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Purification of angiotensin I converting enzyme from pig lung using concanavalin-A sepharose chromatography

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Abstract

Angiotensin I converting enzyme (ACE) plays a major role in blood pressure regulation, catalyzing the conversion of angiotensin I to the vasoconstrictor angiotensin II. In this report we describe a two-step affinity chromatography method for preparative purification of ACE from pig lung using Concanavalin-A Sepharose 4B and affinity chromatography on Lisinopril Sepharose 6B. The same purification scheme was used to obtain Cobalt-ACE, where zinc ion located at the active site is replaced by cobalt. Cobalt-ACE visible spectrum shows a characteristic broad peak from 500 to 600 nm. The shape and maximum absorptivity of this peak changes in presence of ACE inhibitors that bind at the catalytic site.
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1. Introduction

Angiotensin I converting enzyme (ACE) (peptidyl dipeptidase A, EC 3.4.15.1) is part of the renin–angiotensin system and plays an important role in the control of blood pressure [1]. ACE is a zinc metallopeptidase that catalyses the conversion of angiotensin I to the vasoconstrictor octapeptide angiotensin II [2] and inactivates the vasodilator nonapeptide bradykinin [3]. ACE shows a relatively broad specificity and also cleaves dipeptides and tripeptides from the C-terminus of substrates such as luteinizing hormone-releasing hormone and substance P [4].

ACE is present in three main forms: somatic, plasma and testicular. The somatic and testicular forms are two isozymes [5], while plasma circulating ACE is derived by cleavage from the somatic form. cDNA cloning has been used to determine the primary structure of endothelial somatic ACE, revealing the presence of two homologous domains called N and C domains [6]. Each domain contains an active site, characterized by the presence of a zinc-metallopeptidase consensus sequence and whose function has been demonstrated by site-directed mutagenesis. The two ACE active sites hydrolyze angiotensin I and bradykinin [7,8], the two main physiological substrates of ACE involved in the control of blood pressure.

The recognition of the role of this enzyme in the regulation of blood pressure stimulated interest in searching for ACE inhibitors for use as antihypertensive agents. Clinical studies using the snake venom nonapeptide teprotide demonstrated the potential of ACE inhibitors as antihypertensive drugs [9]. Many highly potent inhibitors which can be

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taken orally have been developed during the past two decades [10,11]. The design of potent inhibitors of ACE has been based on its analogy with the active site of other zinc containing peptidases, especially carboxypeptidase A and thermolysin [9]. Structural studies in ACE are limited by the lack of large amounts of protein with the right level of purity and homogeneity to allow the protein to crystallize.

One of the problems encountered in the purification of ACE from tissue is the removal of other proteolytic enzymes that hydrolyse ACE. Early purification procedures involved treatment at 60 °C, or low temperature homogenization followed by differential centrifugation as strategies to eliminate undesirable proteolytic enzymes [12]. The use of ammonium sulphate fractionation of lung homogenates was shown to yield extracts free of angiotensinases, and this procedure was incorporated in most subsequent purification procedures [12,13]. Afterwards the protein is purified to homogeneity by one or more chromatographic steps. Most of the procedures use affinity chromatography with the inhibitor Lisinopril as stationary ligand [14].

All forms of ACE are heavily glycosylated. Somatic ACE has been shown to be only *N*-glycosylated [15], human and bovine ACE possessing 17 and 16 potential sites for *N*-glycosylation, respectively [2,16]. ACE from guinea pig serum has one N-linked high mannose site, while rabbit lung ACE has two to three high mannose glycosylation sites [17]. Glycosylation may play an important role in the release of ACE from membrane, possibly by affecting the folding of the protein and its recognition by other enzymes involved in its transport and cleavage [18]. The extent of ACE glycosylation is important for the enzyme's catalytic properties [15]. As far as we know, Concanavalin-A Sepharose has been not used for large scale purification of ACE [12]. Concanavalin-A interacts with N-linked oligosaccharides in which at least two outer mannose are either unsubstituted or are substituted only at position C-2 by another sugar [19]. Protein is usually eluted using glucose, but tight bound glycoproteins require α-D-methylglucoside or α-D-methylmannoside.

In this article we report a fast and high yield method of ACE purification using Concanavalin-A Sepharose 4B chromatography and Lisinopril Sepharose 6B affinity chromatography. The procedure was slightly modified to obtain Cobalt-ACE. Catalytic and spectral properties of ACE and Cobalt-ACE enzymes are reported.

