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Preparative isolation of angiotensin-converting enzyme from human lung

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ABSTRACT

Angiotensin-converting enzyme from human lung was purified to apparent homogeneity using a five-step purification procedure consisting of ammonium sulfate precipitation, ion-exchange chromatography on DEAE Sephadex A-50, gel permeation on Sephadex G-200, chromatofocusing on a polybuffer exchange (PBE 94) column and high-performance liquid chromatographic gel permeation on a Bio-Sil TSK-250 column. This procedure gave an ≈700-fold purification with a 20% yield compared to a 550-fold purification and a 1% yield with an affinity chromatography-based procedure. The 20-fold greater yield of the five-step procedure offers a major advantage for preparative use in the structural characterization of angiotensin-converting enzyme.

INTRODUCTION

Angiotensin-converting enzyme (ACE) (EN 3.4.15.1), peptidyl dipeptide hydrolase, is a high-molecular-mass (≈150 kDa) glycosylated integral membrane protein located on the luminal surface of the cell membrane. ACE plays a major role in cardiovascular homeostasis by catalyzing the conversion of the decapeptide angiotensin I to the octapeptide angiotensin II, a potent pressor hormone [1], and the hydrolysis of bradykinin, a vasodilator/natriuretic peptide which is also a potent stimulator of vasodilator prostaglandin synthesis [2,3]. Site-directed inhibitors of ACE are effective in the treatment of systemic hypertension and congestive heart failure [4]. ACE is found in a large variety of cells, tissues

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and biological fluids, including plasma [1], semen [5], proximal renal tubular cells [6], intestinal epithelial cells [7], stimulated macrophages [8], brain [9], testis [10], lung [11], vascular endothelium [12], and the medial and adventitial layers of blood vessel walls [13]. ACE associated with vascular endothelium has received the most intensive study both *in vivo* and *in vitro*, in part because of its contribution to the maintenance of blood pressure in normal subjects and to the pathogenesis of systemic hypertension in animal models and man. Although ACE is an abundant membrane protein, it has proven difficult to isolate and purify.

The nucleotide sequence of ACE mRNA has been determined by cloning the DNA complementary to human vascular endothelial cell and mouse kidney ACE mRNA [14,15]. The amino acid sequences deduced from these cDNAs reveal a high degree (83%) of homology between the human and murine enzymes. In both species, a highly hydrophobic sequence was found near the carboxyl terminus of the molecule, likely representing an anchor to the cell membrane. A high degree of internal homology was found between two large domains of the ACE molecule, each of which contains a Zn^{2+} binding region and a catalytic site, suggesting that the ACE gene may represent the duplicated product of a precursor gene [14,15]. Many important questions remain concerning the expression of the ACE gene and the posttranslational processing of this large ($\approx$1300 residues) and complex protein. Further, detailed structural characterization of ACE, including crystallographic examination of the molecule and mapping of the active site, has not yet been performed due to the difficulty in purifying large amounts of enzyme to homogeneity.

Most purification schemes for ACE in current use employ affinity chromatography with lisinopril, N^{α}-[(*S*)-1-carboxy-3-phenylpropyl]-L-lysyl-L-proline, a potent active site-directed ACE inhibitor, as the stationary ligand [16–18]. While these procedures can be used to prepare highly purified enzyme, their yields are low, and they are not practical for preparative use. The affinity of ACE for the Sepharose–spacer–lisinopril matrix ($K_i = 1 \cdot 10^{-5}$ *M*) is weak compared to its affinity for free lisinopril ($K_i = 1 \cdot 10^{-10}$ *M*), so the recovery of ACE from tissue extracts is low. Furthermore, because of its large size, steric hindrance may greatly limit access of the enzyme to the immobilized inhibitor in the affinity column. Development of a ligand and spacer combination with enhanced binding capacity for ACE purification [19] has not greatly enhanced the yield of ACE from human lung in our laboratory. The current study describes a novel method of purifying ACE from human lung using a five-step purification procedure consisting of ammonium sulfate precipitation, ion-exchange chromatography on DEAE Sephadex A-50, gel permeation on Sephadex G-200, chromatofocusing on a polybuffer exchange (PBE 94) column and high-performance liquid chromatographic (HPLC) gel permeation on a Bio-Sil TSK-250 column and compares this method to an affinity chromatographic procedure [19]. Both methods yielded a pure protein of molecular mass $\approx$150 kDa and a specific acitvity of $\approx$40 U/mg; the yield from the five-step purification procedure was 20-fold greater than that from affinity chromatography. Thus, the novel five-step chromatographic procedure is advantageous for preparative use in the structural characterization of ACE.

