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Isolation and Characterization of Native Human Renin Derived From Chinese Hamster Ovary Cells

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ABSTRACT **Transfection of Chinese hamster ovary (CHO) cells with a plasmid containing the cDNA for human preprorenin has provided cell lines that secrete 15 mg of native prorenin per liter of culture medium. Tryptic activation of the prorenin occurs by selective cleavage of the Arg_{66}-Leu_{67} bond (numbering as in preprorenin). The renin product, purified in a single step and in high yield by affinity chromatography, is fully stable for as long as 8 months when stored in solution at 4°C and pH 6.5. Purity of the renin was judged to be greater than 95% by gel electrophoresis, compositional and N-terminal sequence analyses, and specific enzyme activity. An important aspect of the present work is the development of a direct assay for renin which permits accurate and reproducible evaluation of enzyme units and kinetic parameters. Application of methods described herein, combined with appropriate scale-up fermentation capabilities, provides the means for generating gram quantities of human renin and its zymogen.**

Key words: **hypertension, renin production, mammalian expression, affinity chromatography, genetic engineering, prorenin secretion**

INTRODUCTION

A wealth of information suggests that the renin-angiotensin system plays an important role in hypertension (see 1–3 for reviews and references). Renin is an aspartyl proteinase, the sole known function of which is to cleave the tenth peptide bond in its 55,000-dalton substrate, angiotensinogen. The decapeptide product, angiotensin I, is then processed by a converting enzyme to the bioactive octapeptide, angiotensin II, a potent vasoconstrictor and stimulant of aldosterone secretion. For several reasons, renin has been targeted as an intervention point in the development of specific inhibitors for the therapeutic control of hypertension. First, knowledge concerning the enzyme's structure and function can be inferred from other homologous and well-characterized members of the aspartyl proteinases. Second, the renin-catalyzed reaction is the first and rate-limiting step of the cascade and is, therefore, a reasonable target for inhibition. Third, the high selectivity of renin implies that a corresponding degree of specificity might be exhibited by carefully designed inhibitors. Finally, substrate-based peptide inhibitors of renin have proven effective in lowering blood pressure in hypertensive animals.[4–6]

Renins have been isolated from several natural sources including brain,[7] submaxillary gland,[8,9] and kidney,[10–12] but the quantities of enzyme thus obtained have been insufficient for detailed characterization. Nevertheless, application of recombinant techniques has provided the complete amino acid sequences for the preprorenins from mouse submaxillary gland[13–15] and from human kidney.[16–19] Moreover, three-dimensional models of the corresponding renins have been constructed in several laboratories based upon x-ray coordinates derived from studies of highly homologous aspartyl proteinases.[20–24]

Despite this level of sophistication in our understanding of the structure and, by inference, the mechanism of renin, the molecular data are inadequate to allow detailed, reliable modeling for the design of constrained peptide, and nonpeptide, inhibitors. Although preliminary crystallographic data have been reported for the mouse submaxillary gland protein,[25] amounts of human renin available from natural sources are insufficient for crystallographic analysis and for chemical and mechanistic characterization of the enzyme. It is, therefore, of paramount importance to evaluate recombinant DNA methods for production of the mature enzyme or its precursors in large quantities. Efforts thus far in expression of mouse[26] and human[27] renins in *Escherichia coli*, and of human prorenin in *E. coli*,[28,29] in dog tumor cells[30] and in Chinese hamster ovary cells[31] have succeeded to various degrees in generating large amounts of the proteins, but corresponding levels of enzyme activity have not yet been reported. In the last case cited, Fritz et al.[31] demonstrated renin activity in culture

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Abbreviations follow the IUPAC-IUB Tentative Rules on Biochemical Nomenclature, J. Biol. Chem. 247:977 (1972). LeuΨ[CH_2NH]Val is the abbreviation for the compound where the peptide bond between Leu and Val has been reduced to -CH_2NH-.

media from transected Chinese hamster ovary (CHO) cells, but no details were given as to purification, characterization, or yield of the recombinant enzyme.

The present communication describes the engineering of the cDNA for human preprorenin into an expression plasmid appropriate for manipulations in mammalian cells. High-producing clones from CHO cells transfected with this plasmid have yielded culture media containing 15 mg/liter of human prorenin that is folded in its native conformation. The proenzyme has been activated with trypsin, and the resulting renin has been obtained in purity greater than 95% by a single-affinity chromatographic step that is highly selective for human renin. Results from chemical and enzymological characterization of the recombinant renin confirm its identity with the natural human enzyme. During the course of this work, a direct and selective HPLC assay for renin was developed that allows, for the first time, the definition of an enzyme unit which is easily determined and highly reproducible.

