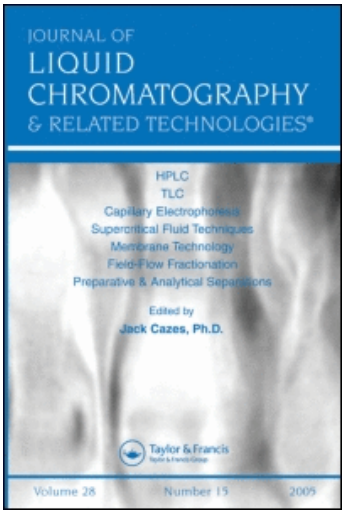

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PREPARATIVE ISOLATION OF A SOLUBLE FORM OF BOVINE LUNG ANGIOTENSIN CONVERTING ENZYME BY AFFINITY AND SIZE EXCLUSION CHROMATOGRAPHY

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ABSTRACT

A high capacity process is described for the preparative purification of a soluble form of bovine lung angiotensin I-converting enzyme by affinity and size exclusion chromatography. The affinity purified enzyme was solubilized by tryptic attack for 1 h at 300C and separated by Sephacryl S-300 HR chromatography. A recovery of 68% was obtained. The purification procedure described here, enables one to obtain 27 mg of enzyme with a specific activity of 26 min^{-1} mg^{-1} from 1 kg of bovine lung. Molecular mass of native soluble ACE form was obtained by size-exclusion high performance liquid chromatography. Molecular mass of membrane-bound enzyme and the ACE form solubilized with trypsin, was found to be 170 kDa and 160 kDa, respectively, using disc gel electrophoresis in the presence of sodium dodecyl sulfate.

INTRODUCTION

Angiotensin I-converting enzyme (ACE; EC 3.4.15.1), is a membrane-bound metallopeptidase that plays an important role in blood pressure regulation. Inhibition of ACE is a widely used approach in the treatment of hypertension.[1] ACE hydrolyzes angiotensin I to the potent vasoconstrictor angiotensin II, and converts the vasodilator bradykinin into an inactive peptide.[2,3]

ACE is a glycoprotein that consists of a single polypeptide chain of an apparent molecular mass, ranging from 130 kDa and 180 kDa[4,5] with two homologous domains, each bearing a potential catalytic site.[6] The difference in the molecular mass is probably due to variations in the glycosilation pattern of the protein from different species.

ACE has been identified in many tissues and is specially abundant in lung. This enzyme is an integral membrane protein anchored to the plasma membrane via its hydrophobic C-terminus.[5] A soluble form of the enzyme also exists in plasma.[7] For its purification, membrane-bound enzyme can be solubilized by detergents or, incubating the membranes with trypsin.[5]

The detergent-extracted, membrane-bound ACE produces aggregates when the detergent is eliminated, while ACE, from the trypsin-extracted samples, is present in non aggregated form even in absence of detergent.[8]

In this work, we describe the trypsin or subtilisin treatment of the purified ACE, to obtain a soluble form of bovine lung ACE in absence of detergent. Purified aggregate ACE was solubilized by tryptic attack for 1 h at 300C. The soluble form was separated by Sephacryl S-300 HR chromatography. A recovery of 68% was obtained. Our purification procedure enables us to carry out a large scale preparation of a soluble ACE form, and to obtain 27 mg of enzyme with a specific activity of 26 min^{-1} mg^{-1} from 1 kg of lung.

MATERIALS AND METHODS

Materials

Standard liquid chromatographic materials used were: Epoxy-activated Sepharose 6B, Sephacryl S-300 HR and Superdex 200HR 10/30 for FPLC, all from Pharmacia. Trypsin from bovine pancreas, subtilisin from Bacillus subtilis, and phenylmethanesulfonyl fluoride (PMSF) were obtained from Boehringer Mannheim. Lisinopril, furanacryloyl-L-phenylalanilglycylglicine, and N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonicacid) (Hepes) were purchased from

Sigma. Ethylenediamine tetraacetic acid (EDTA) was obtained from Merck; other chemicals were bought from either Sigma or Merck. Veal lungs were obtained from a local slaughterhouse immediately after the animals were killed.