EXPERIMENTAL

Materials

Sephadex A-50, Sephadex G-200 and polybuffer PBE 94 were obtained from Pharmacia LKB Biotechnology (Piscataway, NJ, USA). All other chemicals used were of analytical grade and were obtained from Sigma (St. Louis, MO, USA). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) molecular mass marker kits were from Bio-Rad Labs. (Richmond, CA, USA). Lisinopril was a gift from Merck Sharp and Dohme Research Labs. HPLC experiments were performed with a Beckman 165 system (Model 165 variable-wavelength detector, operated at 280 nm; Model 112 solvent-delivery module; Kipp and Zonen BD 41 recorder; Rheodyne Model 340 injector). A Bio-Sil TSK-250 HPLC

gel, 30 mm × 7.5 mm I.D. column (Bio-Rad Labs.) was employed. Standard protein molecular mass markers were obtained from Bio-Rad Labs.

Sample preparation

ACE was purified from human lungs obtained at the time of harvesting hearts and kidneys for organ donation. Lungs were dissected free of major blood vessels and airways, snap frozen in liquid nitrogen and stored at −80°C until used. The protocol was approved by the Institutional Review Board for Human Use of the University of Alabama at Birmingham.

All procedures except HPLC were performed at 4°C. Frozen human lung tissue (200 g) was homogenized in 0.2 *M* Tris, pH 7.8, 0.03 *M* KCl, 0.005 *M* $MgCl_2$ and 0.25 *M* sucrose at 1:5 (w/v). The homogenate was stirred for 2 h and centrifuged at 700 *g* for 20 min. The supernatant was saved and the pellet rehomogenized in the same buffer by the same procedure. After centrifugation, the supernatants were combined. The combined supernatant was adjusted to pH 5.0 with glacial acetic acid and allowed to stir for 30 min, then centrifuged at 15 000 *g* for 20 min. The supernatant was discarded and the pellet resuspended in 0.01 *M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.5, 0.03 *M* KCl to which Nonidet P-40 was added to a final concentration of 0.5% and stirred vigorously for 3 h.

Samples were then subjected to a five-step purification procedure consisting of ammonium sulfate precipitation, ion-exchange chromatography on DEAE Sephadex A-50, gel permeation on Sephadex G-200, chromatofocusing a polybuffer exchange (PBE 94) column and HPLC gel permeation on a Bio-Sil TSK-250 column. The protein content of each column eluate was monitored by measuring absorbance at 280 nm. ACE catalytic activity was measured in each 10-ml fraction by a modification of the spectrophotometric method of Cushman and Cheung [20] with hippuryl histidyl leucine (HHL) as substrate. The reaction mixture (0.5 ml) contained 5 m*M* HHL. One unit of enzyme activity was defined as the amount of enzyme that hydrolyzed 1 μmol of HHL per min at 37°C. Protein concentrations of each fraction were determined by the method of Lowry *et al.* [21] with bovine serum albumin as standard. At the end of each chromatographic step, active fractions were pooled and concentrated in an Amicon cell with a YM30 membrane.

Ammonium sulfate precipitation

Ammonium sulfate was added to a final concentration of 15.8 g per 100 ml (30% saturation), the pH was adjusted to 7.5 with NH_4OH, the mixture was stirred for 30 min, then centrifuged at 30 000 *g* for 30 min and the supernatant was collected. Ammonium sulfate was added to a final concentration of 21.12 g per 100 ml (70% saturation), the pH was adjusted to 7.5 with NH_4OH as above and the mixture was stirred for 30 min. After centrifugation at 30 000 *g* for 30 min, the pellet was dispersed in 0.01 *M* HEPES, pH 7.5, 0.03 *M* KCl and 20 m*M* 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS). The suspension was stirred for 30 min and centrifuged at 30 000 *g* for 10 min and the supernatant was dialyzed against three changes of 1 l each of the buffer.

