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Direct spectrophotometric measurement of angiotensin I-converting enzyme inhibitory activity for screening bioactive peptides

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Abstract

A direct, extraction-free spectrophotometric assay was developed for determination of angiotensin I-converting enzyme activity (ACE) in the presence of ACE inhibitors using hippuryl-L-histidyl-L-leucine (HHL) as the ACE-specific substrate. This method relies on previously published spectrophotometric determination of hippuric acid (HA) content in the urine, the method of which was based on the specific colorimetric reaction of HA with benzene sulfonyl chloride (BSC) in the presence of quinoline. The proposed ACE inhibition assay was applied to the measurement of the ACE inhibitory activity of Captopril. IC_{50} value of Captopril corresponded well with literature data. Furthermore, Alcalase hydrolysates of mung bean and rice protein isolates were assessed for ACE inhibitory activity by this method. These two hydrolysates showed high ACE inhibitory activity. This method proposed here was shown to be direct, sensitive, accurate, reproducible, and less expensive without separation of HA from ACE reaction mixture, and can be used for the screening of ACE inhibitory peptides derived from food proteins.

Keywords: Angiotensin I-converting enzyme; Direct spectrophotometric assay; Bioactive peptides; Protein hydrolysates; Hippuric acid; Benzene sulfonyl chloride

1. Introduction

Angiotensin I-converting enzyme (ACE, dipeptidyl carboxpeptidase, EC 3.4.15.1) plays an important role in the regulation of blood pressure as well as cardiovascular function. ACE converts the inactive decapeptide angiotensin I into the potent vasoconstricting octapeptide angiotensin II and also inactivates vasodilator, bradykinin [1]. Thus, inhibition of ACE results in a decrease in blood pressure. Many potent synthetic ACE inhibitors such as captopril, enalapril, lisinopril, and ramipril have been widely used in the clinical treatment of hypertension and heart failure in humans. However, synthetic ACE inhibitors can have side effects including cough, taste disturbances and skin rashes [2]. Therefore, interest in identifying foods as natural sources of ACE inhibitors has increased.

Many ACE inhibitory peptides have recently been discovered from enzymatic hydrolysates of different food proteins such as casein [3,4], whey protein [5], fish proteins [6], soybean protein [7], and corn gluten [8]. These peptides with in vitro ACE inhibitory activities have been well demonstrated having in vivo inhibitory properties on ACE and antihypertensive effects without side effects in spontaneously hypertensive rats and hypertensive human [9]. These food protein-derived ACE inhibitory peptides show great promise in the development of a novel physiologically functional food for preventing hypertension as well as for therapeutic purposes.

In order to facilitate the identification and isolation of ACE inhibitory peptides, establishment of a simple, sensitive and reliable in vitro ACE inhibition assay is desirable. Numerous methods for the measurement of ACE activity have

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been reported, including spectrophotometric [10–13], fluorometric [14,15], radiochemical [16,17], high-performance liquid chromatography (HPLC) [18–20], and capillary electrophoresis methods [21,22]. These assay methods also were used to obtain information on the inhibitory potency for different ACE inhibitory substances. With respect to radiochemical, HPLC and capillary electrophoresis methods, these methods require specific expensive instruments that are usually not available in common laboratories. The spectrophotometric method described by Cushman and Cheung [10] is the most commonly utilized in the assays for determining ACE activity and inhibition in vitro. It is based on the hydrolysis of hippuryl-L-histidyl-L-leucine (HHL) by ACE to hippuric acid (HA) and histidyl-leucine (HL) as products. The extent of HA release from HHL is directly related to the ACE activity. This method, however, involves several tedious steps including HA extraction with ethyl acetate, evaporation, redissolution in water and then the measurement of absorbance of HA at 228 nm, and moreover, ethyl acetate is able to extract unhydrolyzed HHL, which also absorbs strongly at 228 nm [20], thus overestimating the ACE activity.

In this study, we firstly transformed the method, which was used to determine HA content in urine, into an ACE inhibition assay based on the detection of the production of HA from HHL by the action of ACE in the presence of ACE inhibitors with some modification. This method proposed here was shown to be direct, sensitive, accurate, reproducible, and less expensive without separation of HA from ACE reaction mixture, which will facilitate the screening of ACE inhibitory peptides.