Measurement of Protein Concentration

Protein concentration was measured according to biocinchoninic acid (BCA).[9] For pure lung ACE solutions, the concentration was determined by using rabbit-lung-enzyme specific absorbance at 280 nm of 225,000 M^{-1} cm^{-1}.[10]

Measurement of ACE Activity

ACE enzyme assay was performed by the spectrophotometric method of Holmquist et al.[11] at 25°C, with furanacryloyl-L-phenylalanilglycylglicine as substrate. Reaction mixtures contained 100 μM substrate in 50 mM Hepes, 300 mM NaCl, 10 μM zinc acetate and 0.7 μg/mL to 2 μg/mL of enzyme at pH 7.5. Absorbance measurements at 334 nm were carried out in a Beckman DU-70 spectrophotometer with the cells maintained at 25°C. One unit of activity produces Δ -A_{334}/min of 1.0. The specific activity of the purified protein was 24-26 min^{-1} mg^{-1}.

Purification of ACE

Step 1.- Preparation of the Homogenate. Up to 1 kg of fresh veal lung bought from a local slaughterhouse was taken immediately to our laboratory in crushed ice. From this moment on, the entire procedure was carried out at 4°C. Small portions of the lung were homogeneized in a blender with 10 mM Hepes, 400 mM NaCl, pH 7, at a 5:1 volume:weight ratio.

The homogenate was centrifuged at 9,000xg for 60 min in a Beckmann J2-HS centrifuge. The supernatant was separated and the precipitate was washed twice with the same buffer.

Step 2.- Extraction with Triton X-100. The precipitate was dispersed in 10 liters of a solution containing 10 mM Hepes, 400 mM NaCl, 100 μM $ZnCl_2$ at pH 7 (buffer A), which was made 0.5% in Triton X-100, stirred for 1 h, centrifuged at 9,000xg for 60 min, and the pellet was discarded. We have tested the stirring time of 2 h and 3 h and approximately the same ACE activity was obtained in the supernatant.

Step 3.- Ammonium Sulphate Precipitation. The supernatant was made 34% w/v NH_4SO_4 by addition of solid amonium sulphate, and stirred for 2 h and centrifuged at 9,000xg for 30 min. The supernatant was discarded, and the pellet was redissolved in 750 mL of buffer A and dialyzed against several changes of the same buffer. After dialysis, if any turbidity was still present, a final 30,000xg centrifugation for 30 min in a Beckmann XL-90 ultracentrifuge got rid of it.

Step 4.- Affinity Chromatography. The dialyzed solution was applied to a lisinopril/Sepharose 6B affinity column equilibrated in buffer A. Lisinopril was coupled to the epoxy-activated-Sepharose 6B as described by Bull et al.[12] Approximately 46 mL of the exchanger in a 1.6x23 cm column was used for a purification starting from 1 kg of lung. The flow rate, during chromatography, was 15 mL/h. ACE bound to the lisinopril/Epoxy-activatedSepharose 6B affinity column, the overall retention was 95%. The column was washed with 5 column volumes of buffer A; 3 column volumes of 0.5 M NaCl; 5 column volumes of buffer A and 2 column volumes of 10 mM sodium phosphate at pH 7. After 1.0 bed volume of 10 mM sodium phosphate, 5 mM EDTA at pH 7, at a flow rate of 15 mL/h has passed through the column, the flow was stopped during 24 h.

The enzyme was then eluted with the same buffer, at a flow rate of 50 mL/h. Fractions of 1 mL were collected over 5 mL of 20 mM Hepes, 0.1 mM NaCl, 50 TM $ZnCl_2$ at pH 7, monitoring the absorbance at 280 nm. A protein peak was detected and its fractions were pooled. Phosphate and EDTA was then eliminated by dialysis, against a solution 10 mM Hepes, 0.1 M NaCl, 10 μM $ZnCl_2$, at pH 7. The enzyme was concentrated with an Amicon cell using a XM30 membrane, to a protein concentration of approximately 1 mg/mL. Gel electrophoresis, under denaturating conditions of the purified enzyme, is shown in Fig. 1. Only one band is seen, which clearly demonstrates a high degree of purity for the obtained enzyme. The specific activity of the enzyme was 20 units/mg.

