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Journal of Chromatography A, 950 (2002) 125–130

JOURNAL OF CHROMATOGRAPHY A

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Improved method for direct high-performance liquid chromatography assay of angiotensin-converting enzyme-catalyzed reactions

Jianping Wu, Rotimi E. Aluko*, Alister D. Muir

Agriculture and Agri-Food Canada, Saskatoon Research Station, 107 Science Place, Saskatoon, SK, Canada S7N OX2

Received 29 November 2001; received in revised form 9 January 2002; accepted 9 January 2002

Abstract

A rapid and sensitive assay was developed for determination of the activity of angiotensin-converting enzyme (ACE) in the presence of inhibitory peptides present in soybean protein hydrolysates. The method utilizes reversed-phase high-performance liquid chromatography (HPLC) to separate and quantify hippuryl-histidyl-leucine (HHL) and hippuric acid (HA). HHL and HA were separated on a Symmetry C_{18} column by gradient elution that used mixtures of trifluoroacetic acid (TFA)–acetonitrile and TFA–water as solvents. Analytical time and baseline separation of HA from HHL were improved over previous HPLC methods. In comparison to the standard spectrophotometric method, the new HPLC method obviates the need for ethyl acetate extraction of HA but requires direct injection of the ACE reaction mixture onto the HPLC column.

Keywords: Angiotensin-converting enzyme; Enzymes; Enzyme inhibitors; Proteins; Peptides; Hippuric acid

1. Introduction

Angiotensin-converting enzyme (ACE) is a dipeptidyl carboxypeptidase (EC 3.4.15.1) that catalyzes the conversion of angiotensin I to the potent vasoconstrictor angiotensin II. ACE also catalyzes the degradation of bradykinin, a vasodilatory peptide [1,2]. Inhibition of ACE activity leads to a decrease in the concentration of angiotensin II with a concomitant reduction in blood pressure. The first ACE peptide inhibitors were discovered from snake venom and their significant role in the treatment of hypertension was recognized immediately [3]. Subsequently, potent synthetic inhibitors of ACE, such as captopril, have been used widely as drugs in the clinical treatment of hypertension [4]. Apart from synthetic compounds, certain peptides derived from hydrolysis of food proteins inhibited the activity of ACE in vitro and reduced blood pressure of spontaneously hypertensive rats [5,6]. Therefore, anti-ACE peptides derived from food protein hydrolysates can be used as ingredients in the formulation of functional foods and nutraceuticals that have blood pressure-lowering properties.

In order to facilitate the identification and isolation of peptides with anti-ACE properties, a simple, rapid, and reliable method for assessment of in vitro

*Corresponding author. Department of Foods and Nutrition, University of Manitoba, Winnipeg, Manitoba, Canada R3T 2N2. Tel.: +1-204-474-9555; fax: +1-204-474-7592.

E-mail address: alukor@ms.umanitoba.ca (R.E. Aluko).

0021-9673/02/$ – see front matter
PII: S0021-9673(02)00052-3

ACE inhibition is desirable. Several methods for the measurement of ACE activity have been described including spectrophotometry [7–9], bioassay [10], fluorimetry [11–13], high-performance liquid chromatography (HPLC) [14–17], and internally quenched fluorogenic methods [18]. However, the Cushman and Cheung protocol [7] is the most commonly utilized by the pharmaceutical and food industries. It is based on the hydrolysis of hippuryl-histidyl-leucine (HHL) by ACE to give hippuric acid (HA) and histidyl-leucine as products. The HA is extracted into ethyl acetate and quantified by measuring the absorbance in a spectrophotometer at 228 nm. This method requires several steps and the ethyl acetate extract could be contaminated with HHL, which also absorbs strongly at 228 nm. The objective of this work was to develop an alternative rapid and sensitive HPLC method for direct analysis of the ACE reaction mixture through complete separation of HHL and HA.