2. Experimental

2.1. Chemicals and reagents

ACE (from rabbit lung; 3.4 units/mg of protein), HHL and captopril were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Alcalase 2.4 L FG (a declared activity of 2.4 AU/kg and a density of 1.18 g/ml) was kindly provided by Novo Nordisk (Bagsvaerd, Denmark). HA, quinoline and benzene sulfonyl chloride (BSC) were of analytical grade and purchased from Shanghai Chemical Co. (Shanghai, China). All other reagents used were of analytical grade. Doubly distilled water was used throughout.

2.2. Preparation of protein hydrolysates with ACE inhibitory activity

Mung bean and rice protein isolates (prepared from the defatted mung bean and rice flour, respectively, in our laboratory) were hydrolysed, respectively, in a 250 ml reactor in controlled conditions (temperature, pH and stirring speed) using the pH-stat method [23]. Protein isolates were suspended in distilled water to obtain 4% (w/v) protein solutions. The solutions were then equilibrated at 55 °C and the pH adjusted to 8.0 with 2 M NaOH before addition of the enzyme. Alcalase 2.4 L was then added to the solutions at a ratio of 20 μl/g of protein. After hydrolysis at 55 °C and pH 8.0 for 2 h in both cases, the enzymatic reactions were terminated by heating for 10 min in boiling water. The protein hydrolysates were centrifuged at 10000 × *g* for 20 min, and the resulting supernatants were used for measurement of ACE inhibitory activity. The protein concentrations of the supernatants were determined by the method of UV absorbance difference at 215 and 225 nm [24] using bovine serum albumin as standard.

2.3. Calibration curve of HA

A stock standard solution of HA was prepared by dissolving HA in 100 mM sodium borate buffer (pH 8.3) containing 300 mM NaCl to a concentration of 0.4 mg/ml. A series of working standard HA solutions were obtained from the stock standard solution by dilution with the same buffer over the range from 0.02 to 0.16 mg in a volume of 500 μl placed in 10-ml cuvettes with stopples. Subsequently, 600 μl of quinoline was added to the cuvette followed by mixing on the vortexing mixer for 10 s. After this step, 200 μl of BSC was added to the mixture, the reaction mixture was then immediately mixed on the vortexing mixer for 20 s in the darkness and allowed to stand for 30 °C in a water bath at 30 min in the darkness. Then 3700 μl of ethanol was added to the mixture and the mixture continued to stand for 30 min in the darkness. Finally, 200 μl of the reaction mixture was transferred into the 96-well flat-bottom plate and the absorbance was measured in a microplate reader (Multiskan MK3, Thermo Labsystems Co.) at 492 nm. All measurements were performed in triplicate. A calibration curve was obtained by plotting the absorbance at 492 nm versus HA concentration.

2.4. Assay for ACE inhibitory activity

ACE inhibitory activity was measured by the procedures summarized in Table 1. For each assay, a sample solution of ACE inhibitor (20 μl) with 50 μl of 5 mM HHL in 100 mM sodium borate buffer (pH 8.3) containing 300 mM NaCl was preincubated at 37 °C for 5 min. The reaction was initiated by the addition of 10 μl of ACE solution (100 mU/ml), and the mixture was incubated at 37 °C for 30 min. The reaction was stopped by adding 100 μl of 1 M HCl. Sodium borate buffer was then added to the reaction mixture to a volume of 0.5 ml. The HA released from HHL by ACE was monitored according to the method of Wang et al. [25] which was used to determine HA content in urine with some modification as described above under Section 2.3. All measurements were performed in triplicate.

According to the procedures shown in Table 1, the extent of inhibition was calculated as follows:

$$\text{ACE inhibitory activity (\%)} = \frac{B - A}{B - C} \times 100$$

Table 1
Procedure for assay of ACE inhibitory activity

	A (sample)	*B* (control)	*C* (blank)
100 mU/ml ACE (μl)	0	0	10
1 M HCl (μl)	0	0	100
5 mM HHL (μl)	50	50	50
ACE inhibitor (μl)	20	0	20
100 mM sodium borate buffer (μl)	0	20	0
Incubated at 37 °C for 5 min			
100 mU/ml ACE (μl)	10	10	0
Incubated at 37 °C for 30 min			
1 M HCl (μl)	100	100	0
ACE inhibitor (μl)	0	20	0
100 mM sodium borate buffer (μl)	320	300	320
Quinoline (μl)	600	600	600
BSC (μl)	200	200	200
Incubated at 30 °C for 30 min in the darkness			
Ethanol (μl)	3700	3700	3700
Incubated at 30 °C for 30 min in the darkness and then measured at 492 nm			

where *B* is the absorbance of control (buffer added instead of test sample), *C* the absorbance of the reaction blank (HCl was added before ACE), and *A* the absorbance in the presence of sample.