Step 5.- Trypsin treatment. The protein solution (1 mg/mL) was incubated for 2 h with bovine pancreatic trypsin (0.2 % w/w with respect to protein), in 10 mM Hepes, 0.1 M NaCl, 10 μM $ZnCl_2$, at pH 7 and 30°C. Proteolytic action was stopped by addition of PMSF (1 mM). The trypsin solution (1 mg/mL) was freshly prepared before the experiment by dissolving the enzyme in 0.1 mM HCl.

Step 6.- Sephacryl S-300 HR Chromatography. The ACE solution was finally chromatographed in a gel filtration column. Approximately 140 mL of Sephacryl S-300 HR in a 1.6x70 cm column, was used for a purification starting from 1 kg of lung. The solution that had been incubated with trypsin was applied at a flow rate of 30 mL per hour to the gel filtration column, equilibrated in 10 mM Hepes, 0.1 M NaCl, 10 μM $ZnCl_2$, at pH 7, and the enzyme was eluted with the same buffer.

The active fractions were collected, pooled and usually concentrated with an Amicon cell using a XM30 membrane, to a protein concentration of approximately 3 mg/mL. In this solution the enzyme retains its full activity for at least 6 months at 4°C.

Protein Electrophoresis Under Denaturing Conditions.

Polyacrilamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) of the enzyme, was performed on 7% or 6% gels according to Laemmli[13] using a Bio-Rad Protean II electrophoresis apparatus. Coomassie Brilliant Blue R-250 was used for staining the protein bands. Commercial protein size markers were used for calibration (Pharmacia).

Molecular Mass Determinations

Analytical high performance liquid chromatography (HPLC) was carried out on a Beckman apparatus. Effluents were monitored with a diode array detector (DU168). FPLC column was Superdex 200 HR 10/30 from Pharmacia.

The column was calibrated with tiroglobuline (669 kDa), ferritin (440kDa), alcohol deshidrogenase (150 kDa), bovine serum albumin (67 kDa), ribonuclease (13,7 kDa) and Blue Dextran (2000 kDa) from Sigma. The column was equilibrated with 10 mM Hepes, 0.1 M NaCl, 10 μM $ZnCl_2$ at pH 7.

Proteolysis

Membrane-bound converting enzyme that had been eluted from lisinopril/Epoxy-activated Sepharose 6B affinity column (1 mg/mL), was digested with bovine pancreatic trypsin or subtilisin (1/1000-1/10 w/w with respect to ACE) in 10 mM Hepes, 0.1 M NaCl, 10 μM $ZnCl_2$, pH 7, at 25°C. Proteolytic action was stopped by dilution in 50 mM HEPES, 300 mM NaCl, 10 μM zinc acetate (pH 7.5) and the activity was assayed inmediately.

Proteolysis of ACE was also stopped with PMSF (1 mM) and the peptides were separated by Superdex 200 HR 10/30 HPLC. For the SDS-PAGE experiments, the proteolysis was stopped by precipitation with ice-cold trichloroacetic acid (TCA) (5% final concentration).

