0.05% Tween 20 (Buffer A), then 0.2-ml samples were introduced and incubated overnight at 4°C. Purified porcine renin was used as a standard. Wells were washed extensively with Buffer A, then biotinylated IgG was placed in each well and incubated for 2 h at 25°C. Wells were washed extensively with Buffer A, and the bound biotinylated IgG was incubated for 2 h at 25°C with 0.2 ml of streptavidin-alkaline phosphatase. Wells were washed extensively with Buffer A, then the bound biotinylated IgG-streptavidin-alkaline phosphatase complex was incubated for 30 min at 25°C with 0.2 ml of 5 mg/ml of *p*-nitrophenyl phosphate in 0.1 M Tris-HCl, pH 9.5, containing 5 mM $MgCl_2$. The reaction product, *p*-nitrophenol, was quantified by measuring the absorbance at 405 nm. The sensitivity of the assay method for porcine renin was about 0.1 ng/ml (Fig. 2).

Analytical Procedures—Protein was determined by the method of Bradford (*19*) using bovine serum albumin as the standard. SDS-PAGE was performed on 5-20% acrylamide gradient gel (ATTO Corporation) by the method of Laemmli (*20*). After the electrophoresis, gel was stained with Coomassie Brilliant Blue R-250. Fast protein liquid chromatography of purified recombinant human RnBP (rhRn-

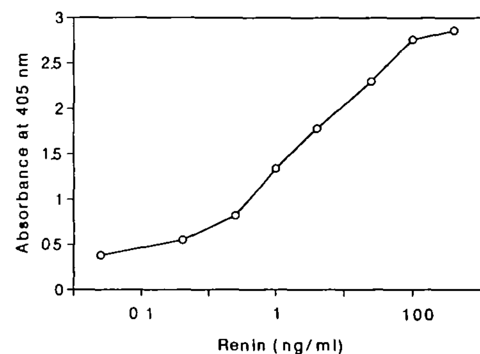

Fig. 2. **Standard curve for ELISA of porcine kidney renin.** The purified porcine kidney renin was used as a standard, and the assay conditions are described under "MATERIALS AND METHODS."

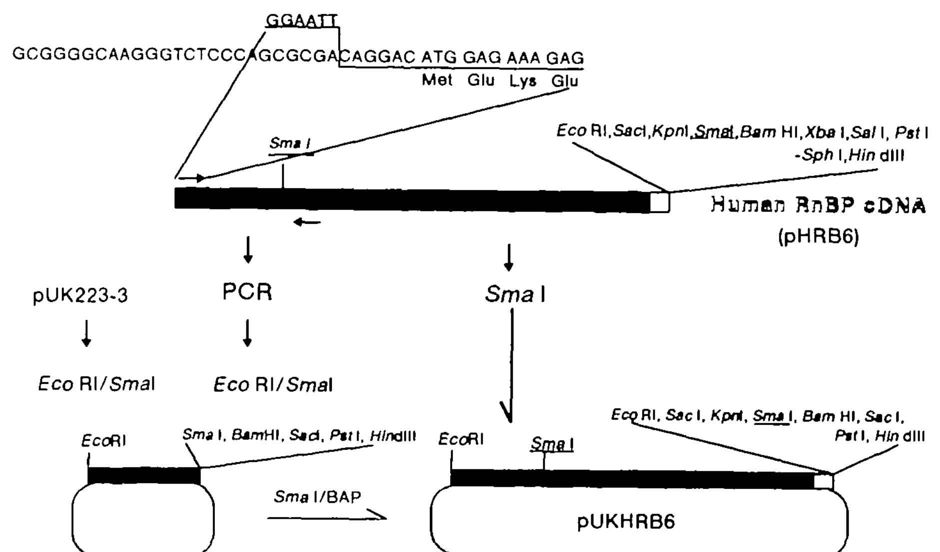

Fig. 1. **Construction of expression plasmid for rhRnBP.** The solid box represent the rhRnBP coding sequence. Details of the plasmid construction are described under "MATERIALS AND METHODS."

