Journal of Chromatography, 461 (1989) 271–275
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1476

Note

Purification of angiotensin-converting enzyme by gel high-performance liquid chromatography

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Angiotensin-converting enzyme (ACE) (peptidyldipeptide hydrolase, E.C. 3.4.15.1) is a high-molecular-weight (*ca.* 150 kDa) glycosolated integral membrane protein, located on the luminal surface of the plasma membrane of endothelial cells and of other cell types, such as the epithelial cells of the kidney proximal tubules, seminiferous tubules of the testis, neurons and gut epithelial cells. Although ACE is an abundant membrane protein, it has proved difficult to isolate and purify.

Several approaches, including extensive chromatographic steps with DEAE-cellulose, Sephacryl, hydroxyapatite, phenyl-Sepharose and chromotofocusing, have been utilized to purify ACE[1,2]. These methods are time consuming and produce low yields of purified ACE, necessitating the use of large amounts of starting material. Affinity chromatography with active site directed inhibitors of ACE, such as lisinopril {N^{α}-[(*S*)-1-carboxy-3-phenylpropyl]-L-lysyl-L-proline}, has also been used to purify ACE[3–5]. Affinity chromatography is superior to conventional chromatographic methods in that it reduces the number of steps and the time required for purification of the enzyme. Further, the yield of purified enzyme is increased following purification by affinity chromatography, possibly owing to the time involved and/or reduced handling steps.

Previous work in our laboratory indicated that exposure of cultured porcine pulmonary artery endothelial cells to chronic normobaric hypoxia causes an increase in cell-associated ACE antigen without an increase in ACE activity[6]. This observation raises the possibility that an inactive form of ACE is synthesized by endothelial cells that are cultured under hypoxic conditions. Purification schemes based on active-site-directed affinity chromatography may therefore yield lower ACE levels than a scheme that does not require the binding of an affinity ligand to the active site of ACE.

The purification scheme illustrated in this study provides a rapid, quantitative method of isolating ACE from cultured endothelial cells. Further, this method

0021-9673/89/$03.50

provides a means of investigating ACE synthesis in small amounts of starting material, thus facilitating the study of regulation of the ACE synthesis.

EXPERIMENTAL

Cell culture

Porcine pulmonary artery endothelial cells were isolated and cultured as previously described[7]. Briefly, pulmonary arteries were dissected from intact porcine heart–lung combinations, obtained from a local slaughterhouse. The vessels were rinsed in Dulbecco's phosphate-buffered saline (DPBS) (pH 7.4) and then filled with 1% collagenase solution in DPBS. The collagenase filled vessels were incubated for 20 min at room temperature. Vessels were then massaged, and the released endothelial cells were removed by pipet and centrifuged at 500 *g* for 5 min at room temperature. Pellets were resuspended in complete medium [Medium 199 (GIBCO, Grand Island, NY, U.S.A.), 200 U/ml penicillin, 150 U/ml streptomycin, 4.76 mg/ml 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES), 2.2 mg/ml sodium hydrogencarbonate, 0.29 mg/ml L-glutamine, 10% heat deactivated fetal calf serum (Armour Pharmaceutical, Tarrytown, NY, U.SA. (pH 7.4)] and seeded into 35-mm dishes, previously coated with fibronectin[8]. The medium was changed every other day, and cells were grown to confluence in an atmosphere of carbon dioxide–air (5:95) at 37°C.

Two days after the cultures reached confluence, two 35-mm dishes were harvested by scraping the cell sheets in 1.5 ml of DPBS (pH 7.4) and 50 U/ml of aprotinin and transferred into a 15-ml centrifuge tube. The dishes were washed with the same buffer and the washings were combined with the previous cell suspension. Cells were centrifuged at 500 *g* for 10 min at room temperature. The resulting pellet was resuspended in 250 μl of buffer, containing PBS (pH 7.4), 0.15 *M* sodium chloride, 1% bovine serum albumin (BSA), 0.5% Nonidet P-40 (Sigma, St. Louis, MO, U.S.A.) and 50 U/ml aprotinin. The cell suspension was kept on ice for 1 h with intermittent mixing, followed by centrifugation at 9000 *g* for 10 min at 4°C. The resulting supernatant was centrifuged at 28 000 *g* for 2 h at 4°C. After centrifugation, the supernatant was dialyzed (10 000 mol. wt. cutoff) overnight against 4 l of the mobile phase 0.041 *M* disodium hydrogenphosphate (Na_2HPO_4)–0.019 *M* sodium dihydrogenphosphate (NaH_2PO_4)–0.15 *M* sodium sulfate (Na_2SO_4), adjusted to pH 6.8 and then injected directly into the high-performance liquid chromatographic (HPLC) column.

Chromatography

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 × 7.5 mm I.D. column (Bio-Rad Labs., Richmond, CA, U.S.A.) was employed. Standard protein samples were obtained from Bio-Rad Labs. and included thyroglobulin (bovine), 670 kDa, γ-globulin (bovine), 158 kDa, ovalbumin (chicken), 44 kDa, myglobin (equine), 17 kDa and vitamin B_{12}, mol. wt. 1350. The mobile phase consisted of 0.041 *M* Na_2HPO_4–0.019 *M* NaH_2PO_4–0.15 *M* Na_2SO_4, adjusted to pH 6.8. All measurements were conducted at ambient temperature (25°C). The purity of the ACE was determined by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting and HPLC.