Table 1

Purification of ACE Starting from 1 kg of Bovine Lung

Purification Step	Volume (mL)	Total Protein (mg)	Total Activity (units)	Specific Activity (units/mg)	Purification (fold)	Yield (%)
Extraction with Triton X-100	9,600	19,560	920	0.04	1	100
Ammonium Sulphate Precipitation	960	4410	830	0.20	5	90
Lisinopril/Epoxy-Sepharose 6B	90	29	635	22	550	70
Trypsin treatment and Sephacryl S-300 HR Chromatography	8	27	700	26	650	68

RESULTS AND DISCUSSION

Purification of ACE

The main features of the ACE purification procedure described in the presvious section are shown in Table 1. As can be seen, 27 mg of ACE, with a specific acitivity of 26 units/mg was obtained from 1 kg of lung. Our procedure is based on the method of Bull et al.[12] for the purification of the ACE from rabbit lung. Before affinity chromatography, a precipitation with ammonium sulfate was performed according to Pantoliano et al.[14] This step reduced the volume to apply on the affinity column ten times. In this way, the fractionation step with 18% w/v ammonium sulphate made by Pantoliano et al.[14] was not necessary. The principal step of the purification is the affinity chromatography. The adsorbed protein was eluted with 10 mM sodium phosphate, 5 mM EDTA at pH 7, and the EDTA was removed by dialysis overnight. If the enzyme is eluted with lisinopril, it is necessary to dyalize the sample for several days to remove the lisinopril.[12,15] The main difference in our procedure is the trypsin treatment and the use of Sephacryl S-300 HR chromatography, as the last purification step. This trypsin treatment enables us to purify a soluble form of the enzyme. Figure 2A, shows the profile of the Superdex 200 HR 10/30 elution, which is similar to that reported by gel

Figure 1. Purified bovine lung angiotensin converting enzyme (15 μg) separated by SDS-PAGE on 7% gels (lane 1). In lane 2 different molecular mass standards from Pharmacia were run, from top to bottom: myosin (212 kDa), α_2-macroglobulin (170 kDa), β-galactosidase(116 kDa), transferrin(76 kDa), glutamic dehydrogenase(53 kDa).

filtration on Sephadex G-200 by Bull et al.[12] When ACE untreated with trypsin, was applied to the column, two protein peaks with ACE activity were obtained: a first major peak near the void volume, corresponding to aggregated angiotensin-converting enzyme with 21 units/mg, and a smaller peak, which traveled with apparent molecular mass around 310 kDa and had a higher specific activity of 26 units/mg. The area ratio was 9:1. Fractions of each peak were pooled and concentrated to approximately 0.4 mg/mL in a centricon 30 (Amicon) filter. The solutions were left one day at 4°C and were rechromatographed on the Superdex 200 HR 10/30 column. In each case, only one peak was obtained with the same elution time of the previous chromatography(Fig. 2, B and C).

Four samples of ACE (375-400 μg of protein) were incubated with increasing amounts of trypsin for 1h at 30°C in 10 mM Hepes, 0.1 M NaCl, 10μM $ZnCl_2$, pH 7, in a final volumen of 500 μL. After the reaction had been stopped with PMSF (1 mM), a fraction of each sample was applied to a column of Superdex 200 HR 10/30 on HPLC, which was equilibrated with the same buffer. Sample 1 (1/1000 w/w with respect to ACE), showed the same area ratio between peaks as that of samples untreated with protease (Fig. 2A).

