Evaluation of Angiotensin I Converting Enzyme Inhibitory Activity by an FIA Combined with Biosensors

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Angiotensin I converting enzyme (ACE) plays an important role in the regulation of blood pressure. Therefore, inactivation or inhibition of ACE is quite effective in lowering blood pressure or preventing the pathogenesis of hypertension.¹ The ACE inhibitors such as captopril and enalapril have made it possible to treat hypertension effectively, for they act as the antihypertensive drugs. In the field of food chemistry, much attention is devoted to discover food materials with antihypertensive efficacy from natural resources. Accordingly, determination of ACE inhibitory activity is important in ACE inhibitor screening. Several methods for the measurement of ACE inhibitory activity have been developed, including colorimetric² and HPLC,³ with that of Cushman and Cheung⁴ being most commonly utilized. But this method requires many steps, including extraction of products and evaporation. Here we describe a method for the assay of ACE inhibitory activity using a flow injection analytical system combined with immobilized enzymes by quantifying the amount of hippuric acid liberated from hippuryl-histidyl-leucine (HHL) by the action of ACE. The high sensitivity of enzymes coupled with the electrochemical signal transducer should ideally result in an enzyme electrode capable of measuring the concentration of the substrate in a medium containing a diverse mixture of other compounds.

Experimental

Reagents

Aminoacylase (EC 3.5.1.14, from pig kidney, 3500 U mg⁻¹) was purchased from Wako Chemicals (Osaka, Japan). D-Amino acid oxidase (D-AOD, EC 1.4.3.3, from hog kidney, 15 U mg⁻¹) were from Boehringer Mannheim GmbH. Glutaraldehyde (25% w/w) and D-alanine were from Nacalai Tesque (Kyoto, Japan). Aminopropyl controlled-pore glass (APCPG, 80 – 120 mesh) and angiotensin I converting enzyme (ACE, EC 3.4.15.1, from rabbit lung, 4 U mg⁻¹) were from Sigma (St. Louis, MO). Hippuric acid and HHL were obtained from Katayama Chemicals (Osaka, Japan) and Peptide Institute, Inc. (Osaka), respectively. Poly sodium 4-styrene sulfonate and poly L-lysine bromate were obtained from Aldrich (Milwaukee, WI). All other chemicals were of analytical reagent grade and were used as received.

Procedure

Enzyme reactors were prepared as reported previously⁵ and were blocked with L-alanine. The reaction scheme, that we used to quantify hippuric acid is shown in Fig. 1. The carrier solution (50 mM phosphate buffer) containing the substrate of D-AOD (0.4 mM D-alanine) was pumped through the system; thus, hydrogen peroxide was constantly produced in the D-AOD reactor, giving a high blank base line, and it was monitored amperometrically on the platinum electrode. The concentration of hippuric acid was determined in an indirect way, by monitoring the decrease of the hydrogen peroxide production by benzoate which acts as an inhibitor on D-AOD, resulting a negative peak from the base line.⁶ Benzoate was produced from hippuric acid by the action of the aminoacylase reactor. To increase the production of benzoate and thus get significant amperometric responses, the reaction time between aminoacylase and hippuric acid was extended using a stopped flow analytical technique that was described in our previous paper.⁷

Flow system

The amperometric measurements were carried out using a stopped flow injection system with an electrochemical flow-through cell. The cell was equipped with a reference electrode (Ag/AgCl), a working electrode (Pt) and a counter electrode (stainless-steel). The carrier solution was pumped through the system by an HPLC pump through an air dumper at a flow rate of 1 ml min⁻¹. The main flow line was divided into two lines by a six way-switching valve. The six way-switching valve was

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Fig. 1 Reaction scheme of the hippuric acid quantification system.

