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SEMI-PREPARATIVE PURIFICATION OF RECOMBINANT HUMAN RENIN AND PRORENIN

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SUMMARY

Chinese hamster ovary (CHO) cells, transfected with a vector containing cDNA coding for preprorenin, have been shown to secrete authentic prorenin into the culture supernatant. Purification of the expressed prorenin and purification of active renin, generated by solid-phase trypsin treatment of the conditioned media, have been achieved by conventional chromatographic methods. Scale-up of the initial steps of these procedures is described, including the use of radial-flow columns and automation with fast protein liquid chromatography valves and pumps. This semi-preparative scheme has allowed hundreds of milligrams of both proteins to be isolated.

INTRODUCTION

Renin is a circulating aspartyl protease, which acts to produce angiotensin II, an important factor in blood-pressure homeostasis and volume control[1]. Inhibitors of renin, expected to be useful in the management of hypertension, have been designed by modification of known peptides (namely angiotensinogen and the naturally isolated inhibitor pepstatin), which have affinity for the active site of renin. However, the techniques of crystallography and molecular modeling will provide a fresh approach to the design of inhibitors, perhaps better enabling the design of small, orally active compounds. At least several milligrams of pure protein are necessary for these studies, yet natural sources contain very low concentrations of renin and its inactive precursor, prorenin. We have utilized recombinant DNA technology to produce larger quantities of these proteins. This article details the methods used for scale-up of the initial purification steps for these proteins.

EXPERIMENTAL

Production of prorenin and renin

Chinese hamster overy (CHO) cell lines were transfected with a plasmid coding for preprorenin as described by Fritz *et al.*[2]. For the preparation of sufficient quantities of material, cells were grown to confluency in roller bottles and then switched to serum-free medium containing inducers of the metallothionein promoter[2]. The

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CHO cell-conditioned medium, containing secreted prorenin, was collected at regular intervals, filtered to remove any cell debris, and stored at 4°C for further purification.

Renin assay

Active and inactive renin were assayed by the method of Atlas *et al.*[3]. Activation of prorenin for assays was performed by addition of trypsin (Boehringer Mannheim, Indianapolis, IN, U.S.A.) at a level of 10 μg/ml. The activity was measured as the rate of angiotensin I produced in the presence of 0.2 μ*M* purified human angiotensinogen[4]. Angiotensin I was quantitated by radioimmunoassay (Travenol-Genentech Diagnostics, Cambridge, MA, U.S.A.).

Gel electrophoresis

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) was performed according to Laemmli[5], with 12.5% acrylamide. Protein was stained with Coomassie Blue G-250 (BioRad, Richmond, CA, U.S.A.), and all gel reagents were electrophoresis-grade (BioRad).

Hydrophobic interaction chromatography (HIC)

Analytical. A volume of 160 ml of filtered supernatant, to which 1.5 *M* sodium chloride had been added, was pumped at 1 ml/min into a hydrophobic interaction high-performance liquid chromatographic (HPLC) column (7.5 × 0.21 cm, Phenyl 5PW, Toyo Soda, Tokyo, Japan) previously equilibrated with 1.5 *M* sodium chloride–50 m*M* Tris–HCl (pH 8.0) (buffer A). The HPLC system consisted of two Beckman/Altex (Berkeley, CA, U.S.A.) 110A pumps with a Model 421 controller, an Eldex (San Carlos, CA, U.S.A.) pump monitor, and a Spectroflow 757 (Applied Biosystems, Ramsey, NJ, U.S.A.) variable-wavelength absorbance detector, set at 280 nm. Bound proteins were eluted with a single stepwise change from buffer A to 50 m*M* Tris–HCl (pH 8.0) (buffer B).