MATERIALS AND METHODS

Construction of Expression Plasmid

Plasmid pHRn321[16] was digested with SacI plus PstI to obtain a 1.4-kb fragment containing the complete coding sequences for human preprorenin, with the GC tail used in cDNA cloning left of the 5'-end. This fragment was cloned between the PstI and SacI sites of pUC12 to give plasmid pRnPS1. To introduce a unique restriction enzyme cleavage site in the sequence coding for the 5'-untranslated sequences of the renin mRNA, an oligonucleotide-directed mutagenesis was performed by using lambda exonuclease[32] to generate a single-stranded template. The details of this method will be published elsewhere. In the resulting plasmid, pDPRn1, position −28 (where +1 is the A of the initiation codon) was converted to a C as expected to generate a BamHI cleavage site, but a deletion of nucleotides −25 to −13 had occurred. Since this deletion caused no loss of preprorenin coding sequence, pDPRn1 was used for the expression experiments.

Plasmid pDPRn1 was digested with SacI and the ends were made blunt by using the Klenow fragment of DNA polymerase I. The plasmid was then digested with BamHI and the 1.4-kb BamHI/blunt fragment was isolated. A 600-bp PvuII/EcoRI fragment containing the polyadenylation signal from the bovine growth hormone (bGH) gene was isolated from pλGH2R2.[33] The plasmid pSV2dhfr[34] was digested with EcoRI plus BamHI, and the larger fragment was isolated. All three of these fragments were ligated to give plasmid pDPRnPA1, which contained the preprorenin cDNA with the bGH polyadenylation site downstream, cloned into pSV2dhfr.

The 325-bp HindIII/PvuII fragment containing the SV40 promoter and origin of replication was isolated from pSV2dhfr, and BamHI linkers were added to its ends. This fragment was then cloned into the BamHI site of pDPRnPA1 such that the SV40 early promoter would direct transcription toward the preprorenin cDNA to generate the plasmid pSVDPRnPA33.

pSVDPRnPA33 DNA was used to transfect dihydrofolate reductase CHO^{dhfr-} cells[35] by calcium phosphate coprecipitation.[36] Cells which had incorporated the plasmid and were $dhfr^+$ were selected by plating in Dulbecco's Modified Eagle's media (MEM) supplemented with 10% fetal bovine serum, 0.1 mM MEM nonessential amino acids, 10 mM HEPES, pH 7.3, 100 units penicillin per ml and 100 μg per ml streptomycin (all media and supplements supplied by Gibco). Clones of cells were then adapted stepwise to growth in methotrexate (Lederle).

Renin Assay

Renin activity was measured relative to the rate of disappearance of model peptide substrates and the appearance of the two N- and C-terminal products by quantitation of the peptides separated by reverse-phase HPLC. Assay mixtures containing 40–100 nmol of peptide substrate in 100 μl of 0.1 M sodium phosphate, pH 6.5, were incubated at 37°C with 10 μl of renin containing 0.01–1 μg of protein. Portions (10 μl) of the assay mixture were removed at various times and mixed with 50 μl of the solvent used for the isocratic HPLC separation. Solvents contained 0.15% trifluoroacetic acid, and variable amounts of acetonitrile, depending upon the substrate employed. Separations were performed at ambient temperature on a Zorbax ODS 5-μm column operated at a flow rate of 1.5 ml/min; effluent was monitored continuously for peptide content at 210 nm. With the standard renin peptide substrate, D-R-V-Y-I-H-P-F-H-L-L-V-Y-S (Vega Biochemicals), the elution times for the tetradecapeptide substrate and the decapeptide and tetrapeptide products were, 5.3, 4.0, and 1.6 min, respectively. Several peptide substrates having both Leu-Leu, and Leu-Val scissile bonds were evaluated in similar systems with similar results. One that was especially useful for kinetic measurements was the undecapeptide K-I-H-P-F-H-L-L-V-Y-S, (Vega Biochemicals), which has superior solubility characteristics in the various solvents employed. With the solvent system described above, but with 30% acetonitrile, the 4-, 7-, and 11-residue peptides emerged at 1.8, 3.2, and 6.1 min, respectively.

Preparation of Affinity Column

Affinity chromatography of human CHO-cell renin and the strategy for ligand design and coupling to activated CH-Sepharose were adapted from a paper by McIntyre et al.[37] The major changes involved the ligand structure which contained a LeuΨ[CH_2NH]Val rather than the reported LeuΨ[CH_2NH]Leu- at the scissile bond site, and the use of 0.1 M sodium acetate, pH 4.3, as the final buffer in gradient elution of renin from the affinity column.