Ion-exchange chromatography

The dialyzed ammonium sulfate fraction (200 ml) was applied at a flow-rate of 1.0 ml/min to a column of DEAE Sephadex A-50 (290 mm × 40 mm I.D.) which had been equilibrated with 20 m*M* Tris, pH 7.2, 20 m*M* NaCl, containing 100 μ*M* pepstatin, 20 m*M* CHAPS and 0.1 m*M* phenylmethylsulfonyl fluoride (PMSF). The column was washed with 5 volumes of buffer until the absorbance at 280 nm of the effluent was < 0.1 a.u.f.s. Elution was carried out with a linear gradient (total volume 3 l) between 20 and 600 m*M* NaCl in the above buffer. Fractions were assayed for ACE catalytic activity. ACE catalytic activity emerged as a single symmetical peak at ≈200 m*M* NaCl.

Gel permeation chromatography

The DEAE fraction was subjected to gel per-

meation through a column (500 mm × 25 mm I.D.) of Sephadex G-200 equilibrated and developed with 20 m*M* Tris–HCl, 20 m*M* CHAPS, pH 7.3, 200 m*M* NaCl, 1 m*M* pepstatin, 0.1 m*M* PMSF at a flow-rate of 30 ml/h. Fractions (5 ml) were collected and assayed for ACE activity as previously described.

Chromatofocusing

A polybuffer exchange column PBE 94 (200 mm × 15 mm I.D.) was equilibrated with 25 m*M* bis-Tris buffer, pH 6.5 (adjusted with HCl), 20 m*M* CHAPS. Concentrated samples from the two peaks of catalytic activity from the gel permeation column were applied to the column individually. Elution was accomplished with a loaded polybuffer solution (10 ml of polybuffer 74 plus 100 ml of distilled water adjusted to pH 3.5 with HCl).

HPLC gel permeation chromatography

The concentrated sample from the chromatofocusing step was applied to the HPLC column and was eluted with the same buffer at a flow-rate of 0.6 ml/min. The column was run at ambient temperature (25°C). Catalytic activity appeared in two peaks at apparent M_r values of 300 and 150 kDa. Gel permeation chromatography on a Bio-Sil TSK-250 gel permeation column was also used to estimate the purity of our ACE preparation. The gel permeation column (300 mm × 7.5 mm I.D.) (Bioanalytical Systems) was equilibrated in 0.041 *M* Na_2HPO_4, 0.019 *M* NaH_2PO_4, 0.2 *M* NaCl, 20 m*M* CHAPS, pH 7.2 and calibrated with molecular mass markers, including: thyroglobulin (bovine), M_r 670 000; γ-globulin (bovin), M_r 158 000; ovalbumin (chicken), M_r 45 000; myoglobulin (equine), M_r 17 000; and vitamin B_{12}, M_r 1350 (Bio-Rad). Purified ACE and the molecular mass markers were injected into the HPLC gel permeation column, eluted at different flow-rates (U) and theoretical plate heights (H) of ACE and the standard proteins were measured [22]. By studying the retention and band broadening of proteins on the TSK column, diffusion coefficients (D_s) of solute in stationary phase were obtained which elucidate the hydrodynamic process of chromatographic resolution of proteins by hydrophilic size-exclusion chromatography. After calculating the correlation between D_s and the molecular mass of the solute, the molecular dimensions of proteins in the process of chromatographic separation can be predicted. Deviations in diffusion coefficient of a protein from the calculated value reflect differences of measured molecular dimensions from molecular volumes predicted from the calibration curve of the gel permeation column. Deviations from $2\lambda d_p$ (where d_p is the particle diameter and λ the tortuosity of the packing material, is ≈ 1, as the microspheres are porous) in the intercept of the theoretical plate height (H) *versus* flow-rate (U) curve from the band broadening equation $H = C_sU + 2\lambda d_p + f(\alpha_M)_T$ (where C_sU represents mass transfer resistance caused by solute diffusion in the stationary phase and $f(\alpha_M)_T$ an added term for polydisperse solutes [23]) reflect impurities in the proteins. Using the band broadening equation [23], the intercepts of the H–U curve were determined and the purity of the ACE samples was evaluated from the values of the intercepts.

SDS-PAGE

Pooled fractions of the two peaks eluted from the HPLC column that contained ACE catalytic activity were concentrated using Amicon cells with a YM-30 membrane treated with SDS and 2-mercaptoethanol, and analyzed by 7% SDS-PAGE using the stacking method of Laemmli [24]. Protein was detected with a silver stain [25].

