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Simultaneous Determination of Angiotensin I-Converting Enzyme Inhibitory Peptides in Tryptic Casein Hydrolysate by High-Performance Liquid Chromatography Combined with a Replicate Heart-Cut Column-Switching Technique

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A replicate heart-cut column-switching HPLC method combined with two switching valves was newly developed for the simultaneous determination of three antihypertensive peptides (Ala-Phe, Tyr-Pro, and Trp-Tyr) in tryptic casein hydrolysate in one run-in assay. After a first separation on an octadecyl silane (ODS) column, heart-cuts of each peptide were individually separated on a subsequent analytical ODS column: 26% acetonitrile for Ala-Phe and Tyr-Pro (32% for Trp-Tyr) in 0.1% trifluoroacetic acid containing 10 mM sodium 1-octanesulfonate at 0.8 mL/min. Ala-Phe, Tyr-Pro, and Trp-Tyr in casein hydrolysate were determined within 70 min to be 0.377 ± 0.037 mg/g, 2.50 ± 0.26 mg/g, and 0.096 ± 0.008 mg/g, respectively.

KEYWORDS: Angiotensin I-converting enzyme (ACE) inhibitor; peptide; column-switching; high-performance liquid chromatography (HPLC); casein hydrolysate

INTRODUCTION

To date, many natural peptides possessing an angiotensin I-converting enzyme (ACE, EC 3.4.15.1) inhibitory action have been identified (*1*); some peptides among these were clinically proven to exert a blood pressure lowering effect in mild hypertensive subjects (*2*, *3*). Hence, some functional food products containing ACE inhibitory peptides have been evidentially developed on the basis of extensive intervention trials.

Although such bioactive peptides have elicited great interest in the field of functional food sciences, the productions require severe control or monitoring of candidates during the process. A common preparation is an enzymatic hydrolysis of natural proteins or fermentation by microorganisms under severe hydrolytic conditions (*1*). For example, sardine muscle hydrolysate, which is one of the antihypertensive products exerting a mild blood pressure lowering in humans (*2*), is prepared with 0.3% *Bacillus licheniformis* alkaline protease under a 17 h incubation at 50 °C (*4*). A shorter incubation was found to reduce the produced amount of the antihypertensive peptide, Val-Tyr, and other ACE inhibitory small peptides (*4*), resulting in a marked degradation of food quality or reduction of the antihypertensive effect. The best way to maintain the quality of a given antihypertensive hydrolysate is to simultaneously monitor the amounts of candidates (predominant ACE inhibitory peptides) produced during the hydrolytic process. However, the specific monitoring for candidates would require a great effort because the hydrolysate may contain a large number of peptides. The convenient chromatographic method of high-performance liquid chromatography (HPLC) for the purification of peptides still requires extensive separation steps to detect a peptide of interest without any interference (*5*–*7*).

Recent analytical methods for peptide determination have been focused on the application of liquid chromatography–tandem mass spectrometry (LC-MS/MS) by which desired peptides can be assayed sensitively and rapidly. However, this method needs an expensive analytical apparatus and tedious procedures for preparing the sample. van Platerink et al. (*8*) have proposed a sensitive and simultaneous assay of plasma levels of 17 small ACE inhibitory peptides using an LC-API-MS method, but its successful application requires great consideration in selecting the quantitative product ions of each peptide or ionization condition and, in particular, the pre-extraction experiments of a given sample because matrices greatly affect the ionization efficacy of peptides. In contrast, a column-switching HPLC technique is a convenient and widely applicable quantification

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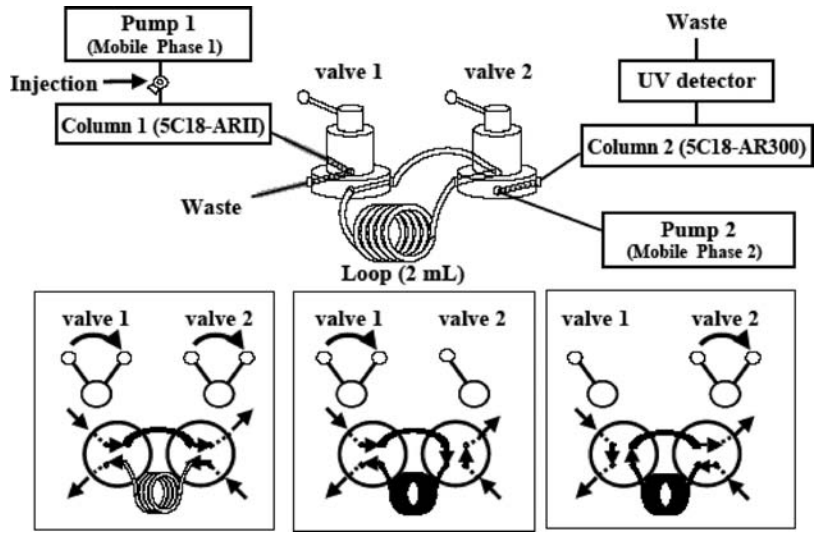

