Sialic acid residue of ovine angiotensinogen does not affect the reactivity to human renin

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ABSTRACT

Recombinant ovine angiotensinogen (oANG) expressed in Chinese hamster ovary cells was purified into five isoforms by ammonium sulfate precipitation, CM-Toyopearl column chromatography and chromatofocusing. These isoforms (A-1, A-2, A-3, A-4 and A-5) had apparent molecular weight of 52,000 and isoelectric points of 6.5, 6.3, 6.3, 6.0 and 5.7, respectively. Each of the isoforms ran in a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and isoelectric focusing. After desialylation by neuraminidase, oANG gave one major band with isoelectric point of 6.9. The isoforms, A-2, A-3 and A-4 had similar K_m and V_{max} values to human renin (95% confidence limit: $K_m = 0.17 \pm 0.07$, 0.15 ± 0.04 and $0.18 \pm 0.01 \mu M$; $V_{max} = 0.90 \pm 0.17$, 0.81 ± 0.07 and 0.88 ± 0.03 nM/min, respectively). These values for desialylated and untreated oANGs were also similar (95% confidence limit: $K_m = 0.11 \pm 0.03$ and $0.15 \pm 0.03 \mu M$; $V_{max} = 0.72 \pm 0.06$ and 0.79 ± 0.06 nM/min, respectively). These results indicate that sialic acid residue of recombinant oANG does not affect the reactivity to human renin.

Angiotensinogen is a plasma glycoprotein and is specifically cleaved by renin (EC 3.4.23.15) to release the N-terminal decapeptide, angiotensin I (AI). AI is subsequently cleaved by angiotensin-converting enzyme (EC 3.4.15.1) to produce the octapeptide, angiotensin II, which regulates blood pressure and electrolyte balance (7).

Many investigators reported that angiotensinogen purified from plasma displays several isoforms different in the molecular weight and isoelectric point (pI), and that the heterogeneity is mainly due to the difference in carbohydrate content (4, 7, 8, 18). In particular, the difference

in sialic acid content causes the charge heterogeneity of angiotensinogen (4, 8, 18). On the other hand, sialic acid residue of glycoprotein is assumed to affect interaction between proteins (5, 9, 12, 17, 20). For example, Imai *et al.* showed that desialylated erythropoietin exhibits a 4-fold higher binding affinity to its receptor than that untreated, which indicates that sialic acid residue of erythropoietin decreases the affinity to the receptor (9). These findings raise the possibility that angiotensinogen with sialic acid residue exhibits a difference in the affinity and/or catalytic activity to renin.

In this study, we used recombinant ovine angiotensinogen (oANG) expressed in Chinese hamster ovary (CHO) cells to examine whether sialic acid residue of angiotensinogen affects the

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affinity and catalytic activity to renin.

Purification of recombinant oANG using chromatofocusing. Recombinant oANG expressed in CHO cells was purified 1.7-fold by 70% saturated ammonium sulfate precipitation (pH 7) and was eluted in a single peak by CM-Toyopearl column chromatography (15). The oANG-containing fraction (fraction A) was chromatofocused to be separated into five isoforms (A-1, A-2, A-3, A-4 and A-5) with elution pH values of 6.6, 6.4, 6.3, 6.1 and 5.8, respectively

Fig. 1 Chromatofocusing of oANG on a polybuffer exchanger 94 column. oANG preparation obtained by CM-Toyopearl column chromatography was dialysed against 25 mM imidazole-HCl buffer, pH 7.4. The dialysate was diluted into 0.06 of absorbance at 280 nm, incubated for 6 h and applied to a column (1.0×23 cm) of Polybuffer exchanger 94 (Pharmacia Biotech AB, Uppsala, Sweden) preequilibrated with the imidazole buffer. The preparation was eluted by a pH gradient generated with Polybuffer 74-HCl, pH 5.0 (dilution factor, 1:8) at a flow rate of 0.17 mL/min.

(Fig. 1). All isoforms ran in a single band with molecular weight of 52,000 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 11). Also the isoforms ran in a single band on isoelectric focusing (IEF) with pI values of 6.5, 6.3, 6.3, 6.0 and 5.7, respectively (data not shown). The resolution of chromatofocusing shown here is similar to that of plasma oANG previously reported by Fernley *et al.* (4). The N-terminal amino acid sequence of all the isoforms was determined to be Asp-Arg-Val-Tyr-Ile, which is identical to that of AI, using a protein sequencer (Procise 491, Perkin-Elmer Co., Applied Biosystems Div., Foster City, CA). Purification of recombinant oANG is summarised in Table 1. The concentration of AI was measured using AI-enzyme-linked immunosorbent assay (AI-ELISA)(15, 19) and total protein concentration was measured as described by Bradford (2). The isoforms were purified about 3-fold with an overall yield of 31% by these procedures. The specific activity of the isoforms averaged 22.4 µg AI/mg protein and the purity is estimated to be 91%, because the theoretical specific activity of pure oANG is calculated to be 24.6 µg AI/mg assuming that molecular weights of AI and oANG are 1,300 and 52,000, respectively.

