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Purification and biochemical characterization of angiotensin I-converting enzyme (ACE) from ostrich lung: The effect of 2,2,2-trifluoroethanol on ACE conformation and activity

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ABSTRACT

This work reports the purification and biochemical characterization of angiotensin I-converting enzyme (ACE) from ostrich (*Struthio camelus*) lung. The molecular weight of the purified enzyme was approximately evaluated to be 200 kDa and the maximum enzyme activity was observed at pH 7.5. The enzyme activity was increased by detergents of Triton X-100 (0.01%), cetyltrimethylammonium bromide (CTAB) (0.1 and 1 mM) and sodium dodecyl sulfate (SDS) (0.1 mM), while decreased by Triton X-100 (1% and 10%) and SDS (1 mM and 10 mM). The secondary and tertiary structure and activity of ACE in the absence and presence of trifluoroethanol (TFE) were investigated using circular dichroism, fluorescence quenching and UV–visible spectroscopy, respectively. Our results revealed that TFE stabilizes ACE at low concentrations, while acts as a denaturant at higher concentration (20%). The K_m, K_{cat} and K_{cat}/K_m values of ostrich ACE towards FAPGG were 0.8×10^{-4} M, 59,240 min^{-1} and 74×10^7 min^{-1} M^{-1}, respectively. The values of IC_{50} and K_i for captopril were determined to be 36.5 nM and 16.6 nM, respectively. In conclusion, ostrich lung ACE is a new enzyme which could be employed as a candidate for studying ACE structure and its natural or synthetic inhibitors.

1. Introduction

Angiotensin-converting enzyme (EC 3.4.15.1) is a peptidyl dipeptide hydrolase presenting a significant role in renin–angiotensin system. It regulates blood pressure and maintains cardiovascular homeostasis through catalyzing the conversion of angiotensin I (Ang I) to angiotensin II (Ang II), as a biologically active, potent vasoconstrictor [1,2]. Additionally, ACE hydrolyses vasodilator factors such as bradykinin and kallidin to inactive components [3]. ACE influences several physiological systems including cardiovascular, reproduction, renal development and hematopoiesis [4]. Somatic ACE (sACE) isozyme is present in plasma as well it is expressed in endothelium, kidney and other somatic tissues [5]. It is composed of two homologous extracellular domains named N- and C-domains, an intracellular domain and a hydrophobic transmembrane domain. Each extracellular domain possesses a functional catalytic site containing an HEXXH zinc binding motif [6]. ACE has been purified froma variety of mammalian tissues such as pig lung [7], rabbit lung, human plasma [8], porcine seminal plasma [9], human lung [10] and hog lung [11] as well as avian lung and small intestine [12,13].

TFE is a chemical compound which has been commonly used as a structure-inducing co-solvent for conformational studies on proteins and peptides. Numerous investigations demonstrated that TFE stabilizes protein secondary structure through stabilizing local H-bonds and weakening hydrophobic interactions [14].

In this study, we have purified and biochemically characterize ostrich lung ACE as a novel source. The effect of TFE on purified ACE activity from ostrich lung was also investigated. Our studies provide insight into the conformational changes of ACE structure in the presence of TFE using circular dichroism (CD) and fluorescence spectroscopy. Furthermore, ACE activity changes were studied in the presence of ionic and nonionic detergents. The effect of different reagents including phenylmethylsulfonyl fluoride (PMSF), metal chelators and captopril on the activity of ostrich lung ACE was evaluated. As well as kinetic parameters (K_m, V_{max}, K_{cat} and K_{cat}/K_m), there activation of apoenzyme in the presence of metal ions was evaluated.

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2. Materials and methods

2.1. Materials

Fresh ostrich lungs were prepared from Mashhad Meat Industrial Complex (Mashhad, Iran). Captopril, N-(3-[2-furyl]acryloyl)-L-phenylalanylglycylglycine (FAPGG), Sephacryl-200 HR, ethylenediaminetetra-acetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF) and trifluoroethanol (TFE) were purchased from Sigma (St. Louis, MO, USA). Fast Flow Q-Sepharose was bought from Pharmacia (Pfizer and Pharmacia, Sweden). Ultrafiltration membrane with a 100 kDa cut-off was procured from Millipore (Bedford, MA, USA).