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BP) was carried out at 25°C. A sample (0.2 ml) was chromatographed on a Superdex 75 column (1.0×30 cm) in 20 mM sodium phosphate buffer, pH 7.0, containing 0.05% 2-mercaptoethanol (Buffer B) and 0.15 M NaCl at a flow rate of 1 ml per min. The apparent molecular weights of renin and renin-RnBP complex were estimated by gel filtration on Sephacryl S-100 HR at 4°C. A sample (0.5 ml) was applied to the calibrated column (1.0×90 cm) and eluted with Buffer B containing 0.15 M NaCl at a flow rate of 3 ml per h. Fractions of 0.5 ml were collected, and the renin concentration of each fraction was assayed by ELISA. Renin activity was measured by the method of Murakami *et al.* (*21*) using Suc-Arg-Pro-Phe-His-Leu-Leu-Val-Tyr-MCA as a substrate. The reaction mixture contained 250 μl of 50 mM sodium phosphate buffer, pH 6.5, containing 0.1 mM $ZnCl_2$, 25 μl of substrate (25 mM in dimethylformamide) and 25 μl of renin preparation. The mixture was incubated for 1 h at 37°C. The reaction was terminated by heating (100°C, 5 min), and 50 mU aminopeptidase M (Pierce) was added to the mixture. The mixture was incubated for 2 h at 37°C, then the product, aminomethylcoumarin, was measured fluorometrically with excitation at 380 nm and emission at 460 nm.

Preparation of Antiserum—One milliliter of emulsion containing 50 μg of the purified rhRnBP in complete Freund's adjuvant was injected subcutaneously into each rabbit. The injection was repeated every 4 weeks, and the rabbits were bled 3 days after fifth injection to prepare the antiserum.

Western Blotting—Western blotting was performed by the method of Towbin *et al.* (*22*). Samples resolved on SDS-PAGE were transferred onto nitrocellulose membrane. After transfer, the membrane was immersed in Buffer A containing 5% skim milk, then incubated for 2 h at room temperature with anti-porcine RnBP or rhRnBP antiserum (1:1,000 dilution). After the incubation, the membrane was washed three times with Buffer A, incubated with alkaline phosphatase conjugated anti-rabbit IgG (Fc) (1:5,000 dilution, Promega), and washed three times with Buffer A. The membrane was allowed to react with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

NH_2-Terminal Amino Acid Sequence—The purified rhRnBP (5 μg) was electrophoresed on SDS-polyacryl amide gel and then electrophoretically transferred onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories). Protein was stained with Coomassie Brilliant Blue R-250 on the membrane. The protein band was directly sequenced with a Shimadzu PPSQ-10 protein sequencer by the method of Matsudaira (*23*).

TABLE I. **Purification of recombinant human RnBP.**

	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)
1. Extract	1,920	710	0.37	100
2. Ammonium sulfate fractionation	1,470	617	0.42	86.9
3. DEAE-Sepharose	215	557	2.59	78.5
4. Sephacryl S-100HR	99.1	386	3.90	54.3
5. 1st Mono Q	9.8	229	23.3	32.3
6. 2nd Mono Q	6.0	215	35.8	30.3

RESULTS AND DISCUSSION

Purification of rhRnBP—The rhRnBP was identified by Western blotting using anti porcine RnBP antiserum and assayed by measuring its GlcNAc 2-epimerase activity.

An overnight culture (6 ml) of JM 109 cells containing the expression plasmid was used to inoculate 300 ml of 2× YT medium (1.6% polypeptone, 1.0% yeast extract, 0.5% NaCl, pH 7.0) containing 1.0% glucose and 0.1 mg/ml ampicillin. The culture was incubated at 30°C for 5 h, then expression of recombinant protein was induced with 1 mM isopropyl-β-D-thiogalactopyranoside. Cells were harvested 3 h after induction. The *E. coli* cells (31.2 g of wet weight) from 3 liters of culture were sonicated with 300 ml of Buffer B containing 1 mM EDTA and 10 μM leupeptin, then centrifuged 20,000×g for 30 min. The supernatant was fractionated with ammonium sulfate at 60% saturation and dialyzed against Buffer B containing 0.1 M NaCl. The dialysate was applied to a column of DEAE-Sepharose CL 4B (22×150 mm) previously equilibrated with Buffer B containing 0.1 M NaCl. The column was washed with the same buffer and recombinant human RnBP (rhRnBP) was eluted with a linear gradient of NaCl concentration from 0.1 to 0.4 M. Fractions containing rhRnBP were pooled and precipitated with ammonium sulfate at 80% saturation. The precipitate was collected by centrifugation and dissolved in 10 ml of Buffer B containing 0.1 M NaCl, then applied to Sephacryl S-100HR (2.2×80 cm). The column was eluted with Buffer B containing 0.1 M NaCl, and the fractions containing rhRnBP were pooled and then dialyzed against Buffer B containing 0.5 mM EDTA and 5 μM leupeptin. The dialysate was applied to a Mono Q HR5/5 column which had been equilibrated with Buffer B. The column was washed with the same buffer, then developed with a linear gradient of 0 to 0.3 M NaCl. Fractions containing rhRnBP were pooled and dialyzed against Buffer B. The sample was rechromatographed on Mono Q HR5/5 column under the same conditions as above. Fractions containing rhRnBP were pooled and stored at 4°C. Table I summarizes the purification. About 6 mg of rhRnBP preparation was obtained from the sonicate of *E. coli* cells. The purified preparation showed a major single protein band on SDS-PAGE (Fig. 3, lane a) with a molecular weight of

