PURIFICATION AND SOME PROPERTIES OF RECOMBINANT SHEEP ANGIOTENSINOGEN EXPRESSED IN CHINESE HAMSTER OVARY CELLS

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ABSTRACT

We established an expression system for large amount of recombinant sheep angiotensinogen (sAngn) in Chinese hamster ovary cells. The recombinant sAngn (21.6 mg) was purified by a single column chromatography to a homogeneous level on sodium dodecyl sulphate-polyacrylamide gel electrophoresis from 2.7l of conditioned medium. Human renin cleaved only a Leu¹⁰-Leu¹¹ bond in sAngn, although Leu¹¹-Val¹² was another candidate for scissile peptide bond. The K_m and k_{cat} values for the reaction of human renin with sAngn were 0.21 μ M and 150 min⁻¹, respectively. Recombinant rat renin could also react with the sAngn. The K_m and k_{cat} for the reaction were 0.84 μ g and 78 min⁻¹, respectively. Purified sAngn was stable at temperature up to 50°C, but rapidly inactivated at that higher than 55°C.

Angiotensinogen (Angn) is a prohormone that is synthesized in the liver and is cleaved by renin (EC 3.4.23.15) to release angiotensin I (Ang I) (17). The decapeptide is processed by angiotensin-converting enzyme (EC 3.4.15.1) to an active octapeptide hormone, angiotensin II, which controls blood pressure and electrolyte balance.

Renin belongs to aspartic protease family (7, 10). Most of aspartic proteases react with their substrates at acidic pH. On the other hand, renin acts with Angn at neutral pH in the blood

circulation. The reaction mechanism of renin is almost unclear, although kinetic studies of renin with synthesized peptide substrates have been reported (2, 5, 14). Kinetic parameters of human renin with peptide substrates were different from those with plasma Angn (2, 6). In order to elucidate the mechanism of renin-angiotensinogen reaction, a sufficient amount of Angn is required. Plasma sheep angiotensinogen (sAngn) is well-known to be a better substrate for human renin, and has therefore been used in clinical study on renin. In this study, we established a Chinese hamster ovary (CHO) cell line that produced a large amount of recombinant sAngn, and determined some of its properties.

MATERIALS AND METHODS

Construction of an Expression Vector of sAngn

A plasmid pBSsAG was digested with *Eco*R I, treated with DNA polymerase I (Klenow fragment), and followed by ligation with *Bam*H I linker. The modified plasmid with *Bam*H I site at the

Abbreviations: sAngn, sheep angiotensinogen; Angn, angiotensinogen; Ang I, angiotensin I; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; CHO, Chinese hamster ovary; dhfr, dihydrofolate reductase; FBS, fetal bovine serum; MTX, methotrexate

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Fig. 1 Structure of an expression vector, pSVsAGD. SV40, early promoter and replication origin of SV40; mDHFR, mouse dihydrofolate reductase gene; bGH poly(A), polyadenylation signal from bovine growth hormone gene; Amp^r, ampicillin resistant gene

5'-end of sAngn cDNA was further digested with *Xba* I and processed again to obtain sAngn cDNA with *Bgl* II site at the 3'-end. The entire sequence of sAngn cDNA with 1,938 bp was ligated into *Bam*H I site of pSVD vector (18) to obtain an expression vector for CHO cells, pSVsAGD (Fig. 1).

Establishment of CHO Cell Line Producing sAngn

The vector, pSVsAGD, was transfected into dihydrofolate reductase (dhfr)-deficient CHO cells (DXB-11) by a calcium phosphate-mediated method using CellPfect Transfection kit (Pharmacia, Uppsala, Sweden). The cells harboring the vector with (dhfr) gene were isolated by culturing in the selection medium, Dulbecco's modified Eagle medium supplemented with 5% dialyzed fetal bovine serum, 100 units/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamin and 100 μ M non essential amino acid. Production of recombinant sAngn was enhanced by methotrexate (MTX, Sigma, St. Louis, U.S.A.). The dose amount was stepwise increased from 0 to 0.5 μ M as shown in Fig. 2. It took for 10 months for the overall procedure of the enhancement. The amount of sAngn secreted into the medium was determined as described previously (13). Briefly, sAngn preparation was incubated with excess amount of recombi-

Fig. 2 Stepwise enhancement of sAngn productivity in CHO cells by MTX. Concentration of recombinant sAngn secreted into the medium was monitored by standard assay method at each step.