Semi-preparative. Supernatants were pumped at 4 ml/min into two semi-preparative HIC columns (15 × 2.15 cm, Phenyl 5PW, Toyo Soda) and eluted as above with a single stepwise change from buffer A to buffer B. A fast protein liquid chromatography (FPLC) system included a Pharmacia (Uppsala, Sweden) LCC 500 FPLC controller,directing two FPLC model P500 pumps in parallel, at a speed of 8 ml/min total, for loading the two separate columns simultaneously through two FPLC MV-7 valves. Two FPLC MV-8 valves were in-line to supply the pumps, which could be directed to allow the pump to distribute either sample, buffer A, buffer B, water, or wash buffer for stripping the columns (either 0.1 *M* sodium hydroxide, or 5 *M* urea–0.1 *M* acetic acid). The outlets of the two columns were connnected to a mixer, and the effluent passed through an FPLC UV-1 absorbance detector, then either to waste or to sample collection with a Frac 100 fraction collector (Pharmacia). Thus, the system automatically carried out consecutive loading, elution, and collection of sample, and washing and equilibration of the columns.

Ion-exchange/affinity chromatography

Analytical. A volume of 4 l of conditioned medium was filtered and loaded at a rate of 2 ml/min into a 50-ml (10 × 2.5 cm) QAE-Sepharose column (Pharmacia), equilibrated with 0.1 *M* sodium acetate (pH 6.5). The effluent was loaded at the same

rate onto a 50-ml (10 × 2.5 cm) Concanavalin A-Sepharose column (Pharmacia), which was similarly equilibrated. Bound protein was eluted with 0.20 *M* α-methyl-D-mannoside. The columns were disposable glass Econo-columns (14 × 2.5 cm, BioRad), loaded by a Minipuls 2 peristaltic pump (Gilson, Middleton, WI, U.S.A.).

Semi-preparative. A volume of 40 l of filtered supernatant was pumped (MasterFlex Model 7553, Cole-Parmer, Chicago, IL, U.S.A.) at a rate of 150 ml/min into two 500-ml radial-flow columns (9.5 cm length × 3 cm bed height, Sepragen, San Leandro, CA, U.S.A.) in tandem, the first containing QAE-Sepharose and the second Concanavalin A-Sepharose, equilibrated as above. The second column was eluted with α-methyl-D-mannoside, as above.

RESULTS AND DISCUSSION

Hydrophobic interaction chromatography

The first step of purification of prorenin from CHO cell conditioned supernatants was an HIC column from which prorenin was eluted in a single-step gradient. The procedure not only gave a ten-fold purification, but also concentrated the volume about ten-fold. This concentration was important for the next step: addition of solid-phase trypsin in order to activate the prorenin to active renin by removal of the 43 amino acid "pro" segment.

Semi-preparative scale-up of HIC was first accomplished by merely replacing the analytical column with the larger HPLC column, containing the same packing material. However, speed was compromised due to the time required for cleaning the HPLC pumps and detector, to flush out the high concentration of sodium chloride used in the sample and buffer A. Therefore, the HPLC columns were placed into an FPLC system since the pumps, tubing, and absorbance detector are salt-resistant.

The two FPLC pumps were operated in parallel, since in this purification step a discontinuous gradient was used. This doubled the capacity of each run. An additional increase in throughput was realized with the use of MV-8 valves, as loading, elution, collection, and wash cycles could be programmed for continuous, automatic operation. Filtered supernatants and collected fractions were kept at 4°C by immersion in a refrigerated water bath.

Table I summarizes the comparison between analytical and semi-preparative chromatography, detailing the increase in throughput, both upon change to larger columns ("manual") and automation of the same procedure (allowing continuous operation). The ten-fold purification [from 4.4 Goldblatt units (Gu) of trypsin-activatable prorenin per mg protein to 42 Gu/mg] and 90% recovery for this step of chromatography were the same for both analytical and semi-preparative chromatography.

Fig. 1 is an SDS-PAGE pattern demonstrating the purification achieved. For comparison, lane 3 contains completely purified renin (500 Gu/mg), obtained after several additional chromatographic steps (data not shown).

