An Assay of Angiotensin-Converting Enzyme Activity Obtained Using Capillary Electrophoresis

MEI-CHAO CHIANG, SHU-CHUAN YANG, KEN-YUON LI AND TSUN-CHUNG TSAI*

Department of Food Science, Tunghai University, 181, Sec. 3, Taichung Port Rd., Situn District, Taichung City 407, Taiwan, R.O.C.

ABSTRACT

The authors developed a simple method to measure angiotensin-converting enzyme (ACE) activity using capillary electrophoresis (CE). With hippuryl-L-histidyl-L-leucine (HHL) as the substrate, we separated and quantified the enzymatic product, hippuric acid (HA) using capillary zone electrophoresis (CZE). CZE was performed with a 65 cm fused-silica capillary (55 cm effective length, 75 μm I.D.), using a 10 or 20 mM phosphate solution (pH 6.0) as the running buffer with an applied voltage of 20 kV at room temperature. The HA was identified and monitored at 228 nm.

Key words: angiotensin-converting enzyme, hippuryl-L-histidyl-L-leucine, hippuric acid, capillary electrophoresis

INTRODUCTION

Hypertension, an increasingly prevalent disease, is today one of the top ten causes of death in Taiwan. Angiotensin-converting enzyme (ACE), a dipeptidyl carboxypeptidase (EC 3.4.15.1), is an important blood pressure regulator that catalyzes the release of His-Leu from the carboxyl terminus of angiotensin I, which, in turn, generates a potent vasopressor octapeptide, angiotensin II. ACE is also involved in the degradation of the vasodilator bradykinin[1].

Many bioactive peptides, e.g., opioid peptides and inhibitory peptides for ACE, have been isolated from the enzymatic digests of food proteins of animal and plant origin[2]. More potent ACE inhibitors and the derivatives of these inhibitory peptides have also been designed and synthesized to treat hypertension effectively[3]. As oral administration of these derivatives frequently results in unwanted side effects, a nutritional approach (consuming functional foods that naturally contain ACE inhibitory peptides) should be the preferred medium by which blood pressure is controlled.

A variety of methods (e.g., spectrophotometry and fluorometry) by which ACE activity can be detected and analyzed are available to researchers[4,5]. High-performance liquid chromatography (HPLC) is widely used because of its effective separation of the substrate and product from the ACE reaction mixture to ensure accurate measurements. HPLC requires the use of a reverse phase C18 column and large amounts of organic solvents (acetonitrile or methanol)[6,7].

The most common electrophoresis buffer used for ACE assays done on a Capillary Electrophoresis (CE) system is sodium borate buffer, due to the dual role the latter plays as the basal solution for the ACE assay mixture[8,9]. It is convenient to inject a reacted ACE mixture directly into the CE system to analyze ACE activity. However, the ACE reaction mixture usually contains high levels of salt (borate buffer and NaCl concentrations were 150 mM and 0.5 M, respectively)[4]. High salt concentrations in the electrophoresis buffer cause elevated electrophoretic current and the joule-heating effect achieved by raising current levels broadens separated peaks and reduces detection sensitivity[10]. Among the efforts underway to alleviate the effects of salt is research into using HEPES (150 mM) as an alternative buffer in kinetic study of ACE[11]. While using acetonitrile to extract ACE products from reacted assay mixtures is another method by which sample salt levels can be reduced, the presence of this chemical in injected samples has been found to cause a dilution effect and reduce detection sensitivity. As a rule of thumb, the amount of buffer concentration used as an CE electrolyte should be below 100 mM. A level of 20-30 mM would be even better.

CE is a powerful analytical technique that provides a high resolution, while requiring lower sample quantities and less running solvent[10]. In this study, we developed a simple capillary electrophoresis method to measure ACE activity in an investigation of inhibitory peptide using a laboratory assembled CE.

MATERIALS AND METHODS

I. *Reagents*

Hippury-L-histidyl-L-leucine (HHL), hippuric acid (HA) and angiotensin-converting enzyme (ACE), obtained from rabbit lungs, were purchased from Sigma (St. Louis, MO, USA). All other reagents were analytical grade.