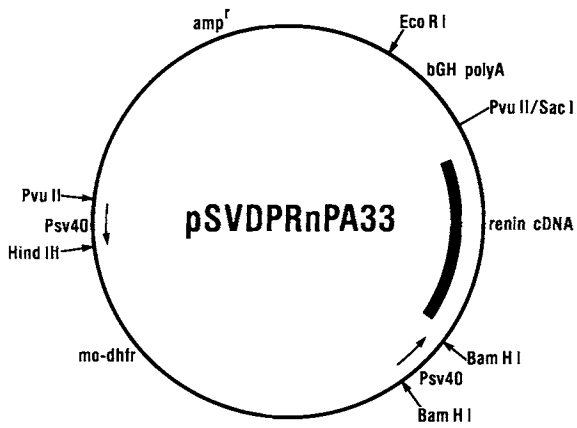

Fig. 1. Structure of plasmid pSVDPRnPA33. The construction of this plasmid is described in Materials and Methods. The internal bar indicates the sequence coding for preprorenin, designated renin cDNA. The region designated bGH polyA includes the polyadenylation signal of the bovine growth hormone gene.[38] P_{SV40} indicates the early promoter of SV40, and the internal arrows indicate the direction of transcription of the early promoter. The regions labeled mo-dhfr and amp^r designate the dihydrofolate reductase and ampicillin resistance genes of the vector pSV2dhfr.[32]

Analytical Methods

Renin samples were assessed for purity by SDS gel electrophoresis according to the procedure of Laemmli.[38] Amino acid analysis of renin and peptide samples hydrolyzed *in vacuo* for 24–72h in 6N HCl at 110°C were performed on a Dionex D-500 analyzer. Automated Edman degradation of renin was carried out on 0.1–1-nmol samples with the aid of an Applied Biosystems Inc. model 470A gas-phase sequencer fitted with an on-line PTH analytical system provided by the manufacturer.[39]

RESULTS

Construction of Cell Lines Producing Prorenin

A schematic representation of the plasmid, pSVDPRnPA33, is shown in Figure 1. This plasmid was transfected into dhfr-negative CHO cells, and dhfr-positive cells were selected. These cells secreted a protein of approximately 43 kd detectable on Western blots with antihuman renin serum. When individual clones from this original pool of transfected cells were analyzed, some were found to produce the protein reactive with antirenin serum. Five such clones were adapted to growth in 300 nM methotrexate, with the idea that such adaptation could lead to amplification of the dhfr gene with coamplification of the renin expression unit.[37] Individual clones from the cells adapted to growth on 300 nM to 1 μM methotrexate were isolated for analysis of renin expression.

Activation of Prorenin

During the course of this investigation, transfected CHO cells adapted to growth on varying concentrations of methotrexate up to 1 μM were analyzed. These clones secrete prorenin at levels from 2 to 15 mg/liter of growth medium. Estimates of prorenin concentration are based upon quantitation and assay of renin generated by treatment of the zymogen with trypsin. Culture media from cells grown in 10% fetal calf serum (about 3.5 mg protein/ml) were treated with trypsin (0.1 mg/ml) for 2 h. At this time, diisopropylphosphofluoridate (125 μM; Aldrich) was added to inactivate trypsin and any other serine proteinases present in the medium. Metalloproteinases and thiol proteinases were also inactivated by addition of EDTA (2 mg/ml) and freshly prepared sodium tetrathionate (1.2 mg/ml), respectively. We have found that renin activity is not diminished by extended exposure to trypsin, suggesting that the activation procedure is selective for the Arg_{66}-Leu_{67} bond (numbering in preprorenin[16]). The activated culture medium was centrifuged at 5,000 rpm for 30 min at 4°C, and supernatants were then passed through a Millipore filter (0.45 μm) prior to purification of the renin.

Purification of Recombinant Human Renin (rh-Renin)

Of the various procedures described for the purification of natural human kidney renin, that of McIntyre et al.[37] appeared to be most promising since it provided material of greater than 80% purity in yields of about 75% in a single affinity chromatographic purification of crude kidney extracts. The ligand in the published method[37] was an isosteric peptide inhibitor of renin, H.77, with a reduced LeuΨ[CH_2NH]Leu scissile bond site. We followed this general procedure but altered the ligand sequence in two positions; Leu-6 was changed to Val, and Val-7 was replaced by Ile. Thus, the sequence of the ligand resembled more closely that of human angiotensinogen.