Western blots

Proteins were separated on SDS-PAGE as described above. Gels were fixed in a solution of 0.1 *M* Tris–HCl, 0.2 *M* glycine, 20% methanol, pH 7.8. Blotting of protein on nitrocellulose was performed electrophoretically by immersing the assembly of gel and paper in a buffer containing 0.02 *M* Tris–HCl, 0.2 *M* glycine, 20% methanol, pH 8.3 (buffer 1). The nitrocellulose was washed three times for 1 min in 0.15 *M* NaCl, 0.01 *M* Tris–HCl, pH 7.5 (buffer 2) and then incubated with 25 ml of 0.05 *M* Tris–Base, 0.005 *M* EDTA,

0.15 *M* NaCl, 0.0035 *M* SDS, 2.5% gelatin, 0.5% Triton X-100 and 5% dried milk, pH 7.5 (buffer 3) at room temperature for 1.5 h. The membrane was immersed in 50 ml of fresh buffer 3 containing 0.25 ml (1%, w/v) of anti-ACE serum (polyclonal antibody from rabbit) and mixed by continuous rocking at 4°C overnight. After rinsing, the membrane was immersed in 25 ml of buffer 3 minus dried milk containing 25 μl of anti-rabbit IgG alkaline phosphatase conjugate and shaken for 1 h at room temperature. The washed membrane was soaked in 20 ml of buffer (100 m*M* Tris–HCl, pH 9.5, 100 m*M* NaCl, 5 m*M* $MgCl_2$), 132 μl of a solution containing nitro blue tetrazolium (50 mg/ml) in 70% dimethylformamide and 66 μl of a solution containing nitro blue tetrazolium (50 mg/ml) 100% dimethylformamide were added and shaken at 37°C in the dark, as described by McGadey [26] and Blake *et al.* [27]. When the color of the bands was developed to the desired intensity, the reaction was stopped by rinsing the mebrane with several changes of deionized water.

Affinity chromatography

Preparation of affinity resin. Epoxy-activated Sepharose 6B (Pharmacia) gel was prepared by coupling 6-[N-(aminobenzoylamino)]caproic acid, as described by Pantoliano *et al.* [19]. The resulting gel was activated to form the N-hydroxysuccinimide ester as outlined by Cuatrecasas and Parikh [28], which was then suspended in dimethylformamide. To this gel slurry, solid N-hydroxysuccinimide (0.5 *M*) and diisopropylcarbodiimide (0.5 *M*) were added, and the mixture was stirred in a sealed system for 70 min. Sepharose-28 (nm)–lisinopril was prepared by reacting 25 m*M* lisinopril with gel in 0.1 *M* Na_2CO_3, pH 10, at 4°C overnight.

Sample preparation and affinity chromatography. A 200-g amount of human lung was dissected, homogenized and subjected to detergent extraction as described above. After membrane solubilization by addition of Nonidet P-40, the sample was centrifuged at 250 000 *g* for 2 h. The supernatant was then dissolved in 20 m*M* HEPES, 0.3 *M* NaCl, 1 m*M* $Zn(C_2H_3O_2)_2$, 20 m*M* CHAPS, pH 7.5 at room temperature and was applied at 0.4 ml/min to a 35 cm × 1.2 cm I.D. column of Sepharose-28–lisinopril, previously equilibrated with the buffer described above. The column was washed with starting buffer until the absorbance at 280 nm of the effluent was < 0.1 a.u.f.s. Bound ACE was then eluted with 50 m*M* sodium borate, pH 9.5, 20 m*M* CHAPS and was concentrated to 5–10 ml in an Amicon cell with a YM-30 membrane.

Gel permeation. The sample was applied at 30 ml/h to a 88 cm × 2.6 cm I.D. column of Ultrogel AcA-34 previously equilibrated with 0.02 *M* potassium phosphate, pH 8.3, 0.1 *M* NaCl, 20 m*M* CHAPS, and eluted. The molecular mass of the catalytically active fraction was determined by HPLC and non-denaturing PAGE as previously described.