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* Author for correspondence. Tel: +886-4-23506747;
  Fax: +886-4-23506747; E-mail: tct1@mail.thu.edu.tw

Standard solutions were prepared by dissolving HHL and HA, respectively, in phosphate buffer.

II. *Apparatus*

Electrophoretic experiments were carried out on a laboratory assembled CE comprising a high voltage power supply (Glassman High Voltage Inc., Whitehouse Station, NJ, USA), UV-detector (Rainin Instrument Inc.,Woburn, MA, USA), and a 65 cm × 75 μm I.D. fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA), with the detection window placed at 55 cm. Samples were applied in a 2 second electrokinetic injection at an applied voltage. Experiments were conducted at room temperature. Both HHL and HA were monitored at 228 nm. Data were collected and peak migration time and area were analyzed using a Chromatocorder 21 (SIC System Instruments Co., Ltd., Rancho Santa Fe, CA, USA)

III. *ACE Assay*

We slightly modified Cushman's method[4], using a 5 mM of HHL substrate solution and 60 mU/mL of ACE solution as our test solutions. Samples were dissolved in 100 mM of borate buffer containing 0.4 M NaCl at pH 8.3. To begin the assay, 5 μL of de-ionized water was added to 45 μL of the HHL solution and incubated in a water incubator at 37°C for 5 min. Afterward, 15 μL of ACE solution was added and incubated at 37°C for 30 min. After mixing the result, further enzymatic reactions were stopped by the addition of 65 μL of trifluoroacetic acid. Five microliter of protein hydrolysate were used instead of de-ionized water to determine inhibitory activity.

RESULTS AND DISCUSSION

I. *Analytic Condition*

Separation was achieved by optimizing the pH and buffer concentrations, applied voltage level and organic modifier. We chose to use a 65 cm capillary tube for convenience in our self-assembled CE apparatus.

We conducted preliminary experiments at 20 mM phosphate buffer, with the following pH values: 5, 6, 7, 8 and 9. Gotti *et al.*[13] and Hillaert *et al.*[14] used a phosphate buffer to separate ACE inhibitors widely used in the treatment of mild to moderate hypertension and heart failure, either alone or in conjunction with other drugs[15]. Figure 1 shows the effect of the running buffer pH on the separation of HHL and HA. Holding applied voltage conditions constant, we achieved complete substrate and product separation at all pH values noted. This is likely due to the large molecular weight and small charge differences. pK histidine values and terminal carboxyl group of HHL and HA would be 6.5-7.4 and 3.5-4.0, respectively[16]. The migration time of these compounds in an electric field is dependent upon electroosmotic flow velocity and charge mobility. The electroosmotic flow velocity from anode to cathode is much greater than the charge mobility. In this case, the HHL charge would approximate zero at pH 5 and become negatively charged above pH 5 and HA would be negative charged under all pH values used in our experiment (see above). The electrophoretic migration due to the charge occurred opposite to the direction of the electroosmotic flow. As the negative charge to the HHL mass was less than that to HA, HHL migrated faster than HA. The electroosmotic flow velocity increased sigmoidally with as
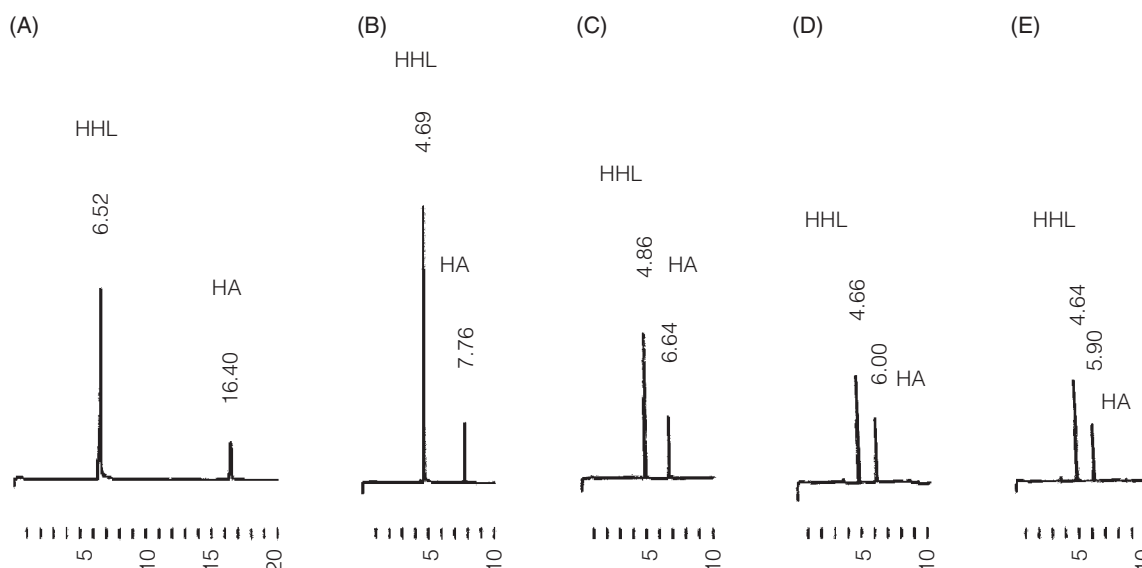