Ion-exchange/lectin affinity chromatography

CHO cells secrete proteases which, under certain circumstances, will cleave the expressed inactive prorenin to active renin in a fashion similar to exogenously added trypsin. Since this proteolytic activity was increased during the conditions used for

TABLE I

HYDROPHOBIC INTERACTION CHROMATOGRAPHY

Mode	*Volume loaded per run (ml)*	*Process capacity (l/week)*
Analytical	170	2
Semi-preparative		
Manual	3200	12
Automated	3200	100

HIC (fortuitously advantageous for the purification of renin), there was a need to design a different purification scheme for isolation of pure prorenin.

It is important to note that the first two chromatographic steps in our procedure could easily be combined into one, as the effluent from the first column, which contains all the prorenin, could be passed directly into the second column. After loading, the columns were disconnected so that the eluent could be pumped directly into the second column, while the first column was washed. The prorenin was then eluted from the second column with α-methyl-D-mannoside.

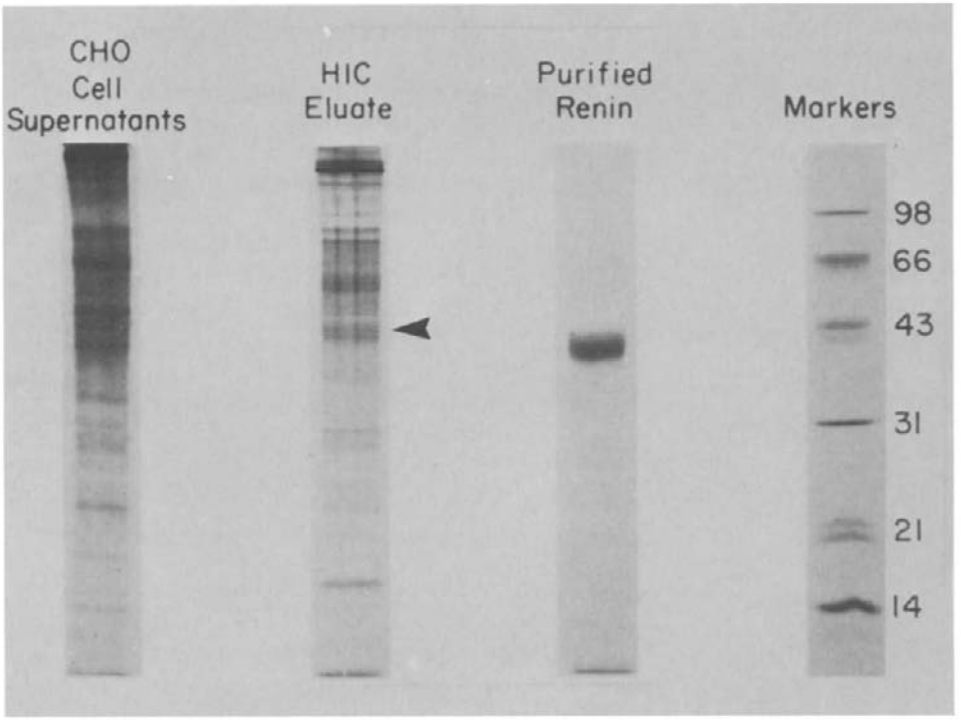

Fig. 1. SDS-PAGE (12.5% gel) of samples from HIC of CHO cell supernatants. Lane 1, starting material; lane 2, eluate (arrow denotes position of prorenin); lane 3, purified renin, from trypsin activation and further purification of material in lane 2; lane 4, molecular weight markers.

TABLE II

ION-EXCHANGE/LECTIN AFFINITY CHROMATOGRAPHY

Mode	*Volume loaded per run (l)*	*Proces capacity (l/week)*
Analytical	4	1
Semi-preparative	30	150

Another important point is that the use of radial-flow columns, rather than conventional columns, allowed a 75-fold increase in throughput with only a 10-fold increase in amount of packing material (Table II). This is due to the small sorbent height in the radial-flow column, which allows a much higher total flow-rate while maintaining the same linear velocity. Thus, the same high recovery (>90%) and almost 20-fold purification (from 4.4 to 80 Gu/mg) were obtained with the semi-preparative procedure as with analytical chromatography.