RESULTS

The results of purification of ACE from human lung by chromatography and gel permeation HPCL are summarized in Table I. After the initial sample preparation and detergent solubilization steps, the ammonium sulfate precipitation steps proved very efficient in removing a large amount of protein with little loss of ACE activity. Ion-exchange chromatography with DEAE Sephadex A-50 yielded a single activity-containing peak eluting at approximately 200 m*M* NaCl. The eluate from the Sephadex G-200 gel permeation column contained two distinct peaks of ACE activity (Fig. 1). Catalytic activity appeared in two symmetrical peaks corresponding to a trough in global protein at a V_e/V_0 (V_0 = the column void volume; V_e = elution volume of the ACE sample) value of ≈ 1.2 and 1.5. From this point on in the isolation scheme, the two peaks were kept separate. When these two peaks were subjected to chromatofocusing individually, a single peak containing ACE activity was observed at pH 5.0 for both samples. These two forms of ACE had apparent M_r values of 300 kDa and 150 kDa, respectively, by HPLC gel permeation and by non-denaturing PAGE. Analysis of the two forms of ACE by denaturing SDS-

TABLE I

PURIFICATION OF ANGIOTENSIN-CONVERTING ENZYME FROM HUMAN LUNG BY CHROMATOGRAPHY AND GEL PERMEATION HPLC

Step	Protein concentration (mg/ml)	Volume (ml)	Total protein (mg)	Specific activity (U/mg)	Total activity (U)	Purification (X-fold)	Yield (%)	
Detergent solubilization	40	250	10 000	0.06	600	1	100	
Ammonium sulfate	29	147	4260	0.12	510	2	85	
DEAE-A50	27	45	1200	0.27	324	4.5	54	
G-200 form I	48	4	190	0.86	163	14.3	27	47[b]
G-200 form II	43	3	130	0.93	120	15.5	20	
Chromatofocusing form I	11	1.6	17.6	9	158	150	26	44[b]
Chromatofocusing form II	11	1.4	15.4	7	108	117	18	
HPLC form I	1.1	1.5	1.7	47	78	783 ≈700[a]	13	22[b]
HPLC form II	1.2	1.2	1.4	36	52	600	9	

[a] Specific activity ratio.
[b] Total activity ratio.

PAGE revealed a single band of M_r 150 kDa (Fig. 2). A single band was found on Western blot (Fig. 3). On HPLC gel permeation chromatography, the two forms of ACE had an intercept on the H–U curve very close to $2\lambda d_p$ (Fig. 4), indicating a highly pure preparation. This purification scheme gave a 700-fold purification of ACE, with a yield of ≈20% of the starting material and a final specific activity of 36 U/mg (Table I).

The results of purification of ACE from human lung by affinity chromatography are summarized in Table II. Enrichment of approximately 360-fold in the purity and specific acitvity of

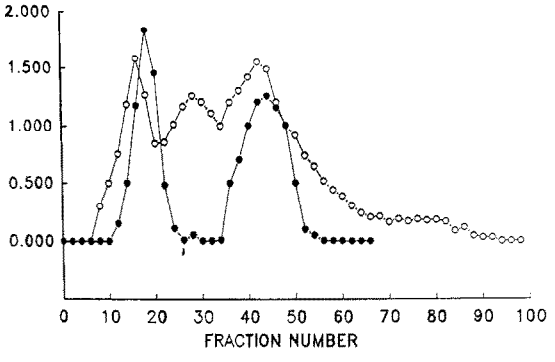

Fig. 1. Sephadex G-200 chromatography. The ACE activity-containing peak from the DEAE Sephadex A-50 column was chromatographed on a Sephadex G-200 column. The volume of each fraction was 5 ml. Fractions 10–25 and 35–50, which contained almost all of the ACE activity, were pooled and concentrated separately in preparation for the chromatofocusing step. (○) Protein absorbance (280 nm); (●) activity (U).

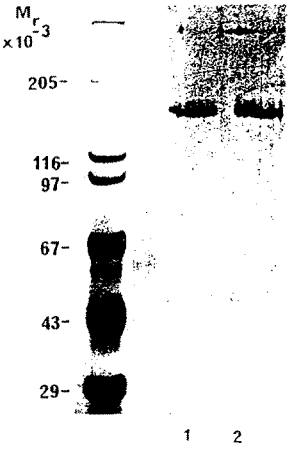

Fig. 2. SDS-PAGE of purified angiotensin-converting enzyme. Angiotensin-converting enzyme (2–4 μg) purified by the five-step chromatographic method was subjected to gel electrophoresis on 7% SDS-PAGE. The 150-kDa form was run in the middle lane (1) and the 300-kDa form in the right lane (2). Molecular mass markers (left lane) were: myosin (M_r = 200 000); β-galactosidase (M_r = 116 250); phosphorylase b (M_r = 97 400); serum albumin (M_r = 66 200); ovalbumin (M_r = 45 000); carbonic anhydrase (M_r = 31 000).