Enzyme assay

Catalytically active ACE molecule were identified and quatitiated by measuring the binding of [^{125}I]351A [N$^{\alpha}$-(1-carbonyl-3-phenylpropyl)-L-lysyl-L-proline] (Merck, Sharp and Dohme Research Labs., West Point, PA, U.S.A.), a highly specific active-site-directed inhibitor of ACE, essentially as described by Tikkanen *et al.*[9]. To duplicate tubes, 100 μl of assay buffer (PBS, 100 μM zinc chloride, pH 7.4), 100 μl of sample and 20 μl of [^{125}I]351A stock solution (*ca.* 200 000 cpm) were added, and the samples were incubated at 37°C in a shaking water-bath for 2 h. After incubation, the tubes were placed on ice and 400 μl of ice-cold dextran-coated charcoal, kept in suspension by a magnetic stirrer, were added and kept on ice for 10 min. Ice-cold assay buffer (500 μl) was then added to each tube and the samples were mixed on a vortex mixer and centrifuged at 1000 *g* for 10 min at 4°C. The supernatants were transferred to clean tubes and their radioactivity was determined in a gamma counter. For the determination of non-specific binding, lisinopril (Merck, Sharp and Dohme) was added to the assay buffer at a final concentration of 1 μM. All other steps were as described above. Electrophoresis was performed in the presence of 0.1% SDS on a slab gel containing 7.5% acrylamide according to the method of Laemmli.[10] Protein was identified by silver staining or transfer to nitrocelluose by Western blotting and probing with a polyclonal antibody against porcine lung ACE.

RESULTS AND DISCUSSION

Fig. 1 is a representative HPLC trace of an extract of confluent porcine pulmonary artery endothelial cells obtained under the conditions described above. The equation for the resolution, R_s, is

$$R_s = 2 \cdot \frac{t_{R1} - t_{R2}}{W_1 + W_2}$$

where W_1 and W_2 are the band widths determined by the intersection of the tangents of the inflection points of the Gaussian peaks with the baseline and t_R is the retention time of a peak measured from the start. The resolution obtained by HPLC is 1.24, indicating satisfactory separation of ACE from other proteins. After collection of the peaks, the ACE activity was determined by using [^{125}I]351A as the ligand. Peak 2 was found to contain catalytically active ACE[3].

Silver stained SDS-PAGE of peak 2 shows a single major band with the same mobility as porcine lung ACE isolated using active-site-directed affinity chromatography. Peaks 1 and 3 show no ACE band by silver-stained SDS-PAGE analysis. Further Western blot analysis using polyclonal antibody against affinity purified ACE shows a single band corresponding to peak 2, which has the same electrophoretic mobility as affinity-purified porcine lung ACE. The molecular weight of the ACE band determined by SDS-PAGE analysis is 150–160 kDa. We obtained the molecular weight of the protein in peak 2 (Fig. 1) (catalytically active ACE) by constructing a calibration graph with proteins from 1350 to 670 000 kDa (Table I). The ACE-containing peak shows a retention volume of 7.40, corresponding to a molecular weight of 150–160 kDa, consistent with that found for ACE isolated using

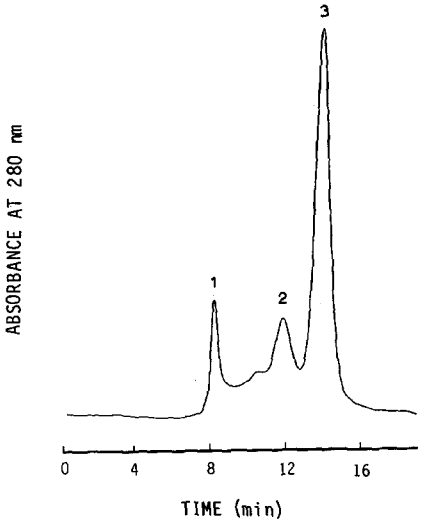

Fig. 1. Separation of angiotensin-converting enzyme (peak 2) from endothelial cells. Column: Bio-Sil TSK-250, 300 mm × 7.5 I.D.; eluent, 0.041 *M* Na_2HPO_4–0.019 *M* NaH_2PO_4–0.15 *M* Na_2SO_4 (pH 6.8); flow-rate, 0.6 ml/min; temperature, 25°C.

active-site-directed affinity chromatography in our laboratory and with the value reported in the literature for lung ACE.

The ACE purification scheme described in this paper provides a rapid, quantitative method of isolating ACE from cultured endothelial cells, although ACE is not purified to homogeneity by a single pass through the gel column. However, this method provides substantial enrichment of ACE, making possible studies of ACE synthesis and metabolism in small amounts of cultured endothelial cells. When combined with pulse labeling of the cultures with [^{35}S]methionine or [^{3}H]leucine, the method described provides a sample well suited for analysis by two-dimensional electrophoresis, followed by autoradiography. Application of this method appears to be limited to cultured endothelial cells. It is not applicable to use with tissue samples because of low yields, owing to the presence of a large proportion of non-ACE-containing cells.

TABLE I
STANDARD CURVE OF MOLECULAR WEIGHT AND RETENTION VOLUME

Substance	*Mol.wt.*	V_R*(ml)*
Thyroglobulin	670 000	4.66
γ-Globulin	158 000	7.38
Ovalbumin	44 000	8.98
Myoglobin	7000	10.24
Vitamin B_{12}	1350	13.13
ACE	150 000–160 000	7.40

ACKNOWLEDGEMENTS

The authors thank Ms. Charlane Crouse for her assistance in preparing the manuscript. [^{125}I]351A and lisinopril were generously supplied by Dr. Martin Hichens of Merck, Sharp and Dohme Research Labs. Endothelial cells were provided by Dr. Francois Booyse. This work was supported in part by NIH/NHLBI Grants HL-07457 and HL-22544.

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