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Flow injection analysis of angiotensin I-converting enzyme inhibitory activity with enzymatic reactors

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ABSTRACT

Assay of angiotensin I-converting enzyme (ACE) inhibitory activity always draws much attention because of diverse applications in the field of antihypertension and related pathogenesis. Recently, the use of a new synthetic substrate, 3-hydroxybutyrylglycyl-glycyl-glycine (3HB-GGG), for the assay of ACE inhibitory activity has been confirmed. To construct a rapid, economical, and automatic determination system of ACE inhibitory activity using 3HB-GGG, a flow injection analysis (FIA) system with enzymatic reactors was developed in this study. Enzyme reactors were composed of aminoacylase and 3-hydroxybutyrate dehydrogenase immobilized separately on CNBr-activated Sepharose 4B. The assay condition was optimized in terms of the conversion of 3HB-G into NADH by the enzymatic reactors when the reaction solution containing 3HB-G generated from 3HB-GGG (after the incubation with ACE) was repetitively injected into the FIA system. Under the optimized conditions, 3HB-G was converted to 3HB, and then 3HB was oxidized by NAD^+ to form NADH. The developed system successfully detected practical ACE inhibitors with a great sensitivity, high sampling frequency (10 samples h^{-1}) and a durable stability of the enzymatic reactors.

1. Introduction

Angiotensin I-converting enzyme (ACE) is a dipeptidyl carboxypeptidase associated with the renin–angiotensin system [1]. One of its key actions is the regulation of blood pressure together with water and salt metabolism, since it cleaves angiotensin I into a potent vasopressor angiotensin II. Besides, ACE inactivates bradykinin, a hypotensive peptide, by sequential removal of two C-terminal dipeptides [2]. The increase in serum ACE activity results in blood-pressure elevation and related diseases, such as sarcordosis, silicosis, and hyperthyrodis [3]. Therefore, the assay of ACE as well as the assay of ACE inhibitory activity was necessary to identify antihypertensive compounds in order to prevent high-blood pressure and related pathogenesis.

In the field of functional food, studies on active components (principally peptides hydrolyzed from various food sources) that can inhibit the ACE activity with the aim to control hypertension through diet have been carried out [2]. In order to facilitate the identification and isolation of ACE inhibitors, numerous methods for the measurement of ACE activity have been reported. In most of those works, the principle of the ACE assays was based on a hydrolysis of the synthetic peptide hippuryl-His-Leu (HHL) [4], which has been used widely. However, this conventional method often encounters interferences because of the use of invisible wavelength 228 nm and the extraction of unexpected components with organic solvent.

A newly synthesized substrate, 3-hydroxybutyrylglycyl-glycyl-glycine (3HB-GGG), has been proposed for ACE inhibition assay [5,6]. It was the measurement of ACE inhibitory activity based on the detection of 3-hydroxybutyric acid (3HB) derived from 3HB-GGG by the action of ACE and aminoacylase. The generated 3HB was detected spectrophotometrically by a commercial F-kit [5]. Subsequently, the costly F-kit was substituted by a more effective measurement of 3HB with a water-soluble tetrazolium salt (WST-1) [6]. Those established assays using 3HB-GGG were applicable for the evaluation of ACE inhibitory activity of practical samples. In addition, they were more selective and convenient than the conventional method using HHL.

In this study, a double-line FIA system with enzymatic reactors for ACE inhibition assay using 3HB-GGG was developed with the aim to construct a rapid, economical, and automatic analysis system. The principle of the assay is shown in Scheme 1. The FIA system with aminoacylase reactor and 3-hydroxybutyrate dehydrogenase (3HBDH) reactor was used to measure 3HB-G derived from 3HB-GGG by the hydrolysis of ACE. Aminoacylase and 3HBDH were immobilized on CNBr-activated Sepharose 4B. In the FIA system, 3HB-G was a sample subjected to a hydrolysis by aminoacylase to form 3HB and Gly. The product, 3HB, was subsequently oxidized by NAD^+ to form NADH and acetoacetate with the catalysis of 3HBDH. The formed NADH was spectrophotometrically detected at 340 nm.