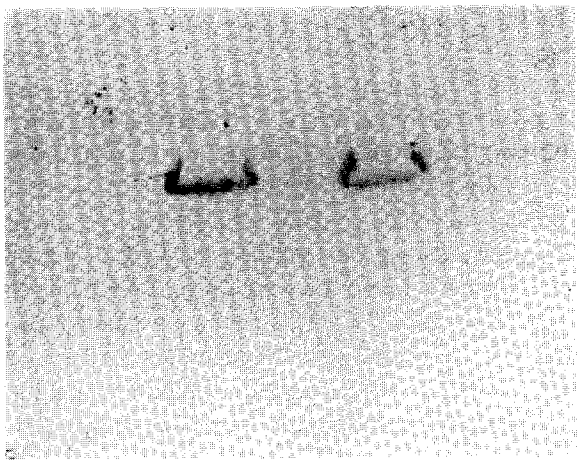

Fig. 3. Western Blot analysis of purified angiotensin-converting enzyme. Left lane: angiotensin-converting enzyme (3–5 μg) purified by the five-step chromatographic method. This was a mixture of the 300-kDa and 150-kDa forms. Right lane: angiotensin-converting enzyme (3–5 μg) purified by Sepharose-28 (nm)–lisinopril affinity chromatography.

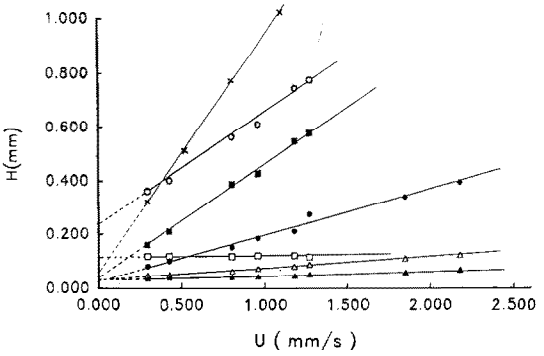

Fig. 4. *H–U* dependence of proteins on TSK-250 gel. Eluent: 0.041 *M* Na_2HPO_4, 0.019 *M* NaH_2PO_4 and 0.15 *M* Na_2SO_4 (pH 6.8). Key to symbols: ○ = γ-globulin; ● = ovalbumin; △ = myoglobin; ▲ = vitamin B_{12}; □ = thyroglobulin; × = angiotensin-converting enzyme I; ■ = angiotensin converting enzyme II. The intercept of the *H–U* curve for angiotensin-converting enzyme was close to 0, indicating that the protein was pure.

ACE was achieved by affinity chromatography alone, but the yield was only ≈ 1% of ACE in the starting material (Table II). Analysis of the protein by silver-stained SDS-PAGE revealed two bands of about equal intensity, one traveling at M_r 150 kDa and the second traveling at M_r 70 kDa (not shown). The second band contained no catalytic activity, suggesting that it represented a contaminant from the affinity matrix. When eluate from the affinity column was subjected to gel permeation chromatography on ACA-34, a single peak containing large amounts of ACE activity was eluted and appeared as a single band on silver-stained SDS-PAGE (Fig. 5). This protein had an M_r of 300 kDa on gel permeation HPLC and non-denatured SDS gel (not shown). A single band was observed on Western blot (Fig. 3).

DISCUSSION

The advent of affinity isolation procedures utilizing active site-directed ACE inhibitors coupled to various supports with secondary chromatog-

TABLE II

PURIFICATION OF ANGIOTENSIN-CONVERTING ENZYME FROM HUMAN LUNG BY AFFINITY CHROMATOGRAPHY

Step	Protein concentration (mg/ml)	Volume (ml)	Total protein (mg)	Specific activity (U/mg)	Total activity (U)	Purification[a] (*X*-fold)	Yield[b] (%)
Detergent solubilization	6.5	1500	9750	0.08	780	1	100
Affinity chromatography	0.01	38	0.38	29	11	362	1.4
ACA-34 eluate	0.2	1	0.2	44	8.8	550	1.1

[a] Specific activity.
[b] Total activity.