2. Experimental

2.1. Materials

All chemicals were bought from either Sigma or Merck. Epoxy-activated Sepharose 6B, Concanavalin-A Sepharose 4B and Superdex 200HR 10/30 from Amersham Biosciences. Pig lung was purchased from a local slaughterhouse and immediately taken to our laboratory in crushed ice. Lisinopril was coupled to the epoxy-activated Sepharose 6B as described by Bull et al. [20].

2.2. Measurement of protein concentration

Total protein concentrations were measured according to Lowry et al. [21]. For pure lung ACE solutions, the concentration was determined on the basis of $\varepsilon_{280\ nm}=2.18\times10^5\ M^{-1}\ cm^{-1}$. The extinction coefficient was obtained as described by Gill and von Hippel [22] using denaturating conditions. The measured coefficient is in good agreement with that of rabbit lung ACE [23].

2.3. Enzyme assay

ACE activity was assayed according to previously published methods [24] with modifications. The assay was buffered with 50 m*M* Hepes, 0.3 *M* NaCl and 10 μ*M* $ZnCl_2$ pH 7.5. A volume of 770 μl of buffer was mixed with 200 μl of 0.5 m*M* 2-furanacryloyl-L-phenylalanylglycylglycine (FAFGG) as substrate. The reaction was initiated by adding 30 μl of enzyme solution (1–3 μg) to the cuvette thermostatted at 25 °C. Absorbance decrease at 334 nm was monitored for a period of 2 min. Each activity value was taken from an average of three to five measurements. One unit of activity is defined as the amount of ACE that produces a ΔA_{334}/min of 1.0. Cobalt-ACE assays were conducted with the same procedure, except that 10 μ*M* $CoCl_2$ was placed in the buffer solution instead of 10 μ*M* $ZnCl_2$.

2.4. SDS–PAGE electrophoresis

Electrophoresis in 7% SDS–polyacrylamide gels was conducted according to the method of Laemmli [25]. It was performed using a constant current of 20 mA for 30 min for the stacking gel and 50 mA for 2 h for the separating gel. The gel was stained with 0.1% (w/v) Coomassie Brilliant Blue R-250, 40% methanol (v/v) with 7% (v/v) acetic acid for 1 h at room temperature and destained with 40% methanol (v/v) with 7% (v/v) acetic acid for 12 h. The molecular mass of purified ACE was estimated by comparison with electrophoretic migration of standard ‘‘High molecular mass’’ from Bio-Rad.

2.5. Size-exclusion chromatography

Size-exclusion chromatography (SEC) analysis was performed in a Beckman System Gold instrument using a Superdex 200 HR 10/30 equilibrated with 10 m*M* Hepes, 0.1 *M* NaCl, 10 μ*M* $ZnCl_2$, pH 7. Chromatography was performed at room temperature at a flow-rate of 0.5 ml/min. Protein elution was monitored at 280 nm using a Beckman diode array detector DU 168.