Figure 1. Effect of running buffer pH value on the migration times of HHL and HA. (A) pH 5.0; (B) pH 6.0; (C) pH 7.0; (D) pH 8.0; (E) pH 9.0. Experiments were carried out using a fused-silica capillary 65 cm (55 cm to the detector) × 75 μm I.D., 20 mM phosphate buffer at varying pH as the running buffer, an applied voltage of 20 kV, electronic injection time of 2 sec at applied voltage, and a detection wavelength of 228 nm. Sample: a solution containing 2.5 mM HA and 2.5 mM HHL in water.

the running buffer pH increased. Both compounds migrated faster initially and then gradually slowed with the increasing pH level. The HHL peak height increased and then decreased slowly with the increasing pH level. The peak HA height increased initially and then remained nearly constant with the increasing pH level.

The pH 6 running buffer produced a reasonable migration time with satisfactory separation. The pH 6 running buffer concentration effect on separation is shown in Figure 2. All running buffer concentrations tested produced complete separation within 10 min. HA migration time increased initially with the increase in running buffer concentration before plateauing after 25 mM. Conversely, HHL migration time remained steady in the 10-30 mM phosphate buffer concentration range. We chose the 10 mM phosphate buffer to test the effect of

applied voltage on reaction mixture separation because it delivered a relatively higher HA sensitivity, due likely to the larger HA quantity introduced during electrokinetic injection in the lower ionic strength buffer.

Five different applied voltage levels were tested in this experiment (Figure 3). Both HHL and HA migration times decreased with increased voltage levels and all applied voltage levels produced complete standard mixture separation. High applied voltage levels may lead to heat production and reduced resolution across successive measurements. In order to complete the experiment within a 10 min time frame, 20 kV applied voltage will be a better choice.