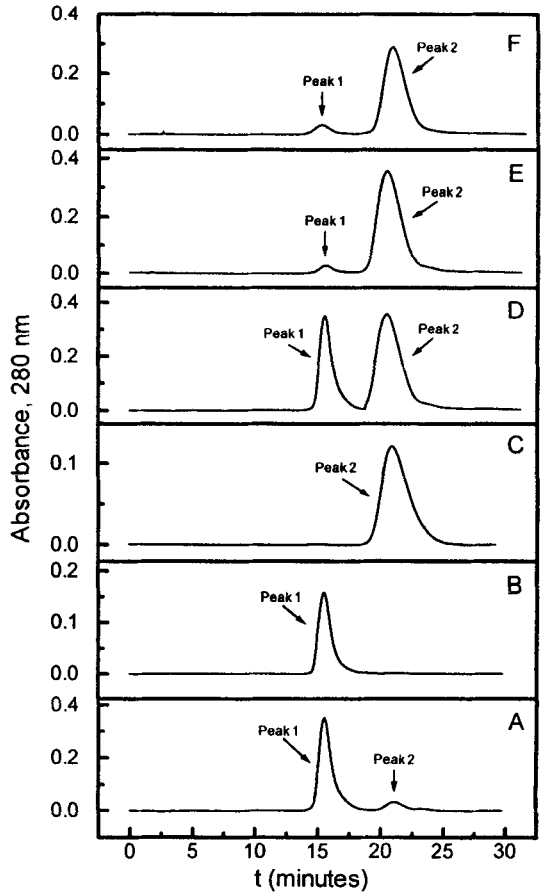

Figure 2. HPLC chromatograms of bovine lung angiotensin converting enzyme. The following samples were injected and absorbance monitored at 280 nm: (A) 500 μL ACE eluted on affinity column; (B) ACE from peak 1 fractions of chromatogram A; (C) ACE from peak 2 fraction in chromatogram A; ACE treated with trypsin: (D) 1 mg/ 500mg protein (E) and 1mg/200mg protein for 1 h at 300C; (F) ACE treated with subtilisin (1mg/200mg protein) for 1 h at 300C. The chromatography was performed on Superdex S-200 10/30 column equilibrated with 10 mM Hepes, 0.1M NaCl, 10 μM $ZnCl_2$ pH 7, at a flow rate of 0.5 mL/h at room temperature.

On increasing the amount of trypsin, the area of peak 2 was increased with respect to that of peak 1 (Fig. 2D). In the experiment to a 1/200 (w/w) trypsin/ACE ratio and higher, the 0.5 : 9.5 area ratio was obtained (Fig. 2E). The solubilization of

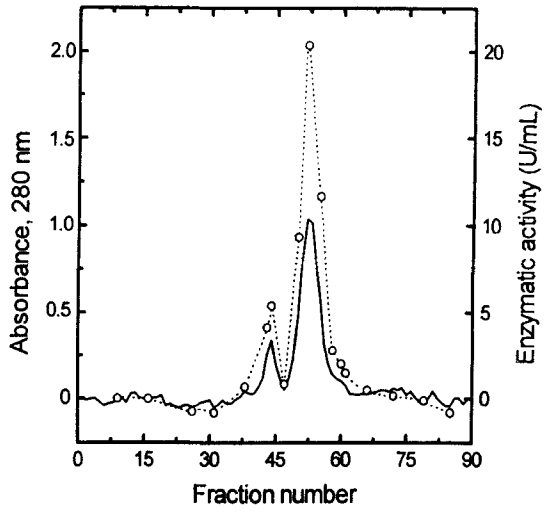

Figure 3. Elution profile in the Sephacryl S-300 HR chromatography; 29 mg of ACE incubated in the presence of trypsin and with a specific activity of 22 units/mg was applied on a 1.6x70 cm column and 1.3 mL fractions were collected. The enzymatic activity is shown with the open circles. The specific activity in the peak 2 pooled solution, containing 27 mg of protein, was 26 units/mg. The chromatography was performed at a flow rate of 30 mL/h at room temperature.

purified ACE was also carried out using subtilisin treatment. Figure 2F, shows an elution profile of a sample of ACE (0.75 mg/mL), incubated for 1 h at 30°C in the presence of subtilisin (1/200 w/w with respect to ACE) . The 1.5:8.5 area ratio was obtained. In all cases, the enzyme activity was 21 units/mg and 26 units/mg for the peak 1 and 2, respectively. The digestion with trypsin or subtilisin [1/200 (w/w) protease/ACE ratio], lets the enzyme to be solubilized. Thus, we introduced a trypsin treatment and the use of size exclusion chromatography as the last purification step. For 1 kg of bovine lung, we used a column of Sephacryl S-300 HR (1.6x70 cm). Fraction (1.3 mL) were collected at a flow rate of 30 mL per hour. As can be seen in Figure 3, the enzyme activity and amount of protein was obtained mostly closely parallel to that of peak 2 (0.5:9.50 area ratio). An elution profile similar to that shown in Fig. 3, was obtained when fractions of peak 1 were pooled, concentrated, incubated with trypsin in the same conditions indicated above and rechromatographed. Lanzillo et al.[8] had showed that the detergent-extracted membrane-bound converting enzyme produced aggregates, while the trypsin-extractedsamples were monomers.