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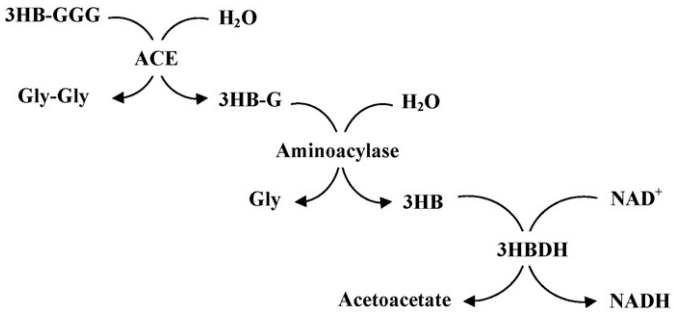

Scheme 1. Principle of assay of ACE inhibitory activity using FIA system with enzymatic reactor.

2. Experimental

2.1. Reagents and chemicals

CNBr-activated Sepharose 4B was obtained from GE Healthcare (Uppsala, Sweden). 3-Hydroxybutyrate dehydrogenase (EC 1.1.1.30, 140 units/mg, from *Pseudomonas sp.*) and aminoacylase (acylase I, EC 3.5.1.14, 4300 units/mg, from pig kidney) were purchased from Wako (Osaka, Japan). The F-kit was purchased from R-Biopharm (Darmstadt, Germany). ACE (EC 3.4.15.1, 3.4 units/mg, from rabbit lung), DL-ß-hydroxybutyric acid sodium salt (DL-3HB), and Val-Tyr were purchased from Sigma (St Louis, MO, USA). 3HB-GGG, 3HB-G, and WST-1 were obtained from Dojindo Laboratories (Kumamoto, Japan). All chemicals and solvents were of analytical grade and were prepared with water purified by a Milli-Q system (Millipore, Tokyo, Japan).

Five kinds of antihypertensive drinks (Tokuho, in Japanese) containing either Val-Tyr or a mixture of Ile-Pro-Pro and Val-Pro-Pro were purchased from local supermarkets and kindly gifted from Senmi Ekisu Co. (Ehime, Japan).

2.2. Apparatus

The diagram of the double-line FIA system (PD-2000, Ogawa Co., Kobe, Japan) with the enzymatic reactors was shown in Fig. 1. The phosphate buffer (0.1 M, pH 8.0) and NAD^+ solution were pumped with two plunger pumps at a total flow rate up to 3.0 mL min^{-1}. The samples were injected into the stream through a manually operated six-way valve. The absorbance was measured at 340 nm with a Chratec colorimeter (KCM-0306, Ogawa Co., Kobe, Japan) and recorded with a potentiometric recorder (Servocorder SR 6211, Graphtec, Tokyo, Japan). The peak height reflected the concentration of 3HB-G in the sample.

The sample solutions were ultrafiltrated with Centricut (cut-off size 10 kDa, Kurabo Industries, Osaka, Japan) before being injected into the FIA system. The ultrafiltration process was performed with a Hitachi compact centrifuge (Himac CF 16 RX, Tokyo, Japan).

2.3. Enzyme immobilization

CNBr-activated Separose 4B was selected as the support for enzyme immobilization, because it provides a convenient way to immobilize ligands by the cyanogens bromide method [7]. Sepharose (0.25 g) was added to 25 mL of 1 mM HCl with occasionally gentle shaking for 15 min in order to swell the gel. After the HCl solution was filtered out, the retained gel was washed with 200 mL of phosphate buffer (0.1 M, pH 8.0). The obtained gel was put into the enzyme solution containing 1.3 mg of 3HBDH or 100 mg of aminoacylase in 1.0 mL of phosphate buffer (0.1 M, pH 8.0). The mixture was shaken adequately and kept at 4 °C overnight for enzyme immobilization. The mixture of enzyme and gel was stuffed into a glass tube (2.0 mm i.d., 150 mm length) by a peristaltic pump (ATTA Co., Tokyo, Japan). The two extremities were closed with glass wool and filter paper. The reactor was washed with phosphate buffer (0.1 M, pH 8.0) to remove the excessive enzyme. The protein concentration of the enzyme solutions was determined by measuring the absorbance at 280 nm before and after the immobilization. The coupling yields were calculated as the percentage disappearance of the amount of protein initially added to the reaction mixture [8]. They were 81% and 21% for 3HBDH and aminoacylase, respectively.