2.6. ACE purification

Small portions of pig lung (50 g) were minced and then homogenized in a blender 10 m*M* Hepes, 0.4 *M* NaCl, pH7, at a volume weight ratio of 5:1. The whole procedure was carried out at 4 °C. The homogenate was centrifuged at 9000 *g* for 60 min in a Beckman J2-HS centrifuge. The supernatant was discarded and the precipitate was washed twice with 10 m*M* Hepes, 0.4 *M* NaCl, pH 7. The final precipitate was resuspended in 10 m*M* Hepes, 0.4 *M* NaCl, 10 μ*M* $ZnCl_2$, 0.5% (w/v) Triton X-100, pH 7, and stirred overnight. The solution was centrifuged at 9000 *g* for 60 min and the pellet was discarded. Solid $MnCl_2$ and $CaCl_2$ were added to the supernatant in order to made the solution 1 m*M* in each salt before loading on a Concanavalin-A Sepharose 4B column (5×30 cm) equilibrated with 20 m*M* Tris, 0.5 *M* NaCl, 1 m*M* $MnCl_2$, 1 m*M* $CaCl_2$, pH 7.4. The column was washed with 20 m*M* Tris, 0.5 *M* NaCl, 1 m*M* $MnCl_2$, 1 m*M* $CaCl_2$, pH 7.4, until the effluent no longer showed absorbance at 280 nm. Bound protein was eluted with 0.5 *M* α-D-methylglucoside in 20 m*M* Tris, 0.5 *M* NaCl, pH 7.4. The preparation was applied on a Lisinopril–Sepharose 6B column (2.6×20 cm) equilibrated with 10 m*M* Hepes, 0.4 *M* NaCl, 10 μ*M* $ZnCl_2$, 0.5% (w/v) Triton X-100, pH 7. The column was washed with 4 column volumes of 10 m*M* Hepes, 0.4 *M* NaCl, 10 μ*M* $ZnCl_2$, 0.5% (w/v) Triton X-100, pH 7; 2 column volumes of 0.5 *M* NaCl; 4 column volumes of buffer Hepes 10 m*M*, NaCl 0.4 *M*, $ZnCl_2$ 10 μ*M*, Triton X-100 0.5% (w/v) at pH 7; and 2 column volumes of 10 m*M* sodium phosphate buffer at pH 7. In order to elute the protein 1 bed volume of 10 m*M* sodium phosphate, 5 m*M* EDTA at pH 7, at a flow-rate of 15 ml/h was passed through the column and the flow was stopped for 24 h. The elution was carried out using the same buffer at a flow-rate of 30 ml/h. Fractions of 1 ml were collected over 5 ml of 20 m*M* Hepes, 0.1 *M* NaCl, 50 μ*M* $ZnCl_2$ at pH 7. Fractions containing ACE were collected, pooled and dialyzed against 10 m*M* Hepes, 0.1 *M* NaCl, 10 μ*M* $ZnCl_2$, pH 7.

2.7. Preparation of Cobalt-ACE

The Cobalt-ACE enzyme was prepared using the same procedure as Zinc-ACE except that effluent from Lisinopril–Sepharose 6B was collected over 5 ml of 20 m*M* Hepes, 0.1 *M* NaCl, 0.05 *M* $CoCl_2$, pH 7. Fractions containing Cobalt-ACE were collected, pooled and dialyzed against 10 m*M* Hepes, 0.1 *M* NaCl, 100 μ*M* $CoCl_2$, pH 7. Cobalt ACE UV–Visible spectra were recorded on a DU-7400 Beckman spectrophotometer over a wavelength range of 250–800 nm. All spectra were recorded against 20 m*M* Hepes, 0.1 *M* NaCl, pH 7.

3. Results and discussion

3.1. ACE purification and molecular mass determination

Lung ACE interacts strongly with Concanavalin-A by means of high mannose and hybrid-type oligosaccharides [15,26]. We have used this lectin affinity chromatography in order to reduce the extract volume and increase the purity of the protein applied to

Lisinopril affinity column. Ten to 20% of the total activity were recovered in the eluate that does not bind to the Concanavalin-A column. Elution was carried out using α-D-methylglucoside with almost 100% recovery of bound protein. Concanavalin-A Sepharose 4B chromatography reduces sample volume from 8 l of extract to 100 ml. α-methyl-D-glucopyranoside eluant was directly applied onto a Lisinopril affinity column. ACE was eluted using 10 m*M* sodium phosphate, 5 m*M* EDTA, pH 7, which removes zinc from the active site and releases the enzyme from the complex formed with the inhibitor [27].

The purification of lung ACE is summarized in Table 1. The purity of purified ACE was assessed in SDS–polyacrylamide gel electrophoresis which revealed one single protein band (Fig. 1). The molecular mass of purified ACE was estimated at about 170 kDa by SDS–polyacrylamide gel electrophoresis analysis. This value correspond well with that of glycosylated lung ACE [15].

The molecular size under non-denaturating conditions was estimated by SEC. ACE elutes as one single peak that correspond to 300 kDa (Fig. 2). The discrepancies between molecular mass obtained by size-exclusion gel chromatography and those obtained by SDS–PAGE can be attributed to hydrodynamic properties of the glycosylated ACE. It has been shown that ACE molecule is clearly elongated in aqueous solutions with a Stokes radius of 6.0 nm [15] that corresponds to the ACE monomer [16]. Pig lung ACE purified by ammonium sulphate precipitation shows an additional peak at the exclusion volume corresponding to the aggregated form of the enzyme (data not shown). The aggregated form shows lower activity (15 Units/mg) than the monomeric form and higher molecular mass (180 kDa by SDS–PAGE). Our results show that pig lung ACE purified by Concanavalin-A chromatography is aggregate free.