The IC_{50} value was defined as the concentration of inhibitor required to inhibit 50% of the ACE activity under the assayed conditions and determined by regression analysis of ACE inhibition (%) versus log (inhibitor concentration) (μM or mg/ml).

2.5. Statistical analysis

All results were expressed as the mean ± S.D. The statistical analysis of data was performed using SAS (1999) statistical software system (SAS institute, Cary, NC, USA). The statistical significance for the IC_{50} value between two methods was determined by Student's *t*-test. Differences were considered significant at $P < 0.05$.

3. Results and discussion

3.1. Choice of the optimal ratio of quinoline to BSC

The effects of various quinoline-to-BSC ratios on the chromogenic reaction were investigated with a specific concentration of HA (0.12 mg per tube) in the present assay system. As can be seen from Fig. 1, when the molar ratios of quinoline to BSC were less then 3, BSC–quinoline caused a dose-dependent intensity of the quinoline–BSC–HA chromogen. But over that ratio, the intensity tended to level off. The results from this study were similar to those of Wang et al. [25]. In the present experiments, a final ratio of 3.2 was used.

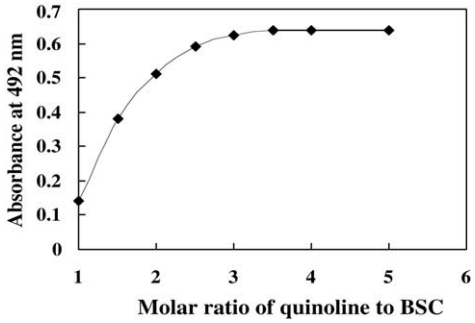

Fig. 1. Effect of molar ratio of quinoline to BSC on the chromogenic reaction. Each ratio of quinoline to BSC was added to the incubation mixture containing 0.16 mg of HA per tube. All measurements were performed in triplicate.

3.2. Effect of temperature on the chromogenic reaction

The effect of temperature on the chromogenic reaction was determined at 20 and 30 °C (Fig. 2). At 20 °C, the absorbance of the quinoline–BSC–HA chromogen increased with incubation time up to 4 h and then tended to level off. However, when incubated at 30 °C, the absorbance reached a plateau at 30 min and no increase of absorbance was observed with increased incubation time. The quinoline–BSC–HA chromogen was found to be quite stable in the incubation medium in the darkness. There were no observable changes in the absorbance of the reaction mixture in the course of the experiment and even after standing for 8 h at room temperature in the darkness.

3.3. Absorption spectra

Most microplate readers use interference filters to select for a wavelength of choices. However, filters for only limited wavelengths are available. To decide on the use of the best filter the absorption spectra of the complex and the reagent blank were measured under conditions of the assay on a UV–vis spectrophotometer (UV-2102 PCS, Unico Co.) with 1.0 cm quartz cell. The absorption spectra of the complex and the reagent blank are shown in Fig. 3. The

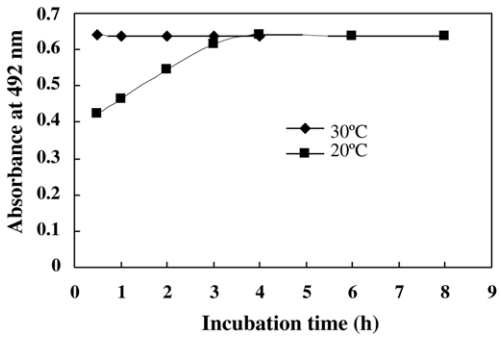

Fig. 2. Effect of temperature on the chromogenic reaction. The incubation mixture contained 0.16 mg of HA per tube. All measurements were performed in triplicate.

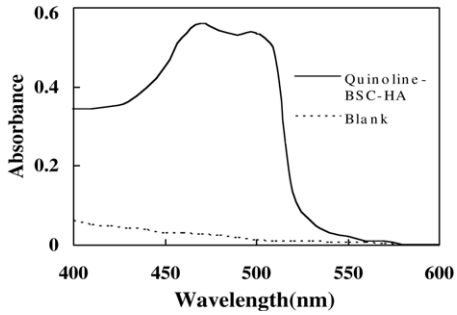

Fig. 3. Absorption spectra of HA complex and reagent blank. Conditions: HA, 0.12 mg in 5 ml solution (per tube), molar ratio of quinoline to BSC, 3.2. Solutions were prepared according to the procedure described in Section 2.