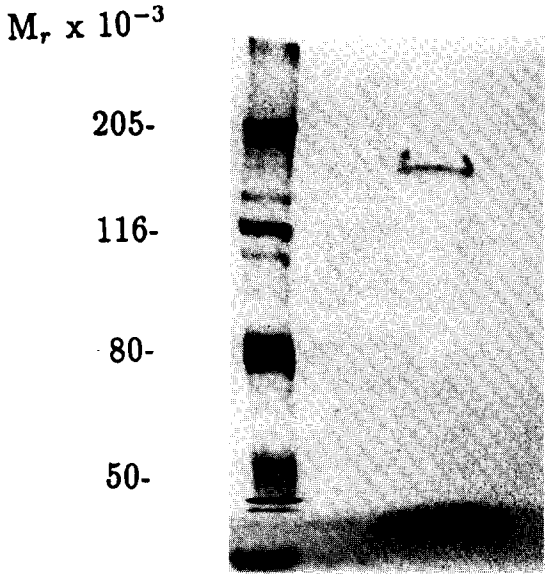

Fig. 5. Angiotensin-converting enzyme (2–4 μg) purified by Sepharose-28 (nm)–lisinopril affinity chromatography subjected to electrophoresis on 7% SDS-PAGE. The silver-stained band has an estimated M_r of 150 kDa. Molecular mass markers were: myosin (M_r = 200 000); β-galactosidase (M_r = 116 250); serum albumin (M_r = 66 200); ovalbumin (M_r = 45 000).

raphy permitted for the first time the isolation of highly purified ACE of high specific activity. The principal disadvantage of affinity isolation procedures for ACE is low yields: the affinity methods reported to date require 1 l or more of resin to purify 20 mg of enzyme. Although placing lisinopril on linker arms of various lengths has been used to improve the yield of ACE purified from rabbit lung [19], the application of this procedure to the purification of ACE from human lung in the current study produced poor yields. A further disadvantage of active site-directed affinity chromatography-based purification schemes is that the ACE isolated by these techniques still has the inhibitor used to remove the enzyme from the affinity matrix bound to its active site, and thus may have an altered conformation. For example, crystals containing this ACE–inhibitor complex may yield misleading information about the three-dimensional structure of the enzyme.

The five-step purification procedure described in the current paper gave a 20-fold greater yield of human lung ACE than the affinity chromatography-based procedure that was used for comparison. The purity and specific activity profiles of proteins obtained by both procedures were comparable. Thus, the five-step chromatographic procedure has major advantages for preparative applications. Our procedure differs in a number of important respects from the only HPLC-based purification scheme for human lung ACE previously reported [29]. The latter procedure employed a combination of detergent extraction with Nonidet P-40 and incubation with trypsin to free ACE from the membrane, followed by hydroxyapatite fractionation and HPLC on Superose 12 and Mono Q. Our yield from the Nonidet P-40 step was 5-fold greater than that of Takeuchi *et al.* [29]. Furthermore, the rationale for use of both trypsin digestion and detergent extraction in the same purification scheme is elusive: inadequate explanation was given for use of trypsin and for the observed effects of trypsin on the Superose 12 gel permeation profile of ACE activity. In the purification scheme of Takeuchi *et al.* [29], a large amount (30 mg) of protein eluted from the hydroxyapatite column was loaded directly onto the Superose 12 HPLC column. These loading conditions would likely either produce very poor separation of proteins or would damage the column. Finally, the specific activity of their ACE preparation (105 U/mg) was more than twice that reported by other groups, including our own.

In the current study, the five-step purification procedure yielded ≈3 mg of pure human lung ACE from 200 g of starting material. This included 1.7 mg of high-molecular-mass (300 kDa) material (form I), specific activity 47 U/mg, and 1.4 mg of lower-molecular-mass (150 kDa) material (form II), specific activity 36 U/mg. When both form I and form II were subjected to PAGE under denaturing conditions (SDS), only one silver-stained band appeared at 150 kDa, indicating that form I was a dimer of ACE. The 150-kDa monomer was pure by criteria of SDS-PAGE and gel permeation chromatography on HPLC and appeared similar to published descriptions of ACE purified by affinity chromatographic tech-

niques [16–19]. The five-step chromatographic procedure is suitable for use with large amounts of starting material for the preparative-scale purification of ACE.

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