2.4. Assay of ACE inhibitory activity

To a 1.5-mL snap-lock microtube, 125 μL of 12.35 mM 3HB-GGG, 15 μL of sample (ACE inhibitors), and 50 μL of 0.2 U mL^{-1} ACE were added. The substrate solution was freshly prepared by dissolving 3HB-GGG in borate buffer containing 380 mM NaCl (pH 8.3), meanwhile the ACE solution was prepared with borate buffer (pH 8.3) [5]. The reaction mixture was stirred thoroughly for 20 s and then incubated at 37 °C for 30 min. Right after the incubation, the reaction was immediately terminated by ultrafiltration with Centricut (cut-off size 10 kDa, Kurabo Industries, Japan) for 10 min at 5000 rpm (10 °C) to remove ACE. The solution containing 3HB-G was then injected into the FIA system as a sample.

2.5. Optimization of the FIA system

Conditions of the FIA system were optimized in terms of the conversion of 3HB-G and 3HB by the action of aminoacylase and 3HBDH. The conversion of 3HB-G and 3HB was calculated based on the calibration curve of 3HB-G, 3HB, and NADH. The standard solutions of 3HB-G, 3HB, and NADH at various concentrations were alternately injected into the FIA system to make the calibration curves between the absorbance and the concentration of substances. The 3HB solution was prepared by dissolving the DL-3HB in buffer (the concentration of D-isomer was half of the DL-3HB

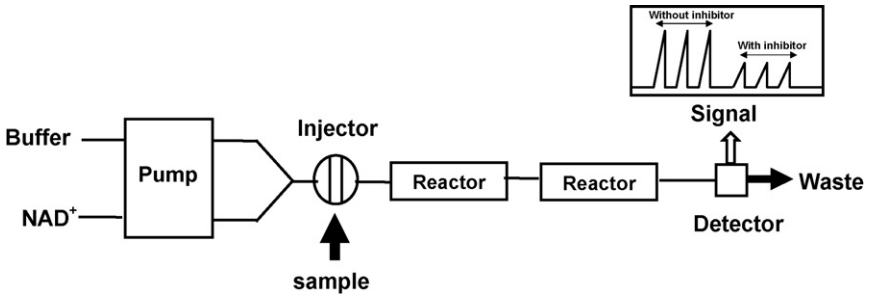

Fig. 1. Flow diagram of FIA system with enzymatic reactors for assay of ACE inhibitory activity. 0.1 M phosphate buffer, pH 8.0; 1 mM NAD^+ in 0.1 mM phosphate buffer, pH 8.0; injection volume, 20 μL.

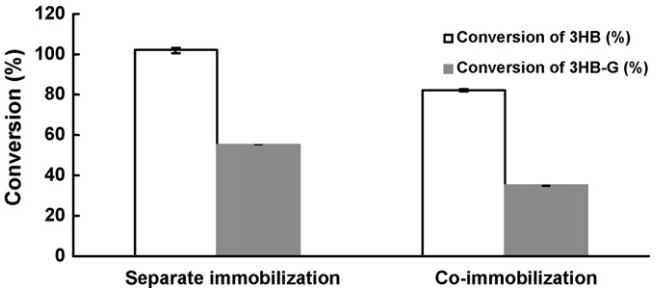

Fig. 2. Dependence of conversion ratios on the immobilization methods of enzymes. 3HBDH, 1.3 mg; aminoacylase, 70 mg; 0.1 M phosphate buffer, pH 8.0; 1 mM NAD^+; flow rate, 0.6 mL min^{-1}.

concentration). All reagents were dissolved in the buffer, and the concentrations of the reagents were expressed as those of the final mixture solutions. The conversion (%) of 3HB by 3HBDH was calculated as the slope of the calibration curve of 3HB per that of NADH. The conversion (%) of 3HB-G was the ratio of the slope of the calibration curve of 3HB-G to that of NADH. The system conditions, including flow rate and enzymatic reactors, were examined to obtain a high conversion rate. In addition, the generation of 3HB-G from 3HB-GGG, which was influenced by the concentration of substrate 3HB-GGG and enzyme ACE, was also considered to get an adequate response when it was introduced into the FIA system. All assays were performed in triplicate.