As much as 2 liters of activated culture medium containing 4–30 mg of renin was passed over a column (0.9 × 5 cm) of the affinity matrix equilibrated in 50 mM Tris/HCl, pH 7.4. The column was next washed with 100ml of 50 mM Tris/HCl, pH 7.4, containing 1 M NaCl, and then equilibrated with 0.1 M sodium acetate, pH 6.0. Elution of bound renin was accomplished with a gradient of decreasing pH in the same salt from pH 6.0 to 4.3 (50 ml each of initial and final buffer) followed by a wash with buffer at pH 4.3. The profile shown in Figure 2 corresponds to a purification of 2 liters of 5-day roller bottle culture medium containing 4 mg of renin. Fractions indicated in Figure 2 were pooled, and the pH was adjusted to 7.2 by the addition of a concentrated solution of Tris. Upon standing overnight or a few days at 4°C some precipitation of inactive, nonrenin protein takes place.

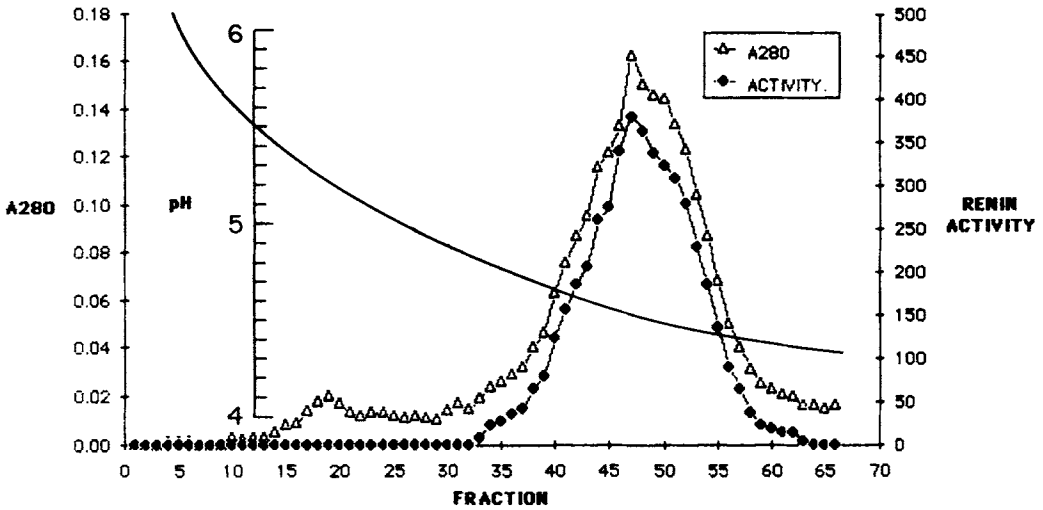

Fig. 2. Elution of rh-renin from an affinity column by a gradient of decreasing pH in 0.1 M sodium acetate. This preparation of enzyme was from 2 liters of cultures of a prorenin-secreting CHO-cell clone.

TABLE I. Amino Acid Analysis of Human Renin From CHO-Cells*

Amino acid	Residues	
	Expected[16]	Found
Asx	29	29.1
Thr	30	29.2†
Ser	33	29.1†
Glx	28	27.8
Pro	13	14.5
Gly	34	34.4
Ala	16	17.0
Val	23	21.2
Met	8	8.0
Ile	22	19.0
Leu	28	28.4
Tyr	17	17.2
Phe	19	17.2
His	6	6.1
Lys	15	15.5
Arg	10	10.0
Cys	6	N.C.
Trp	3	N.C.

*One milliliter from the total 45-ml sample of renin (rh-renin) purified by affinity chromatography was dialyzed against water to remove salts and lyophilized. The dried protein was hydrolyzed *in vacuo* for 24 h at 110°C in 6 N HCl and the hydrolyzate was rotary evaporated to dryness. The residue was dissolved in 50 μl of 0.2 M sodium citrate, pH 2.2, and 10 μl was loaded onto a Dionex 500.
†Uncorrected for decompositional losses.

This precipitate was removed by centrifugation and the supernatant (45 ml in approximately 0.1 M Tris acetate, pH 7.2), hereafter referred to as rh-renin, was stored at 4°C. Various preparations of renin have been stored for months under these conditions at concentrations as dilute as 0.02–0.1 mg/ml with no observed loss in activity.