Fig. 3 A cation exchange chromatography of sAngn on a CM-Toyopearl 650 column. Recombinant sAngn fraction, obtained concentration and dialysis of 2.71 of conditioned medium, was applied into a CM-Toyopearl 650 M column (Tosoh, Tokyo; 2×20 cm) preequilibrated with 10 mM sodium acetate buffer (pH 5.0). Recombinant sAngn was eluted by a NaCl gradient from 0 to 0.3 M at a flow rate of 0.625 ml/min at 4°C.

nant human renin in the presence of 10 mM diisopropyl fluorophosphate, 10 mM EDTA at pH 6.5 and 37°C for 0.5, 1 and 2 h, and Ang I released from sAngn was assayed by Ang I-ELISA (11, 21). The plateau level of Ang I produced was, in this study, represented as the amount of sAngn. Protein concentration was measured using a Protein Assay kit (BIO-RAD, Hercules, U.S.A.). The productivity of sAngn in the cells was increased 10-fold by this MTX enhancement procedure (Fig. 2). The CHO

Table 1 *Purification of Sheep Angiotensinogen*

Purification step	Total protein (mg)	Total Ang I (μ g)	Specific amount (μ g Ang I/mg)	Purification (fold)	Recovery (%)
Conditioned medium	155	838	5.41	1	100
Concentration	137	836	6.01	1.11	99.9
Dialysis	74.6	694	9.30	1.72	82.8
CM-Toyopearl	21.6	455	21.1	3.90	54.3

cell line obtained in this protocol was further cultured under the same conditions as the final enhancement, and was used in this study. The sAngn productivity has almost unchanged for more than 2 years.

Recombinant Human and Rat Renins

Human renin and rat renin used in this study were purified by the methods previously described (18, 23). Both CHO cell lines that express human renin or rat renin were generously gifted from Professor K. Murakami of the Tsukuba University.

RESULTS AND DISCUSSION

Purification of Recombinant sAngn

The 2.7l of conditioned medium was concentrated by polyethylene glycol #20000 and dialyzed against a sufficient amount of 10 mM sodium acetate buffer (pH 5.0). The dialyzate was applied to a CM-Toyopearl 650 M column preequilibrated with 10 mM sodium acetate buffer (pH 5.0). Recombinant sAngn was eluted by a NaCl gradient from 0 to 0.3 M, as shown in Fig. 3. The recombinant sAngn was purified by 3.90-fold with an overall yield of 54.3% by the single step purification as summarized in Table 1.

Molecular Mass of Recombinant sAngn

Purified sAngn was electrophoresed on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (9), with additional sodium thioglycollate (100 mM) in cathodic buffer. As shown in Fig. 4, the migration distance of recombinant sAngn was identical to that of native sAngn (5, 14) and the molecular mass was estimated to be 56 kDa (Fig. 4).

Fig. 4 SDS-PAGE of purified recombinant sAngn. Purified sAngn (5 μ g) was electrophoresed on 7.5% SDS-PAGE and stained by Coomassie Blue R-250.

N-Terminal Amino Acid Sequence of Recombinant sAngn

The purified sAngn, separated by SDS-PAGE under reduced conditions, was transferred onto polyvinylidene difluoride membrane and stained by Coomassie Blue R-250. The sAngn was subjected to automated protein sequencer (model Procise 491, PE-ABI). The N-terminal amino acid sequence was determined to be Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-His-Ser-Lys-Ser-Asn-(Xaa)-Asp-Gln-Leu-Glu-Lys-Pro-Ser-Val-Glu. This result was identical to that of native sAngn previously reported (4). These results indicated that recombinant sAngn was normally processed in the CHO cells and secreted into the culture medium.