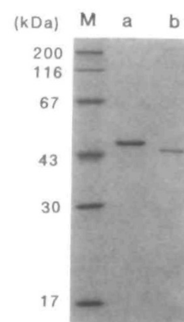

Fig. 3. **SDS-PAGE of the purified rhRnBP.** Lane M, marker proteins (200 kDa, myosin; 116 kDa, β-galactosidase; 67 kDa, bovine serum albumin; 43 kDa, aldolase; 30 kDa, carbonic anhydrase; 17 kDa, myoglobin); lane a, rhRnBP (2 μg); lane b, porcine RnBP (1 μg). After the electrophoresis, proteins were visualized by staining with Coomassie Brilliant Blue R-250.

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45,000. The molecular weight of porcine kidney RnBP was also estimated to be 42,000 (Fig. 3, lane b). The difference in molecular weight between human and porcine RnBP is due to a difference in the amino acid sequences. Indeed, the predicted amino acid sequences for human and porcine RnBPs consisted of 417 and 402 amino acid, respectively (*5, 6*). The molecular weight of rhRnBP estimated by SDS-PAGE is similar to that of human RnBP isolated from human kidney as a complex with porcine renin (*3*). The purified rhRnBP also showed a single protein peak on gel filtration using Superdex 75 (Fig. 4), and its molecular weight was estimated to be 85,000 using aldolase, bovine serum albumin, ovalbumin, and chymotrypsinogen as molecular weight standards. These results indicate that rhRnBP exists as a dimer. The effects of sulfhydryl-oxidizing or -alkylating reagents on rhRnBP as a GlcNAc 2-epimerase activity were also investigated. Monoiodoacetic acid (10 mM), *N*-ethylmaleimide (1 mM), or 5,5′-dithiobis(2-nitrobenzoic acid) (0.1 mM) inhibited more than 95% of GlcNAc 2-epimerase activity, and the molecular weight of monoiodoacetic acid- or *N*-ethylmaleimide-treated rhRnBP was estimated to be 44,000 by gel filtration on Superdex 75 (data not shown). In this connection, our previous studies showed that purified porcine kidney RnBP (*1*) and recombinant rat RnBP (*8*) both existed as dimers. The dimers dissociated into monomers in the presence of sulfhydryl-oxidizing or alkylation reagents (*1, 4, 8*). Moreover, studies of a double aspartyl mutant of porcine RnBP demonstrated that the leucine-zipper motif present in the porcine RnBP molecule was essential for the formation of renin-RnBP heterodimer and an RnBP homodimer (*7*). Thus, the dimerization seems to be essential for the functional activity.

The NH_2-terminal amino acid sequence was determined to be Met-Glu-Lys-Glu-Arg-Glu-Thr-Leu-Gln-Ala-Trp-Lys-Glu-Arg-Val-Gly-Gln-Glu-Leu-Asp-. This sequence agrees with the NH_2-terminal sequence predicted from the human RnBP cDNA (*6*). These results indicate that the rhRnBP had no signal sequence, as was predicted from its hydropathy profile (*6*).