Figure 1. Schematic representation of a replicate column-switching HPLC system for the determination of Ala-Phe, Tyr-Pro, and Trp-Tyr in tryptic casein hydrolysate. A sample (200 μL) was loaded for the C1 column. When each peptide was eluted on the C1 column, each eluting zone of retention times from -1.0 min to $+1.5$ min was heart-cut. Each zone was then transferred to the C2 analytical column by a valve operation, and the introduced eluate was separated on C2 followed by UV detection at 220 nm. Detailed procedures for each peptide are described in the Experimental Procedures section. MP: Mobile phase. V: valve.

method without any expensive instrument or the above considerations, especially for drug assay in biological fluids (*9–12*). Thus, it seems likely that the latter technique makes it possible to avoid cumbersome and laborious fractionation of HPLCs, achieving a selective quantification of a targeted bioactive peptide. In our previous report (*13–15*), we successfully determined an ACE inhibitory peptide, Val-Tyr, in blood by a fluorimetric heart-cut column-switching HPLC method. However, there has been no research for applying the technique to successive assays for some targeted peptides.

In this study, we undertook the development of a replicate of the column-switching HPLC method for the successive determination of some small ACE inhibitory peptides in hydrolysate in one run-in assay. As a small peptide (di- or tripeptide) is mainly responsible for the antihypertensive effect because of its possible absorption via a PEPT1 transporter in the intestinal membrane (*16*), the capability of the present method to allow some small candidates to be determined simultaneously would be of benefit in evaluating the quality of antihypertensive products containing small ACE inhibitory peptides. We targeted small peptides from commercially available casein hydrolysate product by trypsin because researchers allow our proposed method to apply for a given hydrolysate on the basis of the separation information obtained in the casein hydrolysate.

EXPERIMENTAL PROCEDURES

Chemicals. Ala-Phe, Tyr-Pro, and Trp-Tyr were purchased from Kokusan Chemical Works (Tokyo, Japan). Casein hydrolysate prepared by trypsin, which is commercially available from E. Merck (Darmstadt, Germany), was used for this study without purification. Acetonitrile (CH_3CN) was of HPLC grade (Kantokagaku, Tokyo, Japan). The water used was Milli-Q water (Millipore, Tokyo, Japan). Sodium 1-octanesulphonate (SOS) of ion-pair reagent grade was purchased from Nacalai Tesque (Kyoto, Japan). Purified rabbit lung ACE was purchased from Sigma (MO, U.S.A.), and hippuryl-L-histidyl-L-leucine (Hip-His-Leu) as a synthetic ACE substrate was obtained from the Peptide Institute (Osaka, Japan). All other chemicals were of analytical-reagent grade and were used without further purification.

Instruments. A system for the successive determination of bioactive peptides in casein hydrolysate in one run-in assay was developed as depicted in **Figure 1**. The system was composed of a solvent delivery system of two pumps (1 and 2, LC-10A, Shimadzu, Kyoto, Japan) attached with an automated gradient controller (LCV-10AL Gradient Unit, Shimadzu), a column oven (CTO-10A, Shimadzu), two manual four-port switching valves (HPV-4, GL Sciences, Tokyo, Japan), a 2 mL PEEK (polyether ether ketone) loop (GL Sciences, Tokyo, Japan), and a UV–vis detector (UV2075, Nippon Bunko, Tokyo, Japan).

Preparation of Standard Solutions. Stock solutions of the three di-peptides were individually prepared by dissolving each peptide in CH_3CN to a concentration of 1.0 mg/mL and stored at 4 °C. Working standards were prepared daily prior to the experiments by a combination of the three stock solutions and further dilution with a 1% CH_3CN solution to give appropriate standard solutions. The standard solutions containing the three peptides (0.5–10.0 μg/mL for Ala-Phe, 0.1–10.0 μg/mL for Tyr-Pro, and 0.1–1.0 μg/mL for Trp-Tyr) were used to obtain the calibration curves.

Sample Preparation. Tryptic casein hydrolysate was dissolved in 1% CH_3CN to a concentration of 5.0 mg/mL and was applied onto a 0.25 μm Cosmonise Filter (Nacalai Tesque). Aliquots of 200 μL of the filtered solution were injected for HPLC analysis.