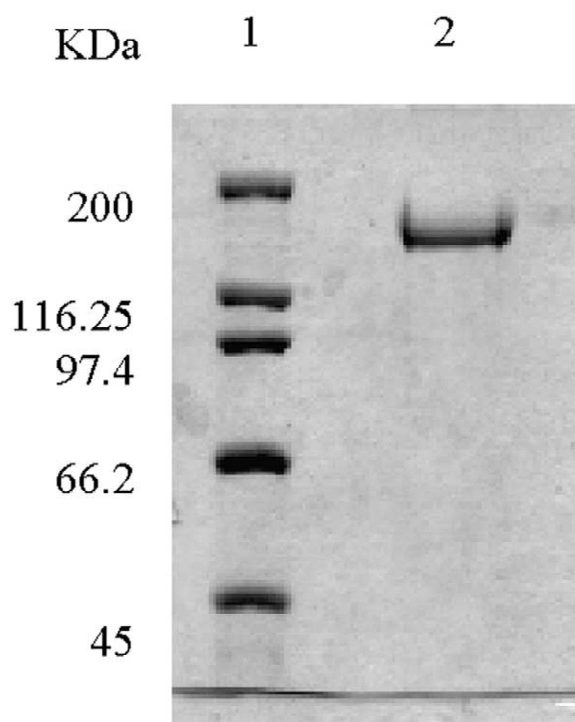

Fig. 1. Purified pig lung angiotensin converting enzyme separated by SDS–PAGE gels (lane 2). In lane 1 protein standards. M_r values are given on the left. Protein standards used are myosin (200 kDa), β-galactosidase (116.25 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa) and ovalbumin (45 kDa). Sample buffer was 0.0625 *M* Tris–HCl, pH 6.8, 10% (w/v) glycerol, 2% (w/v) SDS, 5% (w/v) 2 β-mercaptoethanol and 0.0025% (w/v) bromophenol blue.

3.2. Cobalt-ACE purification

All attempts to obtain zinc-free ACE by dialysis against a zinc chelating agent failed. The protein

Table 1
Purification of angiotensin converting enzyme from 1 kg of pig lung

Fractionation step	Total protein (mg)	Total volume (ml)	Specific activity (Units/mg)	Total activity (Units)	Purification (*n*-fold)	Activity recovered (%)
Extract	19 000	8000	0.07	1330	1	100
Concanavalin-A–Sepharose 4B	1000	100	1.06	1060	15	80
Lisinopril–Sepharose 6B	24	15	37	890	530	67

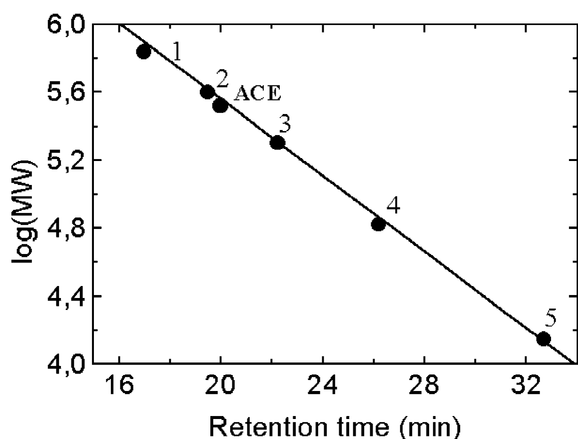

Fig. 2. Molecular mass determination by SEC. Protein standards used are: (1) thyroglobulin (690 kDa); (2) ferritin (440 kDa); (3) β-amylase (200 kDa); (4) bovine serum albumin (66.2 kDa); and (5) ribonuclease A (14 kDa).

became inactivated in a week of dialysis without activity recovery after $ZnCl_2$ addition. A procedure to exchange the metal ion at the active site has been developed by modification of the last step of ACE purification. The protein yields are the same as those for zinc ACE enzyme. Cobalt-ACE shows specific activity of 30 Units/mg when assayed as zinc ACE but with $ZnCl_2$ replaced by $CoCl_2$ in the buffer solution.