2. Experimental

2.1. Reagents

Chemicals and enzymes were obtained as follows: angiotensin-converting enzyme (from rabbit lung), HHL, and pepsin (Sigma, St. Louis, MO, USA); HA and trifluoroacetic acid (TFA) (Acros Organics, NJ, USA), HPLC-grade acetonitrile (Fisher Scientific, Nepean, Canada). All other chemicals were of reagent grade and also obtained from Fisher Scientific. HPLC-grade water was generated by a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Protein hydrolysate preparation

Soybean protein hydrolysate was prepared in a 250-ml reactor with temperature and pH control devices. Soy protein (Archer Daniels Midland, Decatur, IL, USA) was dispersed in distilled water to obtain a 5% (w/v) protein slurry. The slurry was heated to 37 °C, pH adjusted to 2.0 using 2 *M* HCl and pepsin added at 4.0% level (w/w, protein basis). Samples were withdrawn at 30-, 60-, 120-, 180-, 300-min reaction intervals and placed in a boiling water bath for 10 min to inactivate the enzyme. Samples were cooled to room temperature (23–25 °C), centrifuged (16 000 *g*; 10 min; 25 °C) and freeze dried.

2.3. Sample preparation for HPLC analysis

For direct HPLC analysis the total reaction volume was 70 μl, made up of 50 μl of 2.17 m*M* HHL, 10 μl of 2 mU of ACE and 10 μl of different concentrations of soybean protein hydrolysate (all prepared with 100 m*M* borate buffer, containing 300 m*M* NaCl, pH 8.3). The HHL and soybean protein hydrolysates were combined and maintained at 37 °C for 10 min in 1.5 ml polyethylene microcentrifuge tubes. ACE was also maintained at 37 °C for 10 min before the two solutions were combined and incubated at 37 °C in an Eppendorf Thermomixer R (Brinkmann Instruments, New York, USA) with continuous agitation at 450 rpm. The reaction was terminated after 30 min of agitation by addition of 85 μl of 1 *M* HCl and the solution filtered through a 0.45-μm nylon syringe filter for reversed-phase (RP)-HPLC analysis.

2.4. High-performance liquid chromatography

HPLC was performed on a 2690 separation module equipped with a 996 photodiode array detection (DAD) system (Waters, Miliford, MA, USA). Instrument control, data collection and analysis were undertaken using Millennium Chromatography Manager software v 2.15 (Waters). Samples (10 μl) were analyzed on a Symmetry C_{18} column (3.0×150 mm, 5 μm, Waters) and HA and HHL were detected at 228 nm. The column was eluted (0.5 ml min^{-1}) with a two solvent system: (A) 0.05% TFA in water and (B) 0.05% TFA in acetonitrile, with a 5–60% acetonitrile gradient for the first 10 min, maintained for 2 min at 60% acetonitrile, then returned to 5% acetonitrile for 1 min. This was followed by isocratic elution for 4 min at the constant flow-rate of 0.5 ml min^{-1}. External standard HA samples were prepared freshly on a daily basis and used for calculation of the concentration of HA.

2.5. HPLC-DAD mass spectrometry (MS)

Atmospheric pressure chemical ionization (APCI)

mass spectra were obtained on a Quattro liquid chromatograph (Micromass, UK) equipped with an APCI probe, a Z-Spray interface, separation module and a DAD system (Waters). Sample was prepared as described in Section 2.3. Instrument control and data analysis were performed using the MassLynx software (Micromass), while chromatography was carried out on a Symmetry C_{18} column (150×2.0 mm; 5 μm). The same mobile phase (except that 0.05% TFA was replaced with 0.01% formic acid) was applied as described in Section 2.4 at a flow-rate of 0.3 ml min^{-1}. Positive and negative ion intensities were recorded from m/z 50 to 500 with a 1.5-s scan time. The analyzer vacuum was 2.2×10^{-5} Torr (1 Torr=133.322 Pa).