Table 2 Kinetic Parameters of sAngn with Renins of Human and Rat at pH 6.5

Renin species	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\mu\text{M}^{-1}\text{min}^{-1}$)
Human	0.21	150	721
Rat	0.84	78	93

The Cleavage Site of Human Renin in sAngn

Renins of human and rat cleave the peptide bond of Leu¹⁰-Val¹¹ and that of Leu¹⁰-Leu¹¹ in Angns of human and rat, respectively. Additionally, amino acid sequences near the scissile peptide bond of human and rat Angns are His⁹-Leu-Val-Ile¹² (8, 19, 22) and His⁹-Leu-Leu-Tyr¹² (1, 15), respectively. The amino acid sequence from position 9th to 12th in sAngn is His-Leu-Leu-Val (4, 12). Two candidate sites for cleavage by human renin would therefore be Leu¹⁰-Leu¹¹ and Leu¹¹-Val¹² in sAngn. The N-terminal amino acid sequence of the sAngn after incubated with a sufficient amount of human renin was determined to be Leu-Val-His-Ser-Lys-Ser-Asn-Xaa-Asp-Gln-Glu-Lys-Pro-Ser-Val-Glu. The amino acid sequence of this modified sAngn started from the position of Leu¹¹ in the sAngn. Human renin was determined to cleave sAngn at the Leu¹⁰-Leu¹¹ bond to produce Ang I peptide and des(Ang I) Angn. These results indicate that renin reaction prefers the peptide bond of 10th and 11th residue to side chain specificity under these conditions.

Kinetic Parameters of Human or Rat Renin with Recombinant sAngn

The K_m and k_{cat} of human renin with sAngn were 0.21 μM and 150 min^{-1} , respectively (Table 2). This is similar to that for plasma sAngn reported previously (3, 20). On the other hand, The K_m and k_{cat} of rat renin with sAngn were determined to be 0.84 μM and 78 min^{-1} , respectively (Table 2), indicating that recombinant sAngn can also be used for the study of rat renin.

Thermostability of Recombinant sAngn

Thermostability of sAngn was determined by measuring the residual reactivity of Angn with human renin after incubation at various temperatures for 30 min. As shown in Fig. 5, sAngn was stable at temperature up to 50°C and completely inactivated

Fig. 5 Thermostability of recombinant sAngn. The reaction mixture, contained 57 $\mu\text{g}/\text{ml}$ recombinant sheep angiotensinogen, 60 mM sodium phosphate (pH 6.0), 10 mM diisopropyl fluorophosphate and 10 mM ethylenediamine tetraacetate, was incubated at indicated temperature for 30 min, and then cooled on ice to proceed the reaction with human renin.

Fig. 6 Double immunodiffusion analysis of anti-sAngn antiserum against various source of Angns. Native angiotensinogens (2–3 μg) of sheep (well b), human (well c), rat (well d) and porcine (well e) were partially purified from their plasma as previously described (11). Blank well without any serum was set at well f. Anti-sAngn antiserum (1:1,000 dilution) and recombinant sAngn were applied in the center well and well a, respectively.

at 65°C under these conditions.

Production of Anti-sAngn Antibodies and Their Immunochemical Property

Purified recombinant sAngn was emulsified with complete Freund's adjuvant and 100–200 μg quantities of the antigen were intradermally injected into multiple sites of New Zealand White rabbits, followed by boosting at intervals of 1 week with

the protein in incomplete Freund's adjuvant for five weeks. Eighteen weeks later, the same injection procedure was repeated. Anti-sAngn antiserum was obtained one week after the final booster. Ouchterlony double immunodiffusion analysis (16) showed that anti-sAngn antibodies did not bind with human, rat and porcine Angns, but did bind with recombinant and plasma sAngns (Fig. 6). These results show that sAngn antibodies obtained in this study have remarkably high specificity for species. These antibodies would be a useful tool for renin-Angn studies.

In this study, we have established a stable mammalian cell line producing a large amount of recombinant sAngn. This expression system will be useful to elucidate the mechanism of renin-Angn reaction and to further investigate on the renin angiotensin system.

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