quinoline–BSC–HA complex exhibited two absorption peaks at 470 and 500 nm, respectively. The maximum was observed at 470 nm with slightly higher absorbance value than at 500 nm. The reagent blank against ethanol has practically negligible absorption at this region. However, the reagent blank had higher absorbance at short wavelength than at long wavelength. No absorbances were also observed for HHL and a product HL in the assay solution at this region (data not shown), suggesting that the presence of HHL and HL does not interfere with the reaction of HA with quinoline–BSC. Thus, the more widely available 492 nm filter was used in this assay. At this wavelength, the sensitivity was reduced only by 5% when compared to 470 nm.

3.4. Calibration curve, assay sensitivity and precision

Under the assay conditions, calibration curve of HA was obtained by plotting absorbance at 492 nm versus HA concentrations. The calibration curve showed an excellent linearity over the concentration range from 0.02 to 0.16 mg of HA in 5 ml solution. The detection limit (amount causing an identical absorbance to the blank value, thrice the standard deviation) was found to be 5.80 μg/ml in the assay solution. Working standard solutions of HA containing five different concentrations were analyzed in six replicates. The relative standard deviation (R.S.D.) ranged from 0.91 to 5.46% for the assay under the described conditions. This result suggested that the reproducibility and precision of the assay were excellent.

3.5. ACE inhibitory assay and screening for bioactive peptides

The proposed method was applied to the measurement of HA released from HHL by the action of ACE in the presence of Captopril, a widely used synthetic ACE inhibitor, and protein hydrolysates obtained with Alcalase, respectively, to determine the ACE inhibitory activities and IC_{50} values of these inhibitors. The ACE inhibitory activity of Captopril was determined to validate the ACE inhibition assay proposed in this paper. The results are summarized in Table 2 in comparison with those by the conventional method of Cushman and Cheung [10]. As a result, the IC_{50} value of Captopril obtained by the present method was 0.019 μM, which was similar to that of 0.022 μM obtained by the conventional one. The values obtained by the two methods were also very similar to literature data [12,26]. In the case of protein hydrolysates, both inhibition indices and IC_{50} values obtained by the present method were similar to those by the conventional method with no significance ($P < 0.05$). However, the IC_{50} values of all inhibitors obtained by the present method were slightly lower than those by the conventional method. This may be explained by the facts that the ethyl acetate is able to extract unhydrolyzed HHL, which also absorbs at 228 nm and accounts for 12% of the total absorbance of the extract, thus, overestimating the amount of HA produced during ACE-catalyzed reaction in the conventional method [10,20]. As can be seen in Table 2, the R.S.D. ranged from 0.51 to 2.62% for the present assay. These values were lower than those obtained by the conventional procedure probably due to the possibility of simultaneous measurement of large amounts of samples in 96-well plate without extraction of HA from the reaction mixture when compared to the classical UV method of Cushman and Cheung [10] performed with cuvettes. These results indicated that the conventional method has poorer reproducibility and precision than the present method. Furthermore, the present method has another advantage of lower consumption of reagents such as HHL and ACE due to its high sensitivity.

Table 2
Comparison of the results obtained by the present and conventional methods

Samples	Inhibition index (%)[a]		IC_{50} value[b]		R.S.D. (%) for IC_{50} value	
	Present	Conventional	Present	Conventional	Present	Conventional
Captopril	96.54 ± 3.76	93.26 ± 3.95	0.019 ± 0.00[c]	0.022 ± 0.0004[c]	0.51	1.82
Unhydrolyzed mung bean protein	2.49 ± 0.07	2.16 ± 0.08	nd[d]	nd	–	–
Unhydrolyzed rice protein	1.76 ± 0.04	2.13 ± 0.05	nd	nd	–	–
Mung bean protein hydrolysate	76.42 ± 4.73	70.38 ± 5.56	0.621 ± 0.016[c]	0.645 ± 0.025[c]	2.62	3.88
Rice protein hydrolysate	93.27 ± 6.88	89.16 ± 5.74	0.139 ± 0.002[c]	0.146 ± 0.003[c]	1.48	2.10

[a] ACE inhibition was determined with 20 μl of a 0.1 μM inhibitor solution for Captopril, and 10 mg protein/ml for unhydrolyzed proteins and protein hydrolysates.
[b] The unit of IC_{50} is μM and mg protein/ml for Captopril and protein hydrolysates, respectively.
[c] Within the same rows, means with same superscripts are not significantly different ($P > 0.05$).
[d] Not determined.