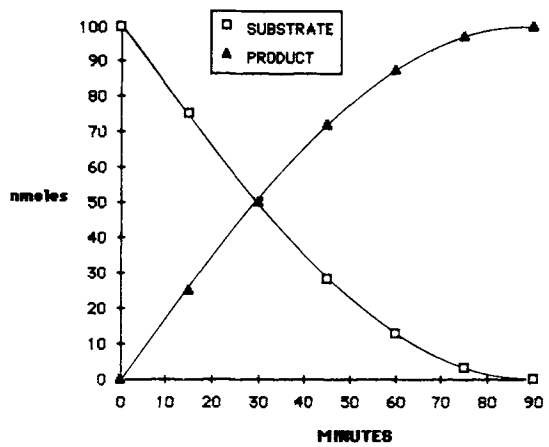

Fig. 3. Time course of disappearance of undecapeptide substrate (L-I-H-P-F-H-L-L-V-Y-S) and generation of heptapeptide product (L-I-H-P-F-H-L) catalyzed by rh-renin. The assay mixture, maintained at 37°C, contained 100 nmol of substrate in 0.1 M sodium phosphate buffer, pH 6.0, and 1 μg of rh-renin (total volume = 100 μl). Aliquots (10 μl) were withdrawn at various times and analyzed by HPLC. The C-terminal tetrapeptide was difficult to measure accurately because it emerges early from the column near the position of salt, and injection artifact peaks.

Estimation of the protein concentration of rh-rhenin was made by amino acid analysis. The composition of rh-rhenin presented in Table I was consistent with that expected[16] for human renin; the total yield of rh-renin based upon this analysis was about 3.5 mg (88%). Activity measurements of rh-renin were in accord with values expected for pure human kidney renin. Based upon the definition that 1 unit of renin produces 120 μg of angiotensin I per milliliter per hour at pH 6.5 and 37°C, we calculate a specific activity of 1,500 units per mg in rh-rhenin. This is based upon our HPLC assays using either the tetra-

decapeptide or undecapeptide substrates or the tridecapeptide containing a Leu-Val scissile bond (Peninsula). Highly purified natural renin shows similar reactivities toward these substrates. A time course showing loss of the undecapeptide and generation of heptapeptide product is shown in Figure 3.

Characterization of rh-Renin

Results described thus far pertaining to the specific activity and amino acid composition of rh-renin were consistent with the view that it was highly purified renin. This material was characterized further by SDS polyacrylamide gel electrophoresis (Fig. 4), which revealed a single, somewhat diffuse band corresponding to protein of molecular weight 40,000 and several very minor bands of higher and lower molecular weight which together account for less than 5% impurity. We suspect that heterogeneity of the carbohydrate component probably contributes to the lack of sharpness of the renin band. The results obtained for 15 cycles of Edman degradation of rh-renin are presented in Table II. Assuming an initial yield of 50%, we calculate that the original preparation of rh-renin contains a total of 3.8mg of renin, a value in good agreement with that obtained from amino acid analysis. Rh-renin gave a single sequence corresponding to that expected for human renin (Table I), except that Asn-5 was not observed. This finding is consistent with earlier speculation[21] that Asn-5 may be glycosylated.

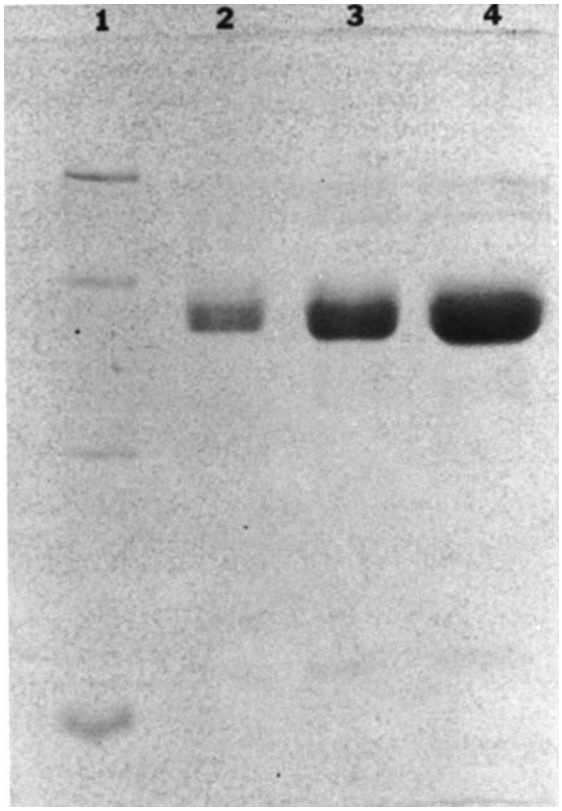

Fig. 4. SDS-polyacrylamide gel electrophoresis[41] of rh-renin (cf. Fig. 2) and 0.2 μg each of four protein standards (Boehringer Mannheim), stained with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories). Lane 1, from top to bottom: bovine serum albumin (68,000 daltons), ovalbumin (45,000 daltons), chymotrypsinogen A (25,000 daltons), and cytochrome c (12,500 daltons). Lanes 2–4: 1.6, 4.2, and 8.3 μg, respectively, of rh-renin.