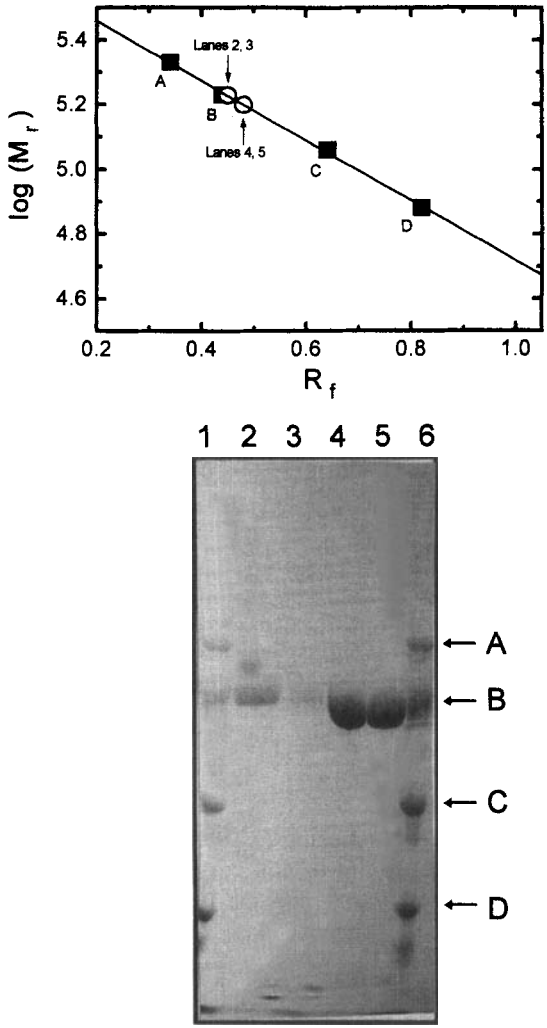

Figure 4. Slab gel electrophoresis of the samples obtained on the chromatographies in the Fig. 2. Trypsin-untreated ACE: lane 2 contained 12 μg of aggregate ACE from Fig. 2B; lane 4 contained 20 μg of soluble ACE form from Fig 2C. Trypsin-treated ACE: lane 3 contained 12 μg aggregate ACE from peak 1, Fig. 2E; lane 5 contained 20 μg of soluble ACE from peak 2 of Fig. 2E. Lanes 1 and 6 contained the following four protein molecular mass standards: A, myosin (212 kDa); B, α_2-macroglobulin (170 kDa); C, β-galactosidase (116 kDa); D, transferrin (76 kDa). Samples were electrophoresed in a 6% polyacrylamide gel under reducing conditions and were stained with Coomassie brilliant blue R-250.

Thus, the peak 1 in Figure 2, would correspond to ACE molecules with its membrane-bound sequence intact, which induces an aggregation among ACE molecules. Peaks 2 in Figure 2 might correspond to ACE molecules with the membrane-bound sequence deleted.

We have checked our purification procedure starting from lungs kept at -20°C for up to four months and have obtained the same results as above.

Molecular mass determination

Molecular mass determination was carried out by size exclusion chromatography on HPLC (SE-HPLC) and disc gel electrophoresis. The results of electrophoresis of chromatographed samples on Superdex 200 HR 10/30 column, under denaturing and reducing conditions, are shown in Fig. 4. Only one band is seen in all cases, the molecular mass of the fractions corresponding to the peak 2 is slightly smaller than those of the fractions of the peak 1.