Replicate Column-Switching HPLC System. A replicate column-switching HPLC system was composed of a clean-up column (C1) and an analytical column (C2) as shown in **Figure 1**. The C1 was a Cosmosil $5C_{18}$-ARII column, and the C2 was a Cosmosil $5C_{18}$-AR300 column (each column: 5 μm, 4.6 mm $\times$ 250 mm, Nacalai Tesque). A Cosmosil $5C_{18}$-ARII guard column and a Cosmosil $5C_{18}$-AR300 guard column (each 5 μm, 4.6 mm $\times$ 10 mm) were connected in front of the C1 and C2 columns, respectively. Both columns were operated at 35 °C. The mobile phase for C1 (MP1) was a CH_3CN–water mixture at a flow rate of 0.8 mL/min. The linear gradient system of MP1 was an increasing concentration of 5% CH_3CN held for 30 min and then to 12% CH_3CN for 30 min. The mobile phase for C2 (MP2) was CH_3CN–water/0.1% trifluroacetic acid (TFA) containing 10 mM SOS at a flow rate of 0.8 mL/min, and a step-wise gradient system was performed 26% CH_3CN/0.1% TFA containing 10 mM SOS for 40 min; then, after changing to 32% CH_3CN/0.1% TFA containing 10 mM SOS for 3 min, the C2 column was run with the mobile phase for 40 min. Optimal elution conditions for the individual separation of the three peptides are investigated in the text. An injection volume to the system was fixed at 200 μL, and the detection of a peptide was conducted at 220 nm throughout the experiment and was recorded with a Shimadzu C-R 5A Chromatopac recorder.

Two heart-cut operation modes could be used as depicted in **Figure 1**. For the separation of two peptides whose retention times on the C1

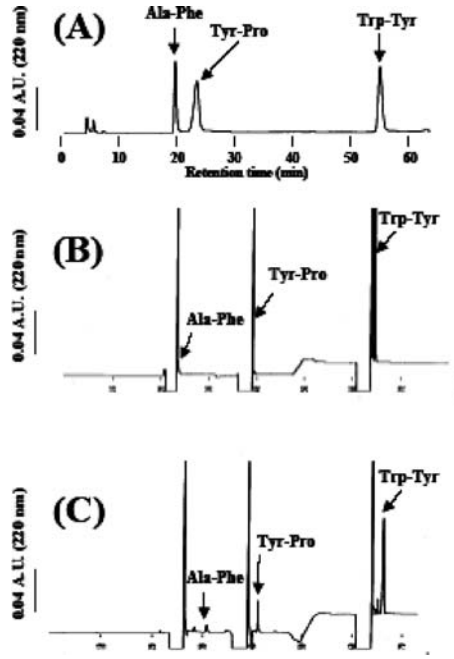

Figure 2. Typical HPLC elution profile of Ala-Phe, Tyr-Pro, and Trp-Tyr on the C1 column (**A**) and the C2 column (**B** and **C**) after column-switching. C1 column: Cosmosil $5C_{18}$-ARII column (4.6 × 250 mm). Elution condition for MP1: 5% CH_3CN held for 30 min, then to 12% CH_3CN for 30 min. C2 column: Cosmosil $5C_{18}$-AR300 column (4.6 × 250 mm). Elution condition for MP2: 26% CH_3CN for Ala-Phe and Tyr-Pro (32% for Typ-Tyr)/0.1% TFA (**B**), 26% CH_3CN for Ala-Phe and Tyr-Pro (32% for Typ-Tyr)/0.1% TFA containing 10 mM SOS (**C**).

Table 1. Intra- and Inter-Day Accuracy of Di-Peptides on C1 Column[a]

	retention time on C1 column (min)			average within-run C.V. (%)	between-run C.V. (%)
	day 1	day 2	day 3		
Ala-Phe	19.0 ± 0.3	19.1 ± 0.3	19.1 ± 0.4	1.6	0.3
Tyr-Pro	22.6 ± 0.4	22.8 ± 0.3	22.8 ± 0.4	1.5	0.4
Trp-Tyr	55.5 ± 0.4	55.2 ± 0.3	55.1 ± 0.1	0.5	0.4

[a] Retention time of each peptide on C1 column (Cosmosil 5C18-AR II, 4.6 × 250 mm) was measured for four replicates of each separated day. Details on HPLC separation conditions are described in the Experimental Procedures. Data represent the mean ± SD ($n = 4$).