Table 3
ACE inhibitory activities of mung bean and rice protein hydrolysates obtained at different hydrolysis times[a]

Hydrolysis time (h)	ACE inhibitory activity (%)	
	Mung bean protein	Rice protein
0.5	65.54 ± 3.26	85.94 ± 4.96
1	67.08 ± 3.87	96.83 ± 5.12
2	76.42 ± 4.73	93.27 ± 6.88
4	71.13 ± 4.15	80.22 ± 3.65
6	65.54 ± 2.11	77.93 ± 4.01
8	55.71 ± 2.56	78.03 ± 2.67
10	52.66 ± 1.89	68.51 ± 3.10

[a] ACE inhibition was determined with 40 μl of a 10 mg protein/ml hydrolysate solution by the present method. All measurements were performed in triplicate.

In addition, the ACE inhibitory activities of mung bean and rice protein hydrolysates obtained at different enzymaticly hydrolyzing times (0.5, 1, 2, 4, 6, 8, 10 h) were measured using the two methods to further validate the ACE inhibition assay proposed in this paper (Table 3). The inhibition indices obtained by the present method were plotted against those obtained by the conventional method (Fig. 4). The regression equation was calculated to be $y = 0.9724x + 0.6201$ with the correlation coefficient of $r = 0.9891$ ($n = 14$). The results obtained by the two methods were similar, which again confirmed that the method proposed here was reliable.

In the ACE inhibition assay, another extraction-free colorimetric method had been proposed by Matsui et al. [12]. This method was based on the specific binding of 2,4,6-trinitrobenzene sulfonate (TNBS) to the primary amine group of HL, the other hydrolytic product of the substrate HHL. The TNBS-dipeptide derivative has a maximum absorbance at 416 nm. However, if the assay sample is a mixture of peptides, as in the case for the protein hydrolysates, there is a possibility that primary amino moiety of peptides other than those from the HL can react with TNBS. Therefore, the present method has a definite advantage over the colorimetric methods described by Cushman and Cheung and Matsui et al., respectively, in terms of its high reproducibility, precision, and specificity without tedious separation of HA from reaction mixture when screening for ACE inhibitory peptides.

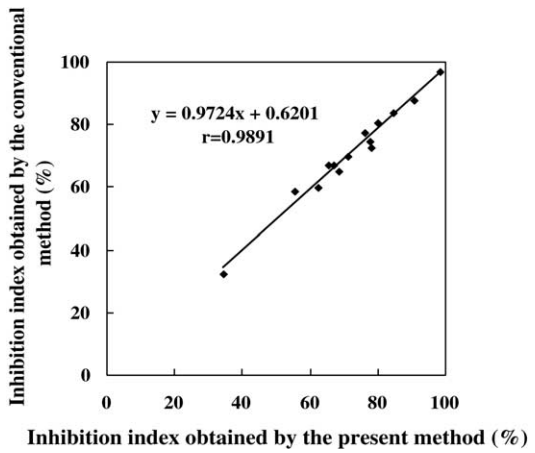

Fig. 4. Correlation between the inhibition index (%) of protein hydrolysates obtained by the present and conventional methods ($P < 0.01$). All measurements were performed in triplicate.

Mung bean and rice protein isolates were subjected to hydrolysis with Alcalase, an alkaline protease produced from *Bacillus licheniformis*, at 55 °C and pH 8.0. The obtained hydrolysates were used for the measurement of ACE inhibitory activity by the proposed method. Before hydrolysis, the mung bean and rice protein isolates showed no inhibitory activity on ACE. ACE inhibitory activities were generated from the proteins after enzymatic hydrolysis. This result suggested that peptides releasing from intact protein by enzymatic hydrolysis were responsible for ACE inhibition. To bring together the results as described above, it can be concluded that the proposed method is suitable to screen for ACE inhibitory peptides derived from food proteins with a possible antihypertensive effect in vivo.

4. Conclusions

In this paper, a direct, extraction-free spectrophotometric method was developed for measurement of angiotensin I-converting enzyme inhibitory activity of ACE inhibitors using HHL as the ACE-specific substrate. This method is shown to be direct, sensitive, accurate, reproducible, and less expensive. Compared to the conventional method, our method does not require tedious separation of HA from reaction mixture. The proposed method is suitable to screen for ACE inhibitory peptides derived from food proteins with a possible antihypertensive effect in vivo.

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