3. Results and discussion

The FIA system with 3HBDH and aminoacylase reactors employed 0.1 M phosphate buffer as the carrier solution. Since the optimum individual working pH is 8.5 for 3HBDH [9] and 7.5 for aminoacylse [10], pH 8.0 was applied as the favorable pH for the activities of both enzymes.

The effect of the loading of 3HBDH on the conversion of 3HB to NADH was surveyed in a range of 1.3–5.0 mg of enzyme loading (data not shown). With this range of enzyme, 3HBDH always showed very high activity even at a flow rate up to 1 mL min^{-1}, and the conversion of 3HB to the final product was always 100%. As a result, the lowest loading of 3HBDH, 1.3 mg, was chosen to be the most favorable enzyme amount in this study.

3.1. Influence of different immobilization methods on the activity of enzymatic reactor

To compare the efficiency of the separate-immobilization method (each enzyme was packed in a separate reactor) and the co-immobilization method (both enzymes were packed in one reactor), two different working systems were prepared. In the system using separately immobilized enzymes, aminoacylase reactor was set into the flow line, followed by 3HBDH reactor. In the case of the co-immobilized enzymes, two reactors containing both aminoacylase and 3HBDH were set in succession. The conversion rates obtained from two different immobilization methods were depicted in Fig. 2. As observed, the separate-immobilization method showed a higher efficiency (102% for the conversion of 3HB and 55% for the conversion of 3HB-G) than the co-immobilization method (82% for the conversion of 3HB and 35% for the conversion of 3HB-G). When the enzymes were packed into separate columns, the activity of 3HBDH was extremely high, showing that the separate-immobilization condition of 3HBDH (1.3 mg of 3HBDH per 0.25 g Sepharose for one glass tube) was optimal. Consequently, the 3HBDH reactor could be used as the optimum reactor for subsequent experiments without any further modification. On the other hand, it was necessary to improve the low conversion ratio of 3HB-G with aminoacylase by a change in the running condition of the FIA system.

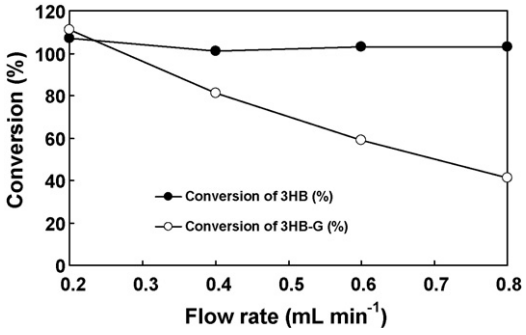

Fig. 3. Effect of flow rate on the conversion ratio of 3HB-G and 3HB. 3HBDH, 1.3 mg; aminoacylase, 70 mg; 0.1 M phosphate buffer, pH 8.0; 1 mM NAD^+; flow rate, 0.2–0.8 mL min^{-1}.

3.2. Effect of flow rate on the activity of enzymatic reactors

To improve the conversion rate of 3HB-G, the flow rate of the FIA system was changed to increase the reaction time between 3HB-G and aminoacylase. The dependence of the conversion of 3HB-G on the flow rate was illustrated in Fig. 3. As observed, 3HB was always converted completely by 3HBDH, meanwhile the conversion of 3HB-G by aminoacylase increased linearly with the decrease in the flow rate. Aminoacylase reactor showed a perfect conversion when the system worked at the flow rate of 0.2 mL min^{-1}. However, in that condition, the sampling frequency was only 6 samples h^{-1}. To satisfy the demand for a higher throughput and conversion, a flow rate of 0.4 mL min^{-1}, at which the output was 10 samples h^{-1} and the conversion by aminoacylase was 81%, was chosen as the promising condition for the FIA system. Concurrently, the aminoacylase reactor needed to be modified in order to get a perfect conversion when it worked at the flow rate of 0.4 mL min^{-1}.