column are close (each elution is within 5 min, e.g., Ala-Phe and Tyr-Pro in **Figure 2A**) the early eluted peptide (Ala-Phe) was sent to the C2 column by switching the V1 and V2 valves. Then, both valves were returned to their original positions. Subsequently, the late eluted peptide (Tyr-Pro) was sent to a 2 mL PEEK loop by switching the valve V1 for 2.5 min and then returning the valve to the original position. After a complete elution of the early eluted peptide was achieved on the C2 column, the valve V2 was then switched so as to send the slower peptide kept in the loop to the C2 column. For the separation of the two peptides whose retention times on the C1 column are apart enough to achieve a complete separation on the C2 column (e.g., Trp-Tyr in **Figure 2A**), the peptide was sent to the C2 column by switching both valves.

The heart-cut of peptides was selected on each corresponding retention time on the C1 column by considering their intra- and inter-day accuracy (**Table 1**). Additionally, the heart-cut of each peptide was selected from 1 min before the retention time and lasted for 1.5 min: Ala-Phe on C1, 18.0–20.5 min; Tyr-Pro on C1, 21.5–24.0 min; and Trp-Tyr on C1, 54.0–56.5 min.

Identification and Selection of Targeted Three Ace Inhibitory Peptides from Tryptic Casein Hydrolysate. Peptides possessing ACE inhibitory activity in the commercial tryptic casein hydrolysate were isolated with a two-step reversed-phase HPLC on a Cosmosil $5C_{18}$-ARII column (4.6 × 250 mm) equipped with a Shimadzu SPD-10AV UV detector at 220 nm. In the first step, tryptic casein hydrolysate was separated on the column with a linear-gradient elution of 5–35% CH_3CN for 60 min at 0.8 mL/min. The fraction with ACE inhibitory activity was then applied onto the $5C_{18}$-ARII column with a linear-gradient elution of 5–40% CH_3CN containing 0.1% TFA for 75 min at 0.8 mL/min. The peptide sequence of each isolate was determined with a protein sequencer (PPSQ-21, Shimadzu).

Recovery. Recoveries of the three peptides were examined by spiking the casein solution with known amounts of the peptides. Because the casein hydrolysate originally contains the three peptides, the recovery test was performed by subtracting the original amount of each peptide in it from the observed amounts in the spiked casein hydrolysate. Specifically, the percentage recoveries of peptides in the casein hydrolysate solution were calculated from the ratio of the net increase in spiked casein solution relative to the standard peptide solution. Intra- and inter-day accuracy and the precision of the assay procedure as well as the recovery test were assessed in spiked concentrations of 4.0 μg/mL for Ala-Phe, 10.0 μg/mL for Tyr-Pro, and 0.1 μg/mL for Trp-Tyr with 5.0 mg/mL of casein hydrolysate solution. Four replicates were analyzed on each of three separate days. All the data obtained in this study are presented as a mean ± SD.

Calibration Curve. Three separate replicates of each of the five different concentrations between 0.1 and 10 μg/mL of the three peptides were conducted for obtaining the calibration curve.

RESULTS AND DISCUSSION

Identification and Selection of Three Targeted ACE Inhibitory Peptides from Tryptic Casein Hydrolysate. Separation of the hydrolysate on a Cosmosil $5C_{18}$-ARII column resulted in a successful identification of ACE inhibitory peptides; though the data are not shown, we identified three ACE inhibitory di-peptides: Ala-Phe (retention time of 29.8 min on the final Cosmosil $5C_{18}$-AR II column chromatography), Tyr-Pro (22.9 min), and Trp-Tyr (46.4 min). By an ACE inhibition assay using Hip-His-Leu as a synthetic substrate against rabbit lung ACE (*5*), Ala-Phe, Tyr-Pro, and Trp-Tyr respectively revealed an IC_{50} value of 165, 720, and 57 μM. Among them, Trp-Tyr was found for the first time as a natural ACE inhibitory peptide. Other identified peptides (Ala-Phe (*17*) and Tyr-Pro (*18*)) have been already isolated from natural protein hydrolysates.