TABLE II. Automated Edman Degradation of Human Renin From CHO Cells

Cycle	Residue expected in human renin([16])	Major sequence		Carryover	
		Residue observed	Yield (pmol)	Residue observed	Yield (pmol)
1	Leu	Leu	751	—	—
2	Thr	Thr†	301	(Leu)	75
3	Leu	Leu	485	(Thr)†	40
4	Gly	Gly	512	(Leu)	112
5	Asn	—*	—*	(Gly)	126
6	Thr	Thr†	383		
7	Thr	Thr†	364	(Thr)†	
8	Ser	Ser†	630	(Thr)†	88
9	Ser	Ser†	583	(Ser)†	
10	Val	Val	298	(Ser)†	269
11	Ile	Ile	240	(Val)	165
12	Leu	Leu	214	(Ile)	79
13	Arg	Arg	43	(Leu)	179
14	Asn	Asn	284	(Arg)	37
15	Tyr	Tyr	263	(Asn)	135

*Asn is the expected residue at this position but was not observed. This position has been suggested as a possible site for glycosylation and the present analysis provides the first evidence that position 5 is, in fact, glycosylated[21].
†Serine and threonine do not give representative values compared to the sensitivity set for most of the PTH amino acids.

DISCUSSION

Identification of prorenin-secreting clones of transfected CHO cells was greatly facilitated by trypsin activation and by our HPLC assay system for renin, which monitors not only proteolysis, but specific cleavage of the renin-sensitive bond in model substrates. This latter point should be emphasized because several preparations that appeared to have renin activity as judged by radioimmunoassay for angiotensin I were shown by our HPLC system to contain nonrenin proteinases. Thus, a definitive criterion for human renin activity is that peptide products obtained by cleavage of model substrates must arise from cleavage of the Leu-Val scissile bond; i.e., they must chromatograph in positions exactly equivalent to those of expected products. We have found that although many renins and aspartyl proteinases such as pepsin show reninlike activity toward peptide substrates with Leu-Leu scissile bonds, these same enzymes, in contrast to human renin, are often sluggish or inactive toward substrates with the Leu-Val cleavage site. Therefore, all preparations were shown to be active against a tridecapeptide containing the Leu-Val scissile bond characteristic of human angiotensinogen.

Trypsin was selected for prorenin activation since earlier work[16,40] had suggested that the prosegment should terminate with Lys_{65}-Arg_{66}; the mature renin hence was suspected and found to begin with Leu_{67}. Neither the time of activation nor the concentration of trypsin seemed to be critical; once maximal levels of renin activity were obtained, very little diminution occurred thereafter. Treatment of activated media with a variety of inhibitors selected to block proteinases from all of the known mechanistic sets, save the aspartyl enzymes, appeared to be effective since model peptides sensitive to such enzymes were not cleaved at sites other than the one expected for renin.

Rh-renin was purified approximately 3,600-fold to near homogeneity in a yield of 88% by a single affinity chromatographic step. This method has been applied over a dozen times with highly reproducible success, and the same affinity column has been used many times with liters of activated CHO-cell culture media. The affinity ligand is designed to be resistant to proteolytic enzymes other than renin, but it does contain a Phe-His bond that is a potential cleavage site for chymotrypsinlike enzymes. Nevertheless, adventitious hydrolysis of the peptide ligand does not appear to have taken place to any significant extent.

We have had ample amounts for the compositional and sequence analyses required to prove the chemical purity of our product. Renin concentrations determined by amino acid analysis correlate nicely with values obtained from Edman degradation, and the latter procedure gave no indication of secondary sequences from contaminants or nicked renin chains. The question of specific enzyme activity is less easily addressed, since this is usually expressed in terms of Goldblatt units, originally defined as to amounts of enzyme required for elevation of blood pressure in dogs[41] and operationally defined relative to some pure human renin standard. Published methods for isolation of pure hog[10] and human[12] kidney renin suggest a value of about 1,000 Goldblatt units per milligram of pure enzyme. Radioimmunoassays for release of angiotensin I performed on rh-renin relative to several human kidney preparations indicate a high level of purity in the recombinant enzyme. The activity of human renin provided by Scripps Laboratories is defined as that amount of enzyme which generates 120 μg (nearly 100 nmol) of angiotensin I per milliliter per hour at 37°C, pH 7.5. One of these units is said to be equivalent to 1 Goldblatt unit. We have provisionally defined our activity in terms of the Scripps definition, i.e., that 1 unit is the amount of enzyme that hydrolyzes 100 nmol of peptide substrate per hour at 37°C, pH 6.5. On this basis, our specific enzyme activity exceeds slightly that expected for pure human kidney renin.