3.3. Optimization of aminoacylase reactor

Effect of aminoacylase reactors with various enzyme contents on the conversion of 3HB-G was depicted in Fig. 4. With the aim to heighten the conversion ratio of 3HB-G, the amount of immobilized aminoacylase in the reactor was increased up to 100 mg. It was shown that the conversion of 3HB-G increased, depending on the aminoacylase amount in the reactor. The conversion reached 100% when 100-mg aminoacylase reactor was used. This aminoacylase reactor satisfied the requirement of a complete conversion

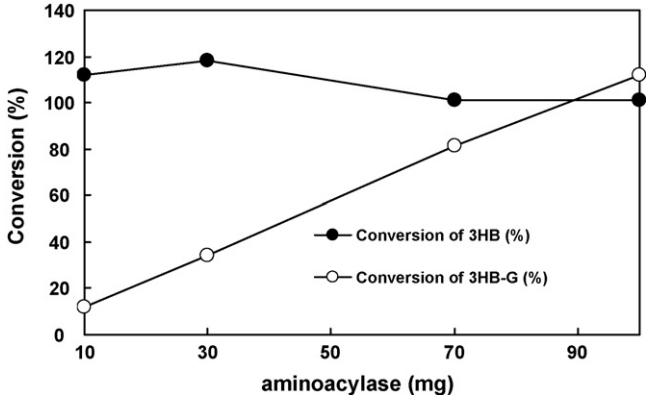

Fig. 4. Effect of aminoacylase on the conversion ratio of 3HB-G. 3HBDH, 1.3 mg; aminoacylase, 10–100 mg; 0.1 M phosphate buffer, pH 8.0; 1 mM NAD^+; flow rate, 0.4 mL min^{-1}.

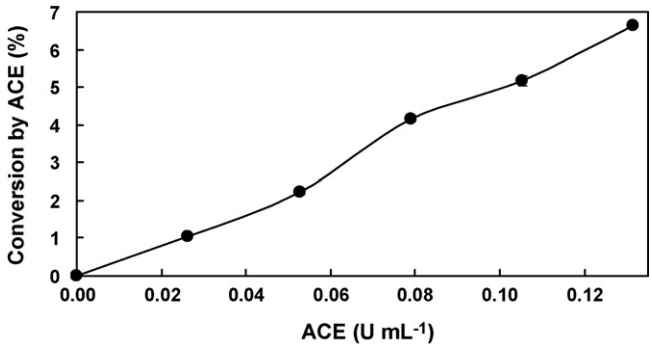

Fig. 5. Effect of ACE concentration on the conversion of 3HB-GGG. 3HB-GGG, 13.2 mM; ACE, 0–0.13 U mL^{-1}; incubation, 37 °C, 30 min; 3HBDH, 1.3 mg; aminoacylase, 100 mg; 0.1 M phosphate buffer, pH 8.0; 1 mM NAD^+; flow rate, 0.4 mL min^{-1}.

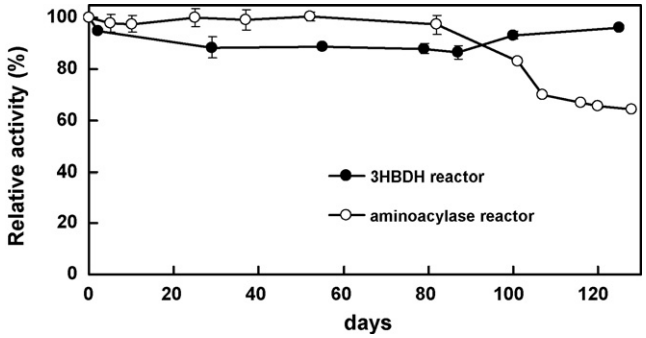

Fig. 6. Stability of the enzymatic reactors. 3HBDH reactor was stored at 4 °C. Aminoacylase reactor was stored in 0.1 M phosphate buffer (pH 8.0) containing 0.5 mM Co^{2+} at 25 °C.

of 3HB-G, thus it was chosen as the most favorable aminoacylase reactor for the FIA system.