Table 1 The IC₅₀ values of some ACE inhibitors measured by the FIA method and by the spectrophotometric method

Inhibitor	IC ₅₀ (μM) ± SD		
	FIA	Spectrophotometry	Other
Captopril	0.039 ± 0.0089	0.044 ± 0.0127	0.04 ^a
Val-Tyr	18.4 ± 0.67	17.2 ± 0.58	22 ^b
Gly-Tyr	256 ± 28.3	268 ± 9.5	259 ^c
Leu-Tyr	76 ± 14.8	81 ± 9.8	79 ^d

a. Ref. 12. b. Ref. 13. c. Ref. 14. d. Ref. 15.

switched to the aminoacylase reactor for hippurate intake or to a blank column for by-pass flow. The blank column was the same size as the enzyme reactor and contained only L-alanine blocked APCPG as a support. The potential of +0.65 V was applied on Pt electrode vs. Ag/AgCl by a potentiostat. The surface of the Pt electrode was covered with a poly ion complex membrane made of poly L-lysine hydrobromide and poly sodium 4-styrenesulfonate.⁸ The flow system was operated at room temperature.

Standard assay method of ACE activity and preparation of samples for FIA

The standard batch assay was performed according to the methods reported by Lieberman⁹ and Yamamoto *et al.*¹⁰ with some modifications. Preparation of samples for FIA was similar to the spectrophotometric method up to the incubation. In the FIA method, after incubation, reactions were terminated by immersing the test tubes in a boiling water bath. Then, samples were appropriately diluted with carrier buffer and injected. The response of hippurate was monitored amperometrically.

Results and Discussion

As described in our previous paper,⁷ this method has been applied to the determination of ACE activity and compared to the spectrophotometric method. A linear relation was obtained between negative peak current and logarithms of hippuric acid concentration in the range of 15 to 1000 μM. The method showed good agreement with the conventional Lieberman's modified method, giving a correlation coefficient of 0.97. In the present study, IC₅₀ values of some ACE inhibitors were measured by the new method and were compared to the spectrophotometric method. The results are summarized in Table 1. The concentration of ACE inhibitor required to inhibit 50% of the ACE activity was defined as IC₅₀. In the spectrophotometric method, the inhibitory ratio (%) is estimated as follows:

$$\text{Inhibitory ratio} = (A_C - A_S)/(A_C - A_B) \times 100.$$

Here, A_C , A_S and A_B represent the absorbance of control, sample and blank, respectively. In the FIA system, converting the peak height to enzymatic activity (mU ml⁻¹) can be accomplished through the transformation of the concentration of the hippuric acid to mU ml⁻¹ directly. Therefore, peak height was used instead of absorbance:

$$\text{Inhibitory ratio} = (PH_C - PH_S)/(PH_C - PH_B) \times 100.$$

Here, PH_C , PH_S and PH_B represent the peak height of control,

sample and blank, respectively. Each datum of the inhibitory ratio to calculate IC₅₀ value was the mean of triplicates. According to Table 1, IC₅₀ value of captopril was found to be nearly 0.04 μM by both method. This was similar to the value that was reported by Ondetti. This method was also applied to measure the IC₅₀ value of some peptides, including Val-Tyr,^{11,13} Gly-Tyr¹⁴ and Leu-Tyr.¹⁵ The results are in good agreement with the results obtained from the spectrophotometric method and from the other methods that were previously reported which are cited in the Refs. 12 – 15. In addition, the performance of the proposed method was judged by calculating the Students's t- and F-tests for each inhibitor. At the 95% confidence level, the calculated t- and F-values do not exceed the theoretical values, indicating that there is no significant difference between the values of each inhibitor that were obtained from the FIA and from the spectrophotometric method. Moreover, the result of the paired t-test was $t = 0.3693$, and no significant difference between two methods under $p < 0.05$ was found.

On consideration of interference, many food components did not interfere with the system. The remains can be prevented by polyion complex on Pt electrode, except for high concentrations of ascorbic acid and ethanol as discussed in our previous papers.^{6,7} In addition, small amounts of interference substances can be neglected as the original samples are diluted more than 40-fold before injection. However, ascorbic acid and polyphenol compounds¹⁶ have to be removed before measurement. In conclusion, FIA system also can be used for determination of ACE inhibitory activity.

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