2.2. Extraction of ACE from lung tissue

Young ostrich lung tissue (0.45 kg) was cut into small pieces. The tissue was suspended in 0.9 L of 5 mM Tris–HC1, pH 7.4 (suspension buffer) and homogenized for 3–4 min at medium speed and then at top-speed for 30 s, followed by centrifugation at 20,000 × *g* for 30 min. The pellet was suspended in an equal volume of cold suspension buffer, and centrifuged in the same manner. The pellet was suspended in 1.2 L of cold 50 mM Tris–HC1, pH 8.5, containing 0.5% (w/v) Triton X-100. The suspension was sonicated using six pulses (15 s each) of a sonicator (QSONICA, LLC series XL-2000, USA), set at power setting 5-W and then centrifuged at 50,000 × *g* for 65 min. All procedures were carried out at 4 °C.

2.3. Ammonium sulfate fractionation

To precipitate protein, solid ammonium sulfate was slowly added to the cooled ACE-containing supernatant to give a concentration of 35% (w/v). After being stirred for 1 h, the mixture was centrifuged at 20,000 × *g* for 30 min. The consequence floating "pellet" was removed by filtration through cheesecloth and the supernatant was collected. The solution was adjusted to 40% (w/v) ammonium sulfate. The precipitates were discarded and the supernatant was adjusted to 70% (w/v) ammonium sulfate, stirred for 1 h and centrifuged as described above. The precipitates were dissolved in 20 mM Tris–HCl buffer (pH 6.0), 0.5 M NaCl, and 0.1 mM zinc acetate and dialyzed twice against the same buffer for 20 h at 4 °C.

2.4. Gel-filtration chromatography

Gel-filtration was performed using a Sephacryl-200 HR (1.5 cm × 90 cm) column equilibrated with 50 mM Tris–HCl and 0.2 M NaCl, pH 8 (equilibrium buffer). Chromatography was performed at a flow rate of 0.2 ml/min and the enzyme was eluted with equilibrium buffer. Protein elution was monitored by absorbance measurements at 280 nm using Optizen 3220 UV spectrophotometer (Daejeon, South Korea). ACE activity was assayed using FAPGG as substrate. Active fractions were pooled and dialyzed in 25 mM Tris–HCl pH 8.6 for 20 h at 4 °C.

2.5. Ion-exchange chromatography

Ultrafiltration is a technique for concentrate protein samples and also is an excellent tool for efficient separation of biological substances according to their molecular weight and size. The continuous dilution and reconcentration was used to remove small protein samples by ultrafiltration (two times); the sample was next concentrated by ultrafiltration membrane with a cut-off of 100 kDa and loaded on an anion-exchange column (Q-sepharose, 2 cm × 5 cm) equilibrated with 25 mM Tris–HCl, pH 8.6. Afterwards, the sample was eluted with a linear NaCl gradient (0.1–1 M) in 25 mM Tris–HC1, pH 8.6, at a flow rate of 1 ml/min. Fractions containing protein were monitored for the absorbance and enzymatic activity. The samples which showed enzymatic activity were collected for further analysis. In all stages, protein content was measured at 595 nm according to Bradford method using bovine serum albumin as standard [15].

2.6. Enzyme activity assay

ACE activity was determined based on the method described by Holmquist [16], with a slight modification, using a spectrophotometer by continuously monitoring the decreased absorbance at 334 nm for a period of 2 min at room temperature. The assay solution contained a volume of 65 μl of 0.5 mM FA-Phe-Gly-Gly and 200 μl of buffer (50 mM Tris–HCl buffer, pH 7.5, 0.3 M NaCl and 10 μM zinc acetate). The reaction was initiated by adding 30 μl enzyme to the reaction mixture. One unit of enzyme activity is defined as the amount of ACE which produces a ΔA_{334} min^{-1} of 1.0 under assay conditions. The specific activity of enzyme was expressed as units/mg of protein [7]. The ACE activity values were determined by triplicate experiments.

2.7. Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on a 5% (w/v) stacking gel and 12% (w/v) separating gel according to the method of Schagger [17]. The gel was stained with silver nitrate. The molecular weight of the purified ACE was estimated from semi-logarithmic plot of standard molecular masses vs. Rf value (relative mobility).

2.8. Determination of optimal pH and temperature stability

ACE activity was assayed in the pH range of 5.5–10.5, using sodium phosphate buffer (pH 5.5–6.5), Tris–HCl buffer (pH 7–9) and borate buffer (pH 9.5–10.5). Purified enzyme was incubated in each buffer (50 mM) for 5 min at 37 °C. All buffers contained 0.3 M NaCl and 10 μM zinc acetate. The maximum activity of ACE was considered as 100% and the percentage of relative activity was plotted against different pH values. To determine thermal stability, the purified ACE was incubated at different temperatures from 20 °C to 60 °C for 30 min at the optimum pH, and then the enzyme was incubated at room temperature for 10 min. The residual activity was calculated under assay conditions.