2.6. Standard Cushman and Cheung method

The ability of soybean protein hydrolysate to inhibit activity of ACE was determined using the spectrophotometric method of Cushman and Cheung [7], except that the total volume was increased to 350 μl from the standard 250 μl. The higher reaction volume was required to provide enough sample for HPLC and spectrophotometric analyses. The same composition as described above (Section 2.3) was used except that 6.5 m*M* of HHL was applied. The reaction was carried out in 15-ml disposable plastic centrifuge tubes at 37 °C in a water bath with constant shaking. The reaction was terminated after 30 min by addition of 1 *M* HCl and 145 μl withdrawn for HPLC analysis. The remaining reaction volume was extracted by vortexing with 1.5 ml ethyl acetate for 30 s to extract HA. After centrifugation (2000 *g*; 5 min), 1.0 ml of the ethyl acetate layer was transferred to a clear glass tube and evaporated by heating at 120 °C for 30 min. The residue was dissolved in 3.0 ml water and the absorbance determined at 228 nm in an Ultrospec 1000 UV–visible spectrophotometer (Amersham–Pharmacia Biotech, Cambridge, UK) using water as blank. After spectrophotometric analysis, the aqueous solution was concentrated to 0.5 ml by heating in a boiling water bath. An aliquot of 10 μl of the concentrated solution was used for HPLC analysis according to the procedure described in Section 2.4 in order to determine whether the ethyl acetate extract contained HHL.

2.7. Data analysis

Analysis of variance and Duncan multiple range tests were performed to determine significant differences among treatments using the Statistical Analysis System software, v 6.10 (SAS Institute, Cary, NC, USA). Linear regression was performed using the Microsoft Excel-97 SR-1 software (Microsoft, USA).

3. Results and discussion

3.1. HPLC separation of HA from HHL

Fig. 1a shows the chromatogram of the ACE

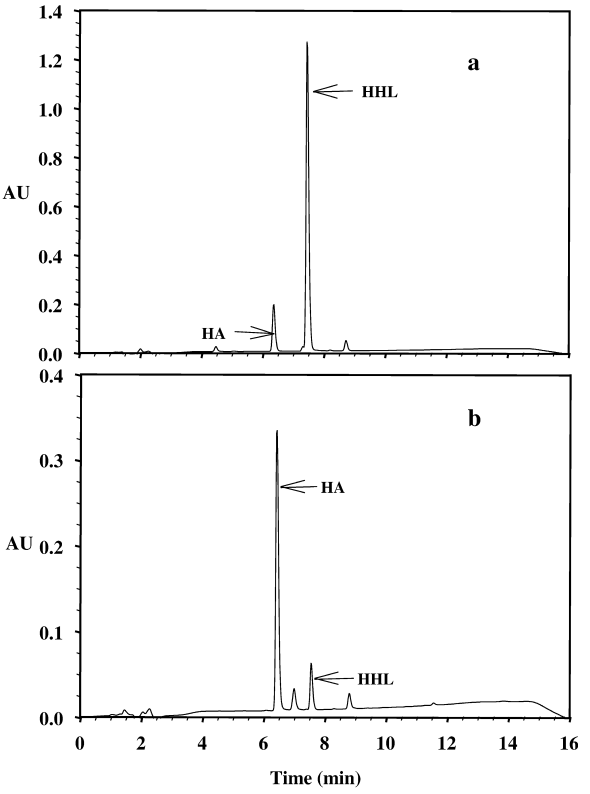

Fig. 1. (a) RP-HPLC separation of ACE reaction mixture that contained soybean protein hydrolysate. An aliquot of 10 μl of the reaction mixture containing 1.55 m*M* HHL was injected. (b) RP-HPLC separation of the aqueous solution of residue from ethyl acetate extraction of ACE reaction mixture that contained soybean protein hydrolysate. An aliquot of 10 μl of the aqueous solution was injected.

reaction mixture that contained soybean protein hydrolysates. Complete baseline separation of HA and HHL was achieved by RP-HPLC on a Symmetry C_{18} column and the detector response for pure HA was found to be linear over the range of 0.01 m*M* to 1.0 m*M*, HA (1.79×10^{-2}–1.79 μg; $y=1\times10^{7}x+18011$, $R^2=0.9998$). The relative standard deviation was 1.40% for determination of a 0.2 m*M* HA solution ($n=10$). In order to further confirm that HA is a product of ACE-catalyzed hydrolysis of HHL, the reaction mixture was acidified with HCl before addition of ACE. Complete inhibition of ACE was obtained in the acidic condition and no HA was detected in the reaction mixture (results not shown). The HA extract obtained from the Cushman and Cheung [7] method was also analyzed by the newly developed HPLC method. Results showed that the ethyl acetate extract contained some levels of unreacted HHL (Fig. 1b), which accounted for 12% of the total absorbance of the extract.