Under these conditions, the subunit of aggregate species (peak 1, Fig. 2, B and E) were calculated to have a molecular mass of 170 kDa (lanes 2 and 3), which agrees with that reported previously.[3] Thus, the peak 1 in Fig. 2E should correspond to ACE molecules, which had not been cleavaged with trypsin. The molecular mass of peak 2 of the samples incubated in the absence (Fig. 2B), or the presence (Fig. 2F) of trypsin, were estimated to be approximately 160 kDa in SDS-PAGE (lanes 4 and 5).

These results seem to indicate, that the peak 2 (Fig. 2A) of samples untreated with trypsin, correspond to protein molecules that had lost a peptide of 10 kDa, probably from hydrophobic C-terminal part. These deletions might be carried out by a endogenous protease during the purification. The tryptic attack also produces the deletion of a peptide of approximately 10 kDa. Recently, Beldent et al.[16] have described that human plasma ACE is secreted from the membrane-bound enzyme, by the deletion of 140 amino acids (14 kDa) at the C-terminal part.

The calculated difference of 10 kDa, approximately in the apparent mass of the soluble ACE forms, separates by gel filtration without (Fig. 2A, peak 2) or with (Fig. 2D, peak 2) tryptic attack, is very close to the result described previously.[16] The discrepancy is probably due to the difficulty in obtaining precise high molecular mass by SDS-PAGE.

The results obtained by SE-HPLC, might indicate that the soluble ACE form obtained by treatment with trypsin, might be in its native state as homodimer. Although, it is necesary to consider that the linked oligosaccharides to polypeptide chain can interact with chromatography gel. This fact can modify its elution time.

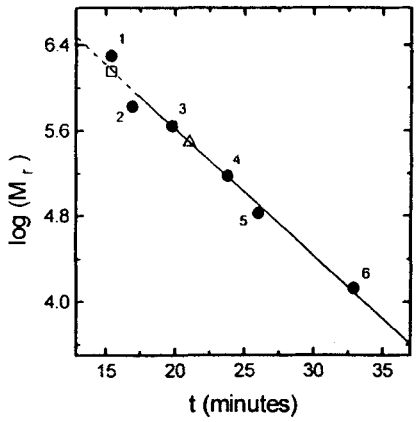

Figure 5. Molecular mass determination of the samples obtained on the showed chromatographies in the Fig. 2. Calibration curve for size exclusion-HPLC. A Superdex 200 HR 10/30 (10x300 mm) was equilibrated with 10mM Hepes, 0.1M NaCl, 10 μM $ZnCl_2$ (pH 7) and operated at 0.5 mL/h. At t=0 500 μL of the protein mixture (in the same buffer) were injected: Blue Dextram (2000 kDa), tiroglobuline (669 kDa), ferritin (440kDa), alcohol deshidrogenase (150 kDa), bovine serum albumin (67 kDa) and ribonuclease (13,7 kDa). Absorbance was monitored at 280 nm. The elution times are plotted vs log mol wt. The peak 1 fractions of the Fig 2B and 2E correspond to aggregates (□) while peak 2 fractions of the Fig 2C and 2E correspond to a soluble ACE form with a molecular mass of 310 kDa (Δ).

CONCLUSIONS

The described purification procedure enables us to obtain 27 mg of a soluble ACE form with a specific activity of 26 min^{-1} mg^{-1}, from 1 kg of bovine lung using affinity and size exclusion chromatography. A 650-fold purification was achieved with a 68% yield. A soluble angiotensin I-converting enzyme form was obtained from purified membrane-bound enzyme form, using trypsin or subtilisin treatment and size exclusion chromatography. The recovery of 93% was acheived. If peak 1 fractions (Fig. 3) were treated again with trypsin, a recovery of 96% can be obtained. The procedure described is more suitable than a tryptic digestion of the membranes, because of its higher yield and better purification. A molecular mass of 310 kDa was obtained for native soluble ACE form by size-exclusion HPLC. Molecular mass of membrane-bound enzyme and of the ACE form solubilized with trypsin, was found to be 170 kDa and 160 kDa, respectively, by SDS-PAGE. Thus, the trypsin solubilizes the protein by deletion of a fragment of 10 kDa, approximately.

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