Organic solvents added to the buffer electrolyte alter the polarity and the viscosity of the mobile phase. As a consequence, both electroosmotic flow and electrophoretic
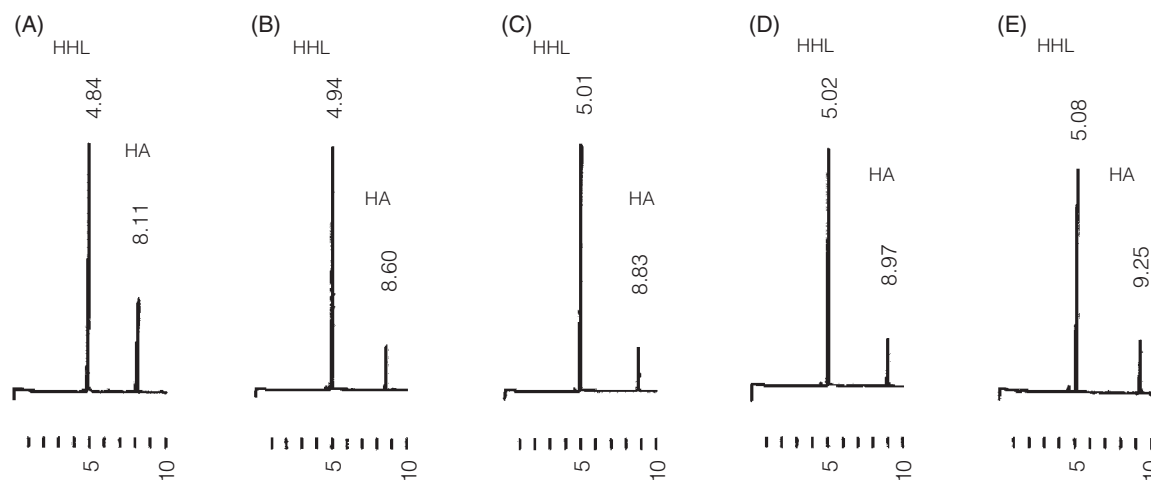

Figure 2. Effect of running buffer concentration on the migration times of HHL and HA. (A) 10 mM; (B) 15 mM; (C) 20 mM; (D) 25 mM; (E) 30 mM. Phosphate buffer at varying concentrations (pH 6.0) were used as the running buffer. Other experimental conditions were the same as those used in Figure 1.
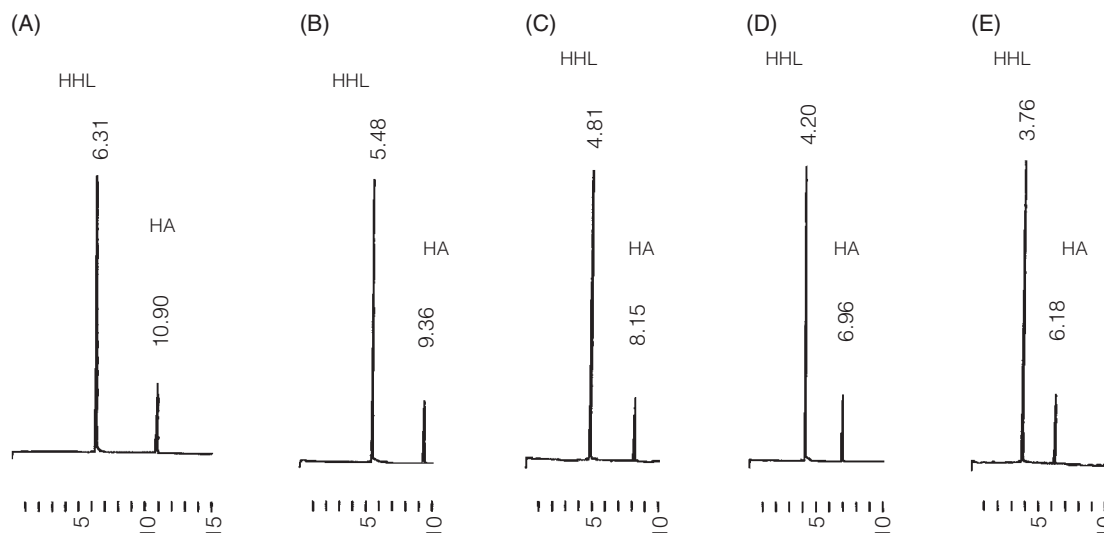