In addition, the conversion of 3HB by 3HBDH reactor was always complete in any conditions. Therefore, 1.3-mg 3HBDH reactor and 100-mg aminoacylase reactor became essential components in the developed FIA system.

3.4. Optimization of the assay of ACE inhibitory activity using the FIA system

After the optimization of working conditions, the FIA system was operated with two consecutive enzymatic reactors, 1.3-mg 3HBDH and 100-mg aminoacylase, at the flow rate of 0.4 mL min^{-1}. This FIA system was applied to detect the 3HB-G generated from the reaction between 3HB-GGG and ACE at 37 °C for 30 min. To obtain an appropriate response, the condition of the reaction between the substrate and the enzyme must be optimized.

One of the factors that influenced the reaction rate was the concentration of substrate 3HB-GGG. The concentration of 3HB-GGG was changed from 2.0 to 13.2 mM in order to survey the effect of the substrate concentration on the conversion rate. However, the conversion ratio of the substrate was almost constant (3–3.6%) when 0.05 U mL^{-1} ACE was used. It meant that ACE was not sufficient for such a large amount of 3HB-GGG. Therefore, the increase in the concentration of ACE incubated with 13.2 mM 3HB-GGG was required to get a higher response as well as a higher conversion. The dependence of the conversion of 3HB-GGG on the concentration of ACE is shown in Fig. 5. The conversion ratio increased as the enzyme concentration increased, and reached the highest value 6.7% at 0.13 U mL^{-1} ACE. However, the concentration of ACE should be minimized because ACE is a costly enzyme. Therefore, 0.08 U mL^{-1} ACE was selected as the optimal enzyme concentration for the reaction with 13.2 mM 3HB-GGG.

3.5. Stability of the enzymatic reactors

When aminoacylase reactor was stored at 4 °C, it quickly lost its activity after several days. This might be because of the change in the conformation of aminoacylase at low temperature. Xie et al. [11] reported that the activity of aminoacylase was very low at low temperature, and totally diminished at 4 °C. Characteristically, aminoacylase is a metalloenzyme consisting of Zn^{2+} at its active sites that may require a metal ion for its activity [12]. On the other hand, as a zinc-dependent enzyme, aminoacylase is quite stable against denaturation induced by heat stress [13]. Thus, it was suggested that this thermostable-enzyme reactor could be kept at ambient temperature (25 °C). In addition, it was reported that Co^{2+} enhanced the stability and the activity of aminoacylase [10,12,14,15]. Thus, the usability of cobalt to stabilize and accelerate the activity of aminoacylase was investigated. When the aminoacylase reactor was stored in the phosphate buffer containing 1 μM to 1.5 mM $CoCl_2$, there was no considerable difference in the decrease in the activity of the aminoacylase reactor during the first week. However, with a longer storage time (2 weeks), the activity of the enzyme reactor fell off at Co^{2+} concentration less than 0.1 mM. On the contrary, the higher concentration of Co^{2+} effectively maintained and activated aminoacylase. The activity of the aminoacylase reactor was slightly increased during the storage. Thus, in the optimal range of $CoCl_2$ concentration from 0.1 mM to 1.0 mM [10], 0.5 mM was selected as the optimal $CoCl_2$ concentration for the storage of the aminoacylase reactor. As a result, when 0.1 M phosphate buffer (pH 8.0) containing 0.5 mM $CoCl_2$ was used for storing aminoacylase reactor at 25 °C, the stability of the enzymatic reactor was improved well (Fig. 6). The aminoacylase reactor retained its activity after 100 runs for 3 months, and reduced to 65% after 4 months. On the other hand, the 3HBDH reactor retained 95% of its activity after 400 runs over 4 months when it was stored at 4 °C. Practically, the 3HBDH reactor showed durable operation stability and great storage stability over half a year with a decrease in the activity less than 10%.

Table 1
IC_{50} of ACE inhibitors obtained by different analytical methods.