2.9. Metal chelators and PMSF

All metal ions in ACE solution were eliminated by dialyzing against 50 mM Tris–HCl (pH 7.5) for 48 h in refrigerator. The dialyzed enzyme (enzyme solution A) was preincubated for 10 min at various concentrations of EDTA and o-phenanthroline (0.5, 0.15, 0.1, 0.01 and 0.001 mM) in 50 mM Tris–HCl and 300 mM NaCl (pH 7.5). The inhibitory effect of PMSF on the enzyme activity was assessed by incubating ACE at various concentrations of PMSF (0.1, 0.01, 0.001 mM) in50 mM Tris–HCl and 300 mM NaCl (pH 7.5) at room temperature for 10 min. The activity of the dialyzed enzyme in the absence of inhibitor was considered as 100% and the percentage of residual activity was plotted against different concentrations of inhibitors. The assay was performed under the conditions previously described.

2.10. Apoenzyme reactivation by metal ions

The effect of metal ions on the enzyme activity was evaluated at three concentrations (0.1, 0.01 and 1 mM) of $CaCl_2$, $ZnCl_2$, $MgCl_2$, $CoCl_2$ and $MnCl_2$ in 50 mM Tris–HCl and 300 mM NaCl (pH 7.5). The apoenzyme solution was prepared by adding of 0.1 mM EDTA to the enzyme solution A and then, the resultant mixture was dialyzed against 50 mM Tris–HCl and 300 mM NaCl (pH 7.5) to eliminate additional reagents. Activity assay was carried out to confirm the enzyme inactivation. The reactivity of the purified enzyme was assayed by incubating the enzyme in the presence of different divalent cations for 15 min at optimum pH and room temperature.

2.11. Statistical analysis

All values are given as mean ± standard deviation (SD). Statistical analysis was performed using SPSS version 16 for Windows. Results were evaluated by one-way ANOVA followed by the Tukey test. The mean difference was considered significant at the 0.05 level.

2.12. Effect of detergents and TFE on the enzyme activity

The effect of ionic and nonionic detergents on the enzyme activity was evaluated. The experiment was performed at different concentrations of SDS (0.1, 1 and 10 mM), CTAB (0.1, 1 and 10 mM) and Triton X-100 (0.01, 0.1, 1 and 10%, w/v) in assay buffer. The enzyme was incubated in each buffer for 30 min at room temperature. The percentage of relative activity for each sample was calculated.

The effect of different concentrations of TFE (0.01, 0.1, 1, 10 and 20%, v/v) on ACE activity was also evaluated. The enzyme solution was incubated in the presence of TFE for 30 min at room temperature. The percentage of relative activity for each concentration of TFE was determined.

2.13. CD and intrinsic fluorescence spectroscopy

CD spectra were recorded on a Jasco's Spectra Manager™ software at ACE concentration of 0.1 mg/ml in 20 mM Tris–HCl, pH 7.5 containing 10 μM $ZnCl_2$ (control). CD spectra were also recorded at 0.1 mg/ml of ACE and in 20 mM Tris–HCl, pH 7.5 containing 10 μM $ZnCl_2$ and TFE (0.1, 1, 10 and 20%, v/v). Spectra were measured within the wavelength range of 195–250 nm using a quartz cuvette of 0.5 mm thickness. Data were collected at time constant of 2 s and a 1-nm band width with a scan rate of 50 nm/min. The CD spectra measure was performed by at least five scans. By considering the total number of amino acid residues (1818) in the enzyme and enzyme concentration (0.5 μM), mean residue molar ellipticity ($[\theta]$) was calculated. The content of secondary structure elements were calculated using software packages as SELCON3 [18], CDSSTR [19] and CONTIN [19].

Fluorescence emission spectra of ACE in the presence of different concentrations of TFE (0.1–20%) were recorded on a Shimadzu spectrofluorimeter (Tokyo, Japan) using 1-cm-pathlength cuvettes. The excitation wavelength was set to 280 nm and the emission spectra were measured in the wavelength range of 300–500 nm.

2.14. Kinetic studies

To determine the kinetic parameters of purified enzyme, ACE activity was examined under enzyme assay conditions, in the presence of different FAPGG concentrations (0.026–0.28 mM). The Michaelis constant (K_m) and maximum velocity of