Figure 3. Effect of voltage on the migration times of HHL and HA at varying voltage: (A) 16 kV; (B) 18 kV; (C) 20 kV; (D) 22 kV; (E) 24 kV. Other experimental conditions were as in Figure 2.
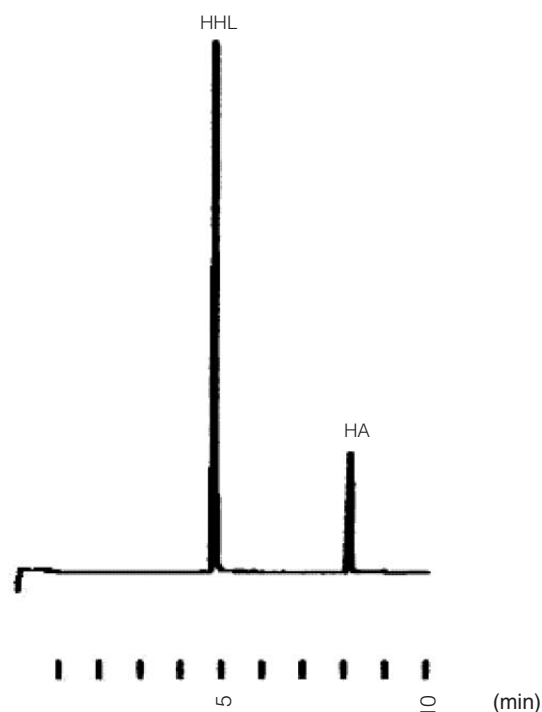
Figure 4. An electropherogram of optimal conditions using the capillary electrophoresis method. (1) standard HHL; (2) standard HA. Experiments were carried out using a fused-silica capillary 65 cm (55 cm to the detector) × 75 μm I.D., 10 mM phosphate buffer at pH 6.0 as the running buffer, an applied voltage of 20 kV, electronic injection time of 2 sec, and a detection wavelength of 228 nm. Sample: a solution of 2.5 mM HA and 2.5 mM HHL in water.

Table 1. RSD (%), linearity, and sensitivity of HA in the CE System

	HA
Migration time RSD (n = 8)	0.196
Peak area RSD (n = 8)	2.885%
Peak height RSD (n = 8)	2.138%
Linearity	1-250 μM
Correlation coefficient	0.9989
Concentration limit detection	1 μM
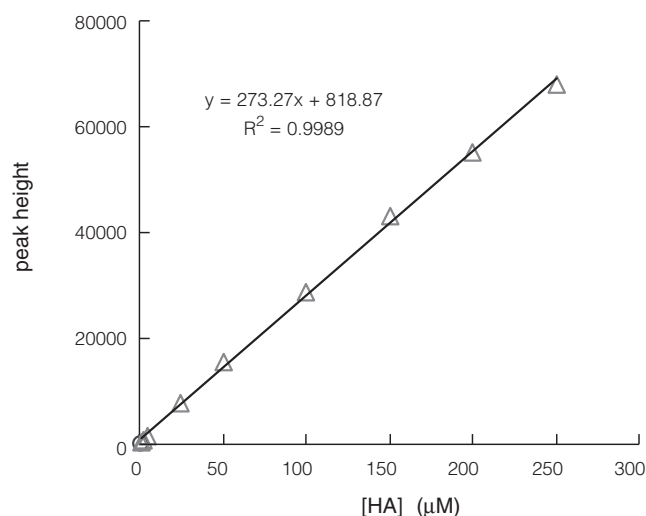
Figure 5. Standard curve for HA obtained from the CE electropherogram.

mobility of analytes are affected. Acetonitrile and methanol were tested in this study to determine their effects on the separation. Both solvents produced longer migration times in both HHL and HA and better separations. Complete standard mixture separation could have been achieved without the addition of an organic solvent. Figure 4 shows the separation of a standard mixture under optimal conditions. Table 1 shows the separation condition analysis. In this study, each CE run was completed in less than 10 min, while the HPLC method with methanol and acetonitrile as the solvents usually required more than 15 and 20 min, respectively. The CE method displayed good reproducibility and linearity over a wide concentration range (Figure 5) and good sensitivity, even at low levels of HA.