Charge heterogeneity of recombinant oANG. Recombinant oANG displayed the charge heterogeneity on IEF, although the molecular weight of all isoforms was 52,000. The molecular weight is higher by 3,000 than that calculated from the amino acid sequence (14), agreeing with the previous data reported by Inui *et al.* (10). The increase of molecular weight is probably due to oligosaccharides attached to the two potential

Table 1 Purification of recombinant ovine angiotensinogen

Purification step	Volume (mL)	Total AI (µg)	Total protein (mg)	Specific activity (µg AI/mg)	Purification (fold)	Recovery (%)
Conditioned medium	2880	844	108	7.84	1.0	100
Ammonium sulfate precipitation	54	578	44.3	13.0	1.7	65
CM-Toyopearl column chromatography	69	337	16.4	20.5	2.6	40
Chromatofocusing						
A-1	5.1	19.0	0.928	20.5	2.6	2.3
A-2	5.1	37.8	1.68	22.5	2.9	4.5
A-3	6.0	53.9	2.20	24.5	3.1	6.4
A-4	6.8	127	5.25	24.2	3.1	15
A-5	6.8	27.3	1.36	20.1	2.6	3.2

N-linked glycosylation sites (10, 14). Moreover, 60% of *N*-linked oligosaccharides of glycoprotein expressed in CHO cells contain sialic acid residues (1). Therefore, sialic acid residue should be a main factor to make the charge heterogeneity of recombinant oANG.

Desialylation of recombinant oANG. oANG (0.5 mg), obtained by CM-Toyopearl column chromatography, was incubated at 37°C for 24 h with 50 mU of neuraminidase from *Vibrio cholerae* (EC 3.2.1.18, Boehringer Mannheim GmbH, Germany) in 100 μ L of 50 mM sodium acetate buffer, pH 5.5 containing 77 mM NaCl and 6.5 mM CaCl_2 . The progress of desialylation was

monitored by native PAGE (3). After desialylation by neuraminidase, oANG gave one major band at pI 6.9 with two minor bands on IEF, whereas untreated oANG gave five bands in the pI range of 5.7 to 6.6 (Fig. 2B, lanes 1 and 2). The increase of pI value to 6.9 is consistent with the release of sialic acid residues. In contrast, incubation of oANG without neuraminidase caused a small shift in pI toward alkaline side (Fig. 2B, lane 3) but fragmentation of the preparation did not occur (Fig. 2A, lane 3), which indicates that a slight amount of sialic acid residue is removed during the incubation without the enzyme. These results indicate that sialic acid residue is a main

Fig. 2 SDS-PAGE and IEF of desialylated oANG. *Panel A*, SDS-PAGE. Lane 1, untreated oANG (2 μ g); lane 2, oANG incubated with neuraminidase (2 μ g); lane 3, oANG incubated without neuraminidase (2 μ g); lane 4, neuraminidase (no oANG). Molecular weight (M_r) of marker protein was shown to the left. Proteins were stained by Coomassie Brilliant Blue R-250 (CBB R-250). *Panel B*, IEF. A flat bed gel containing 6% (w/v) acrylamide and 2% (w/v) ampholytes (Pharmalyte 3-10, Pharmacia Biotech AB, Uppsala, Sweden) was used to develop a pH gradient of 3.0 to 10. Proteins separated by IEF were transferred to a polyvinylidene difluoride membrane after a 30 min immersion of the gel in the transfer buffer containing 0.1% (w/v) SDS. Transferred proteins were stained by CBB R-250. Lane 1, untreated oANG (5 μ g); lane 2, oANG incubated with neuraminidase (5 μ g); lane 3, oANG incubated without neuraminidase (5 μ g); lane 4, neuraminidase (no oANG). The pI value of marker protein was shown to the left.

factor to cause the charge heterogeneity of recombinant oANG. The remaining charge heterogeneity of desialylated oANG (Fig. 2B, lane 2) is probably attributed to other posttranslational modifications such as phosphorylation, sulfation (6), sialylation of *O*-linked oligosaccharide (5) or truncation of C-terminal amino acid (21).

Kinetic parameters of intact and desialylated recombinant oANGs. Various concentrations (0.0–0.5 μ M) of oANG were incubated with recombinant human renin at 37° C for 30 min in 74 mM sodium phosphate buffer, pH 6.5 containing 7.4 mM diisopropyl fluorophosphate and 7.4 mM EDTA (13, 16). The recombinant human renin used in this study was produced as prorenin in CHO cells and activated by trypsin (16). The rate of AI production was determined by AI-ELISA. The Michaelis constant (K_m) and maximum velocity (V_{max}) were estimated with the software, UltraFit (Biosoft, Cambridge, UK) using Michaelis-Menten equation as a fitting equation and were expressed as 95% confidence limits. The K_m values for reaction of human renin with fractions A-2, A-3, A-4 and fraction A were 0.17 $\pm$ 0.07, 0.15 $\pm$ 0.04, 0.18 $\pm$ 0.01 and 0.17 $\pm$ 0.05 μ M, respectively, and the V_{max} values were 0.90 $\pm$ 0.17, 0.81 $\pm$ 0.07, 0.88 $\pm$ 0.03 and 0.77 $\pm$ 0.08 nM/min, respectively. These kinetic parameters are almost similar among fractions A-2, A-3, A-4 and fraction A. On the other hand, all these fractions were sialylated and probably have the different content of sialic acid, because a single sialic acid residue causes the difference in pI value of angiotensinogen (4, 8). The result of kinetic parameter indicates that sialic acid residue of oANG does not affect the affinity and catalytic activity to human renin.

The K_m values for desialylated and untreated oANGs were 0.11 $\pm$ 0.03 and 0.15 $\pm$ 0.03 μ M, respectively, and the V_{max} values were 0.72 $\pm$ 0.06 and 0.79 $\pm$ 0.06 nM/min, respectively. No marked differences are observed in the kinetic parameters between desialylated and untreated oANGs, which indicates that the removal of sialic acid residue from oANG does not change the affinity and catalytic activity to human renin. These two results of kinetic parameter suggest that sialic acid residue of angiotensinogen does not affect the reactivity to renin.

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