RP-HPLC analysis of HA has been previously demonstrated using several different column chromatography techniques [15–17,19]. However, several of these methods involved analysis of HA after ethyl acetate extraction as in the Cushman and Cheung [7] method. For example, a micro assay of ACE in serum utilized hippuryl-glycyl-glycine as substrate and the HA produced was extracted into ethyl acetate [14]. The solvent was subsequently evaporated to dryness and the residue dissolved in buffer and injected into the chromatograph to quantify HA [14]. In the Chiknas method [19], an alkaline HPLC solvent was used with a flow-rate of 2 ml min^{-1} with a standard C_{18} RP column, while Meng et al. [16] used a neutral pH solvent system and a phenyl-RP column. In both cases, a relatively high concentration of HHL was used (5 vs. 2.17 m*M* used in this study) and there were large HHL peaks in the HPLC trace of the ethyl acetate fraction. This was surprising since the basis of the Cushman and Cheung [7] method is that HA is selectively extracted into the ethyl acetate phase. Direct HPLC analysis of ACE activity in serum and tissue extracts was also reported utilizing a shielded hydrophobic phase column [15]; however, the incomplete baseline separation of HA from HHL could lead to an incorrect estimation of the potency of ACE inhibitory compounds. In the most recent report of the use of HPLC to analyze ACE inhibition, inhibition of ACE by peptide fractions obtained from ultrafiltration or Sephadex G-25 column chromatography was determined using C_8 RP columns [17]. As in earlier methods, a high concentration of HHL was used, a larger reaction volume (225 μl compared to 70 μl in this study), and it was unclear whether or not ethyl acetate extraction was used as a large HHL peak was present in their chromatograms [17].

In comparison to previous methods, the HPLC method developed in this study used the crude protein hydrolysates as part of the ACE reaction mixture in a total reaction volume of 70 μl. Baseline separation of HA from HHL was achieved in 8 min using a commonly available C_{18} reversed-phase column and a mobile phase composed of acetonitrile and water containing TFA. The reduced analytical time and improved baseline separation of HA from HHL when compared to previous reports [15,16] may be due to differences in the type of columns and solvents used. In the present report, the use of a smaller pore size C_{18} reversed-phase column seemed to be more efficient than the shielded hydrophobic phase [15] and phenyl silica gel [16] columns. Moreover, our method used a mobile phase that contained a mixture of acetonitrile and TFA under gradient and isocratic elution conditions in comparison to the acetonitrile–ammonium phosphate buffer and solely isocratic elutions of the previous reports [15,16]. Although baseline separation of HA from HHL was achieved in less than 8 min (Fig. 1), gradient elution for 17 min was applied to wash out all UV-absorbing substances and eliminate any adsorbed peptides, prior to a new separation. No clogging and concomitant deterioration of column performance was detected after HPLC analysis of 1000 samples of ACE reaction mixtures that contained soybean protein hydrolysates.

3.2. Identification of HA peak by MS

The presence of HA in the enzyme reaction mixture was confirmed by HPLC–MS analyses. The peak at 6.90 min in the HPLC reconstructed total ion current chromatogram (Fig. 2a) of a typical ACE-catalyzed reaction inhibitory activity assay showed a quasi-molecular ion $[M-H]^-$ at m/z 178 (Fig. 2b) and $[M+H]^+$ at m/z 180 (Fig. 2c), which were