Fig. 2 demonstrates the purification obtained, in comparison with purified prorenin (lane 3), obtained after several additional chromatographic steps (specific activity of 500 Gu/mg after trypsin activation).

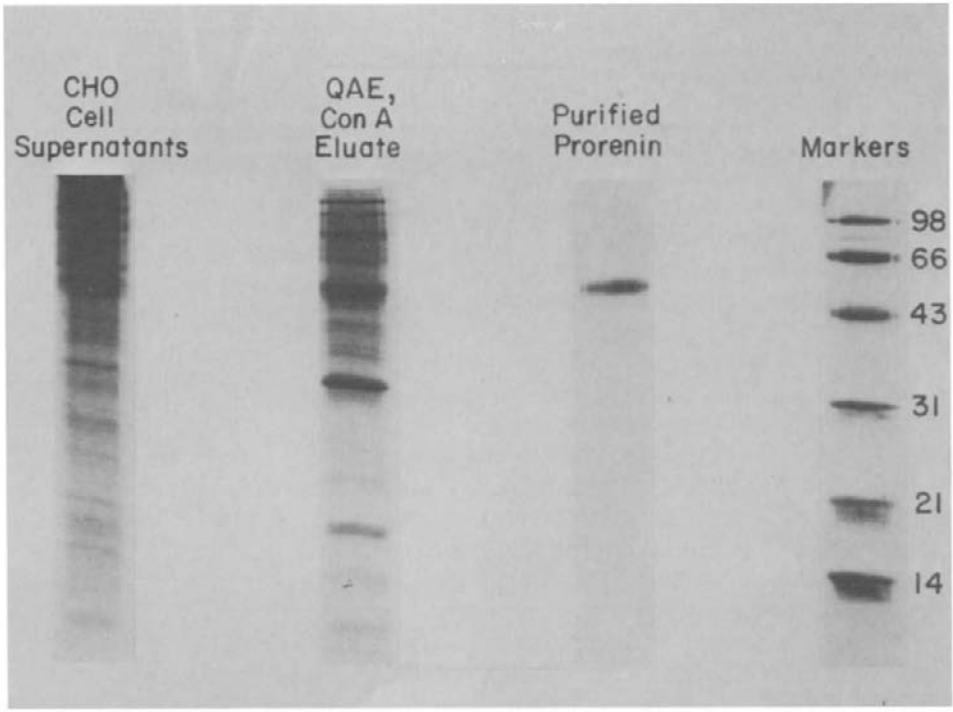

Fig. 2. SDS-PAGE (12.5% gel) of samples from tandem QAE-Sepharose, ConA-Sepharose chromatography. Lane 1, starting material; lane 2, eluate from ConA; lane 3, purified prorenin, from further purification of material in lane 2; lane 4, molecular weight markers.

CONCLUSIONS

Purification of recombinant human renin and prorenin from cultured mammalian cells has been achieved. The first step of each purification scheme is presented, demonstrating greater than 90% recovery and 10- to 20-fold purification from the crude supernatants of cells expressing a vector containing cDNA coding for preprorenin.

The data presented here demonstrate several novel approaches for semi-preparative scale-up of protein purification. The use of FPLC valves for automation of chromatographic runs, together with the ability to operate columns in parallel by virtue of the use of a step gradient for elution, result in greater through-put than that possible by a mere scale-up to larger columns. Radial-flow columns were shown to provide a similar increase in production capacity, without the necessity for changing to high-performance gel matrices and without compromise in the purification or recovery of material. Furthermore, the use of a chromatographic material that does not bind the protein of interest in the first purification step allows increased efficiency by virtue of the tandem operation of two columns simultaneously.

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