3.3. ACE and Cobalt-ACE UV–Visible spectra

ACE shows a typical protein UV absorption spectra with a maximum peak at 280 nm (ε=2.18× 10^5 M^{-1} cm^{-1}), and no absorption was detected at visible wavelengths. Nevertheless Cobalt-ACE visible spectrum showed a broad peak with a maximum at 580 nm that can be attributed to cobalt d–d transitions [24] and with a molar absorptivity of 117.89 M^{-1} cm^{-1} comparable to that of Cobalt-carboxypeptidase (maxima at 555 nm and 572 nm) and Cobalt-thermolysin (maximum at 555 nm) [28]. The Cobalt-ACE visible absorption spectrum is sensitive to enalaprilat and captopril binding as

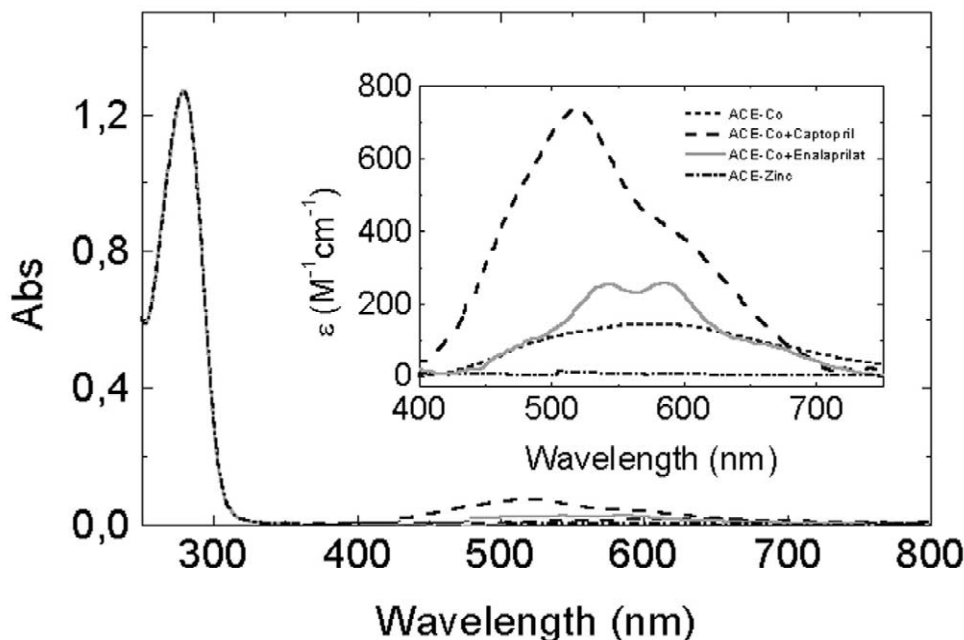

Fig. 3. Absorption spectra of Cobalt(II)-ACE and the enzyme inhibitor complexes with captopril and enalaprilat. Conditions: ACE 12.02 μM, 0.3 M NaCl, 10 mM Hepes, 100 μM Cobalt(II) chloride, pH 7, and captopril (0.1 mM) and 100 μM Cobalt(II) chloride and enalaprilat (0.1 mM) and and 100 μM Cobalt(II) chloride. Addition of captopril or enalaprilat to Cobalt(II) Chloride caused no change the absorption spectrum within the range 250–800 nm at this pH.

Table 2
Kinetic parameters of the hydrolysis of FAFGG by native ACE and Cobalt ACE

Enzyme	k_{cat} (min^{-1})	K_{max} (mM)	k_{cat}/K_{max} (min^{-1} mM^{-1})
ACE	7590	0.23	33 000
Cobalt-ACE	5520	0.25	22 000

shown in Fig. 3. Enalaprilat Cobalt-ACE displays two bands at 543 and 585 nm with absorptivities of 210 and 211 M^{-1} cm^{-1}, respectively. Captopril Cobalt-ACE shows a more intense band at 519 nm and a shoulder at 595 nm with absorptivities of 608 and 325 M^{-1} cm^{-1}, respectively. The spectra of these complexes with the high-molecular absorptivity at around 550 nm suggest a coordination number of 4 or 5 for cobalt (II) [29] and the same conclusion has been drawn from electronic resonance spectra for ramiprilat Cobalt-ACE [30].

3.4. Michaelis–Menten parameters

Zn-ACE and Cobalt-ACE kinetic parameters for FAFGG were measured (Table 2). Values of K_{max} indicate a similar affinity of both enzymes toward the substrate. Zn-ACE shows a slightly higher k_{cat} than Cobalt-ACE, which results in an increased efficiency of the former to hydrolyze FAFGG (k_{cat}/K_{max}). These results are similar to those previously reported for rabbit lung ACE [24,31]

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