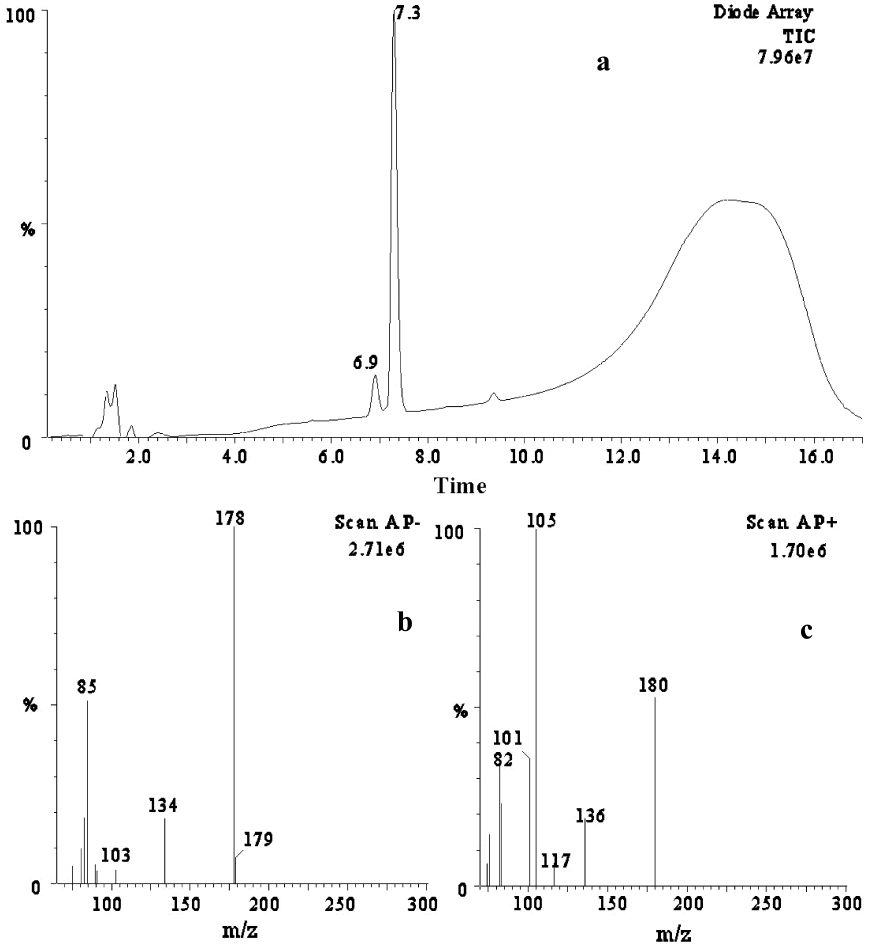

Fig. 2. (a) RP-HPLC-reconstructed total ion current chromatogram of the ACE-catalyzed reaction mixture that contained soybean protein hydrolysate. An aliquot of 10 μl of the reaction mixture containing 1.55 m*M* HHL was injected. (b) Negative ion APCI mass spectrum of the HA peak eluting at 6.9 min in (a). (c) Positive ion APCI mass spectrum of the HA peak eluting at 6.9 min in (a).

identical to that observed for authentic HA. Results of the MS analysis showed that there was no interfering compound (such as a peptide or HHL) embedded in the HA peak.

4. Conclusions

Results from the present study have shown that the Cushman and Cheung protocol [7] does not completely separate the ACE substrate (HHL) from the product (HA). Since both HHL and HA absorb at 228 nm, the Cushman and Cheung method [7] overestimates the amount of HA produced during ACE-catalyzed reactions. The HPLC method shown in this report completely eliminates interference from HHL during quantification of the HA peak. The new method eliminates the time-consuming process of extraction of HA into ethyl acetate that is required by the standard Cushman and Cheung method [7]. Analytical time and baseline separation of HA from HHL was improved when compared to previous HPLC methods. The new method provides a simple, rapid and accurate method for the assay of ACE-catalyzed reactions, especially in the presence of inhibitor compounds such as protein hydrolysates. The new method involves use of a C_{18} reversed-phase column and a mobile phase (acetonitrile–

water–TFA), which can be adapted readily for on-line automatic assay. The standard spectrophotometric assay required at least 250 μl of reaction mixture, compared to the 70 μl used in this HPLC assay.

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