Experiments are in progress to prove further the equivalence of natural human renin and that derived from transfected CHO cells. In the meantime, amplified cell lines currently in hand are capable of providing large quantities of human renin necessary for *in vivo* testing of renin inhibitors which may prove useful in the treatment of hypertension. Moreover, chemical and physical characterization of the enzyme is now a realistic undertaking. In fact, preliminary work has shown that rh-renin crystallizes readily both in the presence and absence of inhibitors,[42] thus holding promise for success in deriving a detailed structure of the enzyme and its active state.

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REFERENCES

1. Ondetti, M.A., Cushman, D.W. Enzymes of the renin-angiotensin system and their inhibitors. Annu. Rev. Biochem. 51:238–308, 1982.
2. Nakanishi, S., Kitamura, N., Ohkubo, H. Structure, regulation, and evolution of the genes for the renin-angiotensin and the kallekrein-kinin systems. Bio/Technol. 3:1089–1098, 1985.
3. Navar, L.G. Symposium: Physiological role of the intrarenal renin-angiotensin system. Fed. Proc. 45:1411–1453, 1986.
4. Evans, B.E., Rittle, K.E., Bock, M.G., Bennett, C.D., DiPardo, R.M., Boger, J., Poe, M., Ulm, E.H., LaMont, B.I., Blaine, E.H., Fanelli, G.M., Stabilito, I.I., Veber, D. A uniquely potent renin inhibitor and its unanticipated plasma binding component. J. Med. Chem. 28:1755–1756, 1985.
5. Tree, M., Atrash, B., Donovan, B., Gamble, J., Hallett, A., Hughes, M., Jones, D.M., Leckie, B., Lever, A.F., Morton, J.J., Szelke, M. New inhibitors of human renin tested *in vitro* and *in vivo* in the anesthesized baboon. J. Hypertension 1:399–403, 1983.