ACE inhibitors	3HB-GGG and FIA	3HB-GGG and WST-1 [6]	3HB-GGG and F-kit [5]	Conventional method [6]	Reference
Captopril (nM)	5.72 ± 0.13	6.87 ± 0.24	6.14 ± 0.06	6.39 ± 0.40	5–23 [16]
Val-Tyr (mM)	0.01 ± 0.00	0.03 ± 0.000	0.04 ± 0.00	0.03 ± 0.00	0.01 [17], 0.04 [18]
Tokuho 1 (%)	1.71 ± 0.04	1.36 ± 0.01	1.21 ± 0.05	2.37 ± 0.33	
Tokuho 2 (%)	0.58 ± 0.01	1.40 ± 0.03	1.06 ± 0.04	1.28 ± 0.21	
Tokuho 3 (%)	0.97 ± 0.02	1.49 ± 0.01	1.71 ± 0.07	1.21 ± 0.09	
Tokuho 4 (%)	0.17 ± 0.00	0.31 ± 0.00	0.44 ± 0.02	0.37 ± 0.03	
Tokuho 5 (%)	0.24 ± 0.00	0.64 ± 0.00	0.55 ± 0.02	0.78 ± 0.04	

Values are reported as mean ± standard deviation.

3.6. Application to the screening of ACE inhibitors

In order to assess the efficacy of the newly developed ACE inhibition assay, ACE inhibitory activity of captopril, Val-Tyr, and five kinds of Tokuho products was evaluated in terms of IC_{50} values. The obtained IC_{50} values by the present FIA system, by the previously established assay using 3HB-GGG with F-kit and WST-1 [5,6], and by the conventional method using HHL [6] are listed in Table 1. As can be seen, the investigated samples exhibited the ACE inhibitory activity, which was displayed as the decrease of the response signal. Captopril showed very strong inhibitory activity at nanomolar level. Val-Tyr, an ACE-inhibiting compound derived from food, had a moderate inhibitory activity. The IC_{50} values of captopril and Val-Tyr agreed with the data obtained from published literatures [16–18] as well as the results from previously established methods [5,6]. In addition, the experiments with Tokuho products containing ACE-inhibiting peptides also showed a satisfactory results, because the data corresponded well to those obtained from previous methods. Among five kinds of Tokuho, the Tokuho 1 contains the mixture of Ile-Pro-Pro and Val-Pro-Pro, meanwhile the other Tokuho products contain Val-Tyr. The Tokuho 1 had the lowest inhibitory activity. On the contrary, the Tokuho 4 containing the highest concentration of peptides exhibited the strongest inhibitory activity.

In addition, the performance of the proposed method compared with HHL method was judged by calculating the Student *t*- and *F*-tests for each inhibitor. At the 95% confidence level, the calculated *F*-values did not exceed the theoretical values, indicating that there was no significant difference in the precision of the two methods. Moreover, the result of the paired *t*-test of each sample showed that no significant difference between two methods under $p < 0.01$ was found. The paired *t*-test of samples between FIA method and the other methods was almost impracticable because of the difference in the precision of those methods. The reason for such difference in precision was due to the deviation of the results obtained from distinct assays because of the difference in the assay conditions, the variation of the principles of the assays, and the calculation methods. Nevertheless, among examined ACE inhibition assays, the significant linearities were recognized between the developed FIA method and the other methods. The correlation coefficient was 0.982, 0.984, and 0.994 ($n = 7$, $p < 0.01$) for the regressions between the FIA method and the F-kit method, the WST-1 method, and the conventional method, respectively. The data obtained by the FIA method tended to be lower than those obtained by previously established methods because of the remarkable difference in the ratio between the substrate and the enzyme of each method. Moreover, the system has a detection limit of 4 μM 3HB-G, comparable with that of previously developed methods [6]. Besides, it had a high reproducibility with a coefficient of variation less than 2.3% ($n = 3$). It was suggested that this sensitive ACE-inhibition assay with the FIA system could detect precisely ACE inhibitory activity of ACE inhibitors even at minor concentrations. The FIA system with enzymatic reactor was successfully developed with higher sensitivity and throughput, continuous measurement, and economical enzyme consumption. Promisingly, it can be applied to measure ACE inhibitory activity of diverse samples for the purpose of quality control and diversification of antihypertensive products in the functional food industry.

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