Immunochemical Properties of Human and Porcine RnBPs—The purification of rhRnBP was monitored by Western blotting using rabbit anti-porcine kidney RnBP antiserum. As shown in Fig. 5, when rabbit anti-porcine RnBP was used for the Western blotting, 40 ng of rhRnBP (lane c) and 10 ng of porcine RnBP (lane d) gave nearly the same intensity. On the other hand, when the rabbit anti-rhRnBP antiserum was used for Western blotting, about 1 ng of rhRnBP (lane a) was comparable to 20 ng of porcine RnBP (lane b). These results indicate that rabbit antibodies against rhRnBP and porcine kidney RnBP are relatively specific for rhRnBP and porcine RnBP, respectively.

We have already established ELISA for rhRnBP using the highly specific antibody. The sensitivity of rhRnBP was about 100 pg/well (data not shown). The ELISA for rhRnBP will be useful for study of the distribution and expression of human tissue RnBP.

Interaction of Renin with rhRnBP—Our previous studies showed that porcine and rat RnBPs inhibited porcine and rat renin activities, and formed a complex called HMW renin (*1, 8*). Purified human kidney renin is not commercially available. The purification and characterization of human renin from cadaver kidney and pathological samples have been reported (*24-28*), but it is very difficult to use

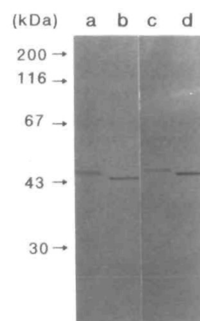

Fig. 5. **Western blot analysis of rhRnBP.** Rabbit anti-rhRnBP antiserum (lanes a and b) or anti-porcine RnBP antiserum (lanes c and d) was used for the Western blotting. Lane a, rhRnBP (1 ng); lane b, porcine RnBP (20 ng); lane c, rhRnBP (40 ng); lane d, porcine RnBP (10 ng).

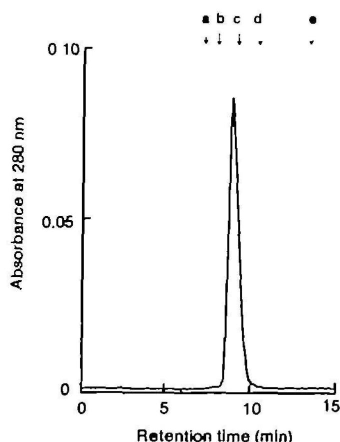

Fig. 4. **Gel filtration of purified rhRnBP.** The purified rhRnBP (20 μg) was chromatographed on Superdex 75. Blue dextran 2000 (a), aldolase (b), bovine serum albumin (c), ovalbumin (d), and chymotrypsinogen (e) were used as molecular weight markers.

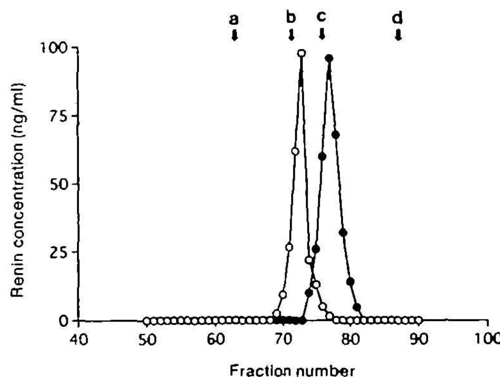

Fig. 6. **Gel filtration of porcine renin and porcine renin-rhRnBP complex.** Samples applied to a Sephacryl S-100HR column were as follows: closed circles, purified porcine renin (1 μg); open circles, mixture of porcine renin (1 μg) and rhRnBP (40 μg). The mixture was incubated for 30 min at 37°C, then applied to the column. Renin was determined by ELISA as described under "MATERIALS AND METHODS."

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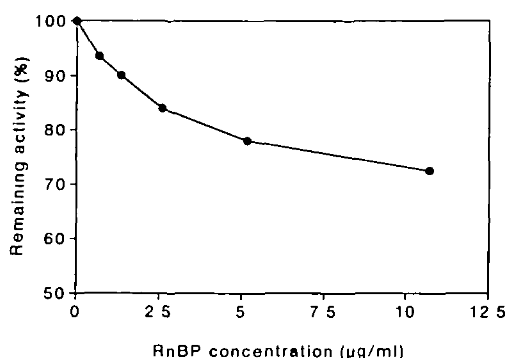

Fig. 7. **Inhibition of porcine renin by rhRnBP.** The purified porcine renin was incubated with the indicated amount of rhRnBP at 37°C for 10 min, then the remaining renin activity was measured as described under "MATERIALS AND METHODS."

purified human kidney renin because of the limitation of materials and the extremely low concentrations of the enzyme in the kidney. In the present study, we used purified porcine kidney renin (*1*) to clarify the binding ability of rhRnBP to renin.