II. *Application to Enzymatic Reaction Analysis*

Using the method proposed, all tested running buffer concentrations from 10 mM to 40 mM produced satisfactory HHL and HA separation in standard mixtures, with an optimal running buffer concentration of 10 mM phosphate buffer for the standard mixture. Figure 6A shows the application results from a inhibitory enzymatic sample analysis of α-zein hydrolysate. Because of the high salt concentration (0.4 M NaCl and 100 mM borate) used in

fixing enzymatic reaction reagents, highly concentrated trifluoroacetic acid was used to stop the enzymatic reaction. If 10 mM phosphate running buffer was used, the high ionic strength and low acidity in sample would skewed HA peak. Therefore, the enzymatic reaction solution could be two-fold diluted with de-ionized water before injection or, alternatively, a 20 mM phosphate running buffer could be used instead of a 10 mM buffer (Figure 6C) to avoid undesirable effects. Unlike the CE method developed by Zhang *et al.*[9], the capillary used in our proposed procedure does not require successive rinsing with 0.1 N NaOH and deionized water after the end of each run, nor does it require rinsing with buffer prior to each run. The proposed method saved time over alternatives and proved satisfactory in the analysis of inhibitory peptides in enzymatic α-zein hydrolysates.

CONCLUSIONS

Compared with the spectrophotometric and HPLC methods, capillary electrophoresis is simpler and faster and results in significantly improved separation. The proposed method requires neither the application of organic solvents nor the use of an ODS column. Also, the CE method required much smaller amounts of sample and buffer to render accurate results. The proposed method was especial-
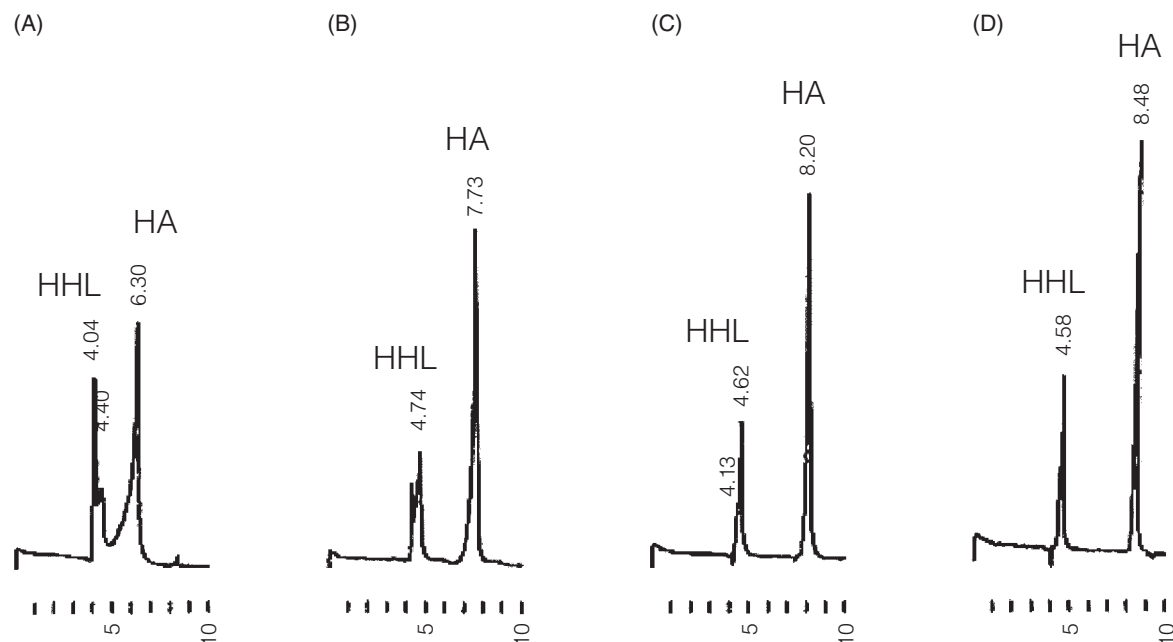
Figure 6. Effect of running buffer concentration on HHL and HA separation. (A) 10 mM; (B) 20 mM; (C) 30 mM; (D) 40 mM. Refer to the ACE assay text for a description of enzyme reaction conditions.

ly good for ACE inhibitory peptide screening. The low buffer concentration used as the electrolyte in this method gives much better sensitivity and linearity than any other previously developed CE method for measuring ACE activity. The proposed method can be applied directly to analyze an ACE reaction mixture without product organic extraction prior to injection.

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