6. Geiger, R. Chemistry of the inhibitors of the renin-angiotensin system. Arzneimittelforsch. 35:1386–1398, 1984.
7. Hirose, S., Ohsawa, T., Inagami, T., Murakami, K. Brain renin from bovine anterior pituitary: Isolation and properties. J. Biol. Chem. 257:6316–6321, 1982.
8. Misono, K.S., Inagami, T. Characterization of the active site of mouse submaxillary gland renin. Biochemistry 19:2616–2622, 1980.
9. Poe, M., Wu, J.K., Florance, J.R., Rodkey, J.A., Bennett, C.D., Hoogsteen, K. Purification and properties of renin and γ-renin from the mouse submaxillary gland. J. Biol. Chem. 258:2209–2216, 1983.
10. Inagami, T., Murakami, K. Pure renin: Isolation from hog kidney and characterization. J. Biol. Chem. 252:2978–2983, 1977.
11. Slater, E., Strout, H.V. Jr. Pure human renin: Identification and characterization of two major molecular weight forms. J. Biol. Chem. 256:8164–8171, 1981.
12. Yokosawa, H., Holladay, L.A., Inagami, T., Haas, E., Murakami, K. Human renal renin: Complete purification and characterization. J. Biol. Chem. 255:3498–3502, 1980.
13. Rougeon, F., Chambraud, B., Foote, S., Panthier, J.J., Nageotte, R., Corvol, P. Molecular cloning of a mouse submaxillary gland renin cDNA fragment. Proc. Natl. Acad. Sci. USA 78:6367–6371, 1981.
14. Panthier, J.J., Foote, S., Chambraud, B., Stosberg, A.D., Corvol, P., Rougeon, F. Complete amino acid sequence and maturation of the mouse submaxillary gland renin precursor. Nature 298:90–92, 1982.
15. Masuda, T., Imai, T., Fukushi, T., Sudoh, M., Hirose, S., Murakami K. Molecular cloning of DNA complimentary to mouse submandibular gland renin mRNA. Biomed. Res. 3:541–545, 1982.
16. Imai, T., Miyazaki, H., Hirose, S., Hori, H., Hayashi, T., Kageyame, R., Ohkubo, H., Nakanishi, S., Murakami, K. Cloning and sequence analysis of cDNA for human renin precursor. Proc. Natl. Acad. Sci. USA 80:7405–7409, 1983.
17. Soubrier, F., Panthier, J.J., Corvol, P., Rougeon, F. Molecular cloning and nucleotide sequence of a human renin cDNA fragment. Nucleic Acids Res. 11:7181–7190, 1983.
18. Hobart, P.M., Fogliano, M., O'Conner, B.A., Schaefer, I.M., Chirgwin, J.M. Human renin gene: Structure and sequence analysis. Proc. Natl. Acad. Sci. USA 81:5026–5030, 1984.
19. Hardman, J., Hort, Y.J., Catanzaro, D.F., Tellam, J.T., Baxter, J.D., Morris, B.J., Shine, J. Primary structure of the human renin gene. DNA 3:457–468, 1984.
20. Blundell, T., Sibanda, B.L., Pearl, L. Three-dimensional structure, specificity, and catalytic mechanism of renin. Nature 304:273–275, 1983.
21. Murakami, K., Hirose, S., Miyazaki, H., Imai, T., Hori, H., Hayashi, T., Kageyama, R., Ohkubo, H., Nakanishi, S. Complementary DNA sequences of renin: State-of-the-art review. Hypertension 6:I-95–I-100, 1984.
22. Akahane, K., Umeyama, H., Nakagawa, S., Moriguchi, I., Hirose, S., Iizuka, K., Murakami, I. Three-dimensional structure of human renin. Hypertension 7:3–12, 1985.
23. Sibanda, B.L., Blundell, T., Hobart, P.M., Fogliano, M., Bindra, J.S., Dominy, B.W., Chirgwin, J.M. Computer graphics modelling of human renin: Specificity, catalytic activity, and intron-exon junctions. FEBS Lett. 174:102–111, 1984.
24. Carlson, W., Karplus, M., Haber, E. Construction of a model for the three-dimensional structure of human renal rein. Hypertension 7:13–26, 1985.
25. Navia, M.A., Springer, J.P., Poe, M., Boger, J., Hoogsteen, K. Preliminary x-ray crystallographic data on mouse submaxillary gland renin and renin-inhibitor complexes. J. Biol. Chem. 259:12714–12717, 1984.
26. Masuda, T., Nakano, E., Hirose, S., Murakami, K. Synthesis of enzymatically active mouse submandibular gland renin in *Escherichia coli.* Agric. Biol. Chem. 50:271–278, 1986.
27. Kaytes, P.S., Theriault, N.Y., Poorman, R.A., Murakami, K., Tomich, C.C. High level expression of human renin in *E. coli.* J. Biotech., 4:205–218, 1986.
28. Imai, T., Cho, P., Takamatsu, H., Hori, H., Siato, M., Masuda, T., Hirose, S., Murakami, K. Synthesis of human prorenin in *Escherichia coli.* J. Biochem. 100:425–532, 1986.
29. Tomich, C.C., personal communication.
30. Weighouse, T.F., Cornette, J.C., Sharma, S.K., Tarpley, W.G. Secretion of enzymatically active human renin from mammalian cells using an avian retroviral vector. Gene, in press, 1986.
31. Fritz, L.C., Arfsten, A.E., Dzau, V.J., Atlas, S.A., Baxter, J.D., Fiddes, J.C., Shine, J., Cofer, C.L., Kushner, P., Ponte, P.A. Characterization of human prorenin expressed in mammalian cells from cloned cDNA. Proc. Natl Acad. Sci. USA 83:4114–4118, 1986.
32. Little, J.W. An exonuclease induced by bacteriophage λ: II. Nature of the enzymatic reaction. J. Biol. Chem. 242:679–686, 1967.
33. Woychik, R.P., Camper, S.A., Lyons, R.H., Horowitz, S., Goodwin, E.C., Rottman, F.M. Cloning and nucleotide sequencing of the bovine growth hormone gene. Nucleic Acids Res. 10:7197–7210, 1982.
34. Subramani, S., Mulligan, R., Berg, P. Expression of the mouse dihydrofolate reductase cDNA in SV-40 vectors. Mol. Cell. Biol. 1:854–864, 1981.
35. Urlaub, G., Chasin, L.A. Isolation of Chinese hamster cell mutants deficient in dihydrofolate reductase activity. Proc. Natl. Acad. Sci. USA 77:4216–4220, 1980.
36. Graham, F.L., van der Eb, A.J. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology 52:456–467, 1973.
37. McIntyre, G.D., Leckie, B., Hallett, A., Szelke, M. Purification of human renin by affinity chromatography using a new peptide inhibitor of renin, H.77 (D-His-Pro-Phe-His-Leu^R-Leu-Val-Tyr). Biochem. J. 211:519–522, 1983.
38. Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685, 1970.
39. Hunkapiller, M. PTH amino acid analysis. Appl. Biosystems User Bull. 14:1–27, 1985.
40. Hirose, S., Kim, S.-J., Miyazaki, H., Park, Y.-S., Murakami, K. *In vitro* biosynthesis of human renin and identification of plasma inactive renin as an activation intermediate. J. Biol. Chem. 260:16400–16405, 1985.
41. Haas, E. Renin from kidney. In: "Methods in Enzymology. Vol. II." Colowick, S.P., Kaplan, N.O. (eds.) New York: Academic Press, 1955.124–135.
42. Watenpaugh, K.D., Einspahr, H.M., personal communication.