When the purified porcine renin was chromatographed on a Sephacryl S-100HR column, renin was eluted as a single peak with a molecular weight of 40,000 (Fig. 6, closed circles). The purified renin incubated with rhRnBP was also showed a single peak, and the molecular weight was estimated to be 58,000 (Fig. 6, open circles). The molecular weight of the complex agrees with that of the heterocomplex isolated from human kidney extract and purified porcine kidney renin (*3*). Thus, the rhRnBP has binding ability to porcine renin.

The effect of rhRnBP on porcine renin activity was also investigated. The rhRnBP inhibited porcine renin activity in a dose-dependent manner (Fig. 7), but the inhibition level was lower than that of porcine (*1*) or rat (*8*) RnBP. This was presumably due to the low affinity of porcine renin toward rhRnBP.

GlcNAc 2-Epimerase Activity of rhRnBP—To directly confirm that rhRnBP is the enzyme GlcNAc 2-epimerase, the GlcNAc 2-epimerase activity of an extract from *E. coli* cells expressing rhRnBP and of the purified preparation of rhRnBP was measured. The *E. coli* strains harboring pUK223-3 or pUKHRB6 in 10 ml of 2×YT medium containing 0.1 mg/ml ampicillin were grown at 30°C overnight. The overnight culture (6 ml) was used to inoculate 300 ml of 2×YT medium containing 1% glucose and 0.1 mg/ml ampicillin. Cells were grown at 30°C for 5 h, then incubated with 1 mM isopropyl-β-D-thiogalactopyranoside for 3 h. When the expression vector pUK223-3, which had no insert, was used to transform *E. coli* JM 109 cells, no GlcNAc 2-epimerase activity was detected in the cell extract (data not shown). On the other hand, GlcNAc 2-epimerase activity was detected in the extract from *E. coli* JM 109 cells carrying pUKHRB6 with the specific activity of 0.37 U/mg protein (Table I). The enzyme was purified about 100-fold from the cell extract with apparent homogeneity (Fig. 3) and its specific activity was determined to be 35.8 U/mg protein (Table I). These results clearly indicate that rhRnBP has GlcNAc 2-epimerase activity.

Substrate Specificity of rhRnBP as a GlcNAc 2-Epimerase—Table II shows the substrate specificity of rhRnBP as a GlcNAc 2-epimerase. The human enzyme did not react with *N*-acetyl-D-galactosamine, mannosamine, glucosamine, mannose, or glucose. It catalyzed only the interconversion between GlcNAc and ManNAc with apparent K_m values of 21.3 mM for GlcNAc and 12.8 mM for ManNAc. ATP was essential for the GlcNAc 2-epimerase activity as an effector, and the apparent K_m value was estimated to be 0.13 mM. The K_m values for GlcNAc 2-epimerase isolated from porcine kidney were reported to be 7.4 mM for GlcNAc, 6.3 mM for ManNAc, and 0.18 mM for ATP as an effector (*14*). In contrast to human enzyme, the porcine enzyme did not require ATP for GlcNAc 2-epimerase activity (*14*).

TABLE II. **Substrate specificity of recombinant human RnBP.**

Substrate	Relative activity (%)
ManNAc	100
GlcNAc	12.6
GalNAc	0
Mannosamine	0
Glucosamine	0
Mannose	0
Glucose	0

In the present study, we established a functional expression and purification method for human RnBP in *E. coli* cells. The purified rhRnBP inhibits porcine renin activity through formation of a heterocomplex. Moreover, the purified preparation showed GlcNAc 2-epimerase activity with the specific activity of 35.8 U/mg of protein. These results strongly imply that rhRnBP is the enzyme GlcNAc 2-epimerase. To understand the physiological role of human RnBP as a GlcNAc 2-epimerase, further elaborate studies, *e.g.*, transgenic animal studies or gene targeting studies, are necessary.

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