Optimization of a Replicate Column-Switching HPLC System. Because the three ACE inhibitory di-peptides of interest in this study possess a similar structural property and tryptic casein hydrolysate is a mixture of diverse peptides, the selection of packaging materials to separate each di-peptide from the hydrolysate was of great importance. In our reports on isolation of ACE inhibitory peptides from natural proteins, C_{18} reversed-phase materials such as C_{18} and phenyl–C_{18} in combination with a CH_3CN–water mobile phase system were found to be the most retentive for small peptides (*5*, *17*). In this study, we examined some C_{18} phases, and the $5C_{18}$-AR II phase was found to be feasible for the overall separation of each di-peptide (**Figure 2A**). **Table 1** shows the intra- and inter-day accuracy of each peptide on the $5C_{18}$-AR II column. A reproducible elution of each peptide was observed on the C1 column; retention times of Ala-Phe, Tyr-Pro, and Trp-Tyr on C1 were 19.0 ± 0.3, 22.6 ± 0.4, and 55.5 ± 0.4 min, respectively. Under the established conditions, each peptide eluted on the C1 column with high reproducibility of within-run C.V. (coefficient of variation) values of 1.6%, 1.5%, and 0.5% for Ala-Phe, Tyr-Pro, and Trp-Tyr, respectively, and between-day C.V. values of 0.3%, 0.4%, and 0.4% for Ala-Phe, Tyr-Pro, and Trp-Tyr, respectively (**Table 1**).

To accomplish a complete separation of each peptide in the casein hydrolysate on the C2 column after their heart-cuts, more

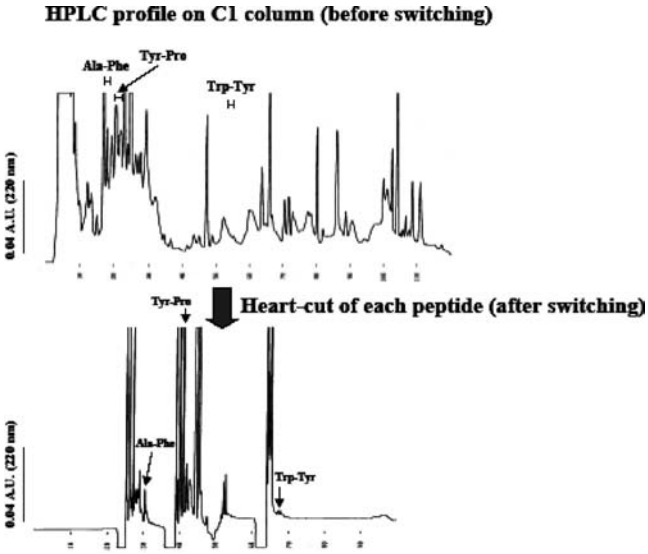

Figure 3. Application of a replicate column-switching HPLC method to determine Ala-Phe, Tyr-Pro, and Trp-Tyr in commercial tryptic casein hydrolysate in one run-in assay. Two hundred microliters of 5.0 mg/mL of casein hydrolysate was applied to the HPLC system, and each peptide was successively detected by a proposed replicate column-switching technique on a Cosmosil $5C_{18}$-AR300 column (4.6 × 250 mm) at a flow rate of 0.8 mL/min.

enhanced hydrophobic interaction between each peptide and ligand on the stationary phase was required than that on the C1 column. On the reversed-phase materials, a $5C_{18}$-AR300 packing fulfilled this demand because of its higher end-capped silanol groups than in the $5C_{18}$-AR II packing. In addition, the addition of acid, TFA, into the MP2 solvent system would be a useful way to enhance the hydrophobicity of a peptide by suppressing the ionization of the carboxyl group of the peptide and shielding the ionized amino group ($-NH^{3+}$) by an ion-pair formation of it (NH^{3+}) with TFA^-. Taking into account the separation of the three di-peptides on the C2 column, we were challenged to establish an optimal replicate heart-cut HPLC condition. As shown in **Figure 2B**, a CH_3CN/0.1% TFA mobile phase system (concentration of 26% for Ala-Phe and Tyr-Pro retentions and 32% for Trp-Tyr retention) did not improve a poor separation due to closeness or overlap of each peptide with the ghost-pressure peak induced by the second switching. Thus, our next challenge was to add an ion-pair reagent, SOS, to achieve a more hydrophobic interaction of the peptide with the column packaging. As a result, the addition of 10 mM SOS into the CH_3CN–water mobile phase acidified by 0.1% TFA was effective for the highest retention of each peptide on the $5C_{18}$-AR300 column as shown in **Figure 2C**. Consequently, we optimized the elution conditions as a CH_3CN concentration of 26% for Ala-Phe and Tyr-Pro and 32% for Trp-Tyr. The successive detection and separation of the three ACE inhibitory peptides in one run-in assay would prove the validity of the present replicate column-switching HPLC method for simultaneous determination of peptide levels in a given hydrolysate, as shown in **Figure 3**. The analysis time, established on the basis of the above switching conditions, was within 70 min, which is comparable to that in the ACE inhibitory peptide assay using an LC-MS/MS method (*8*).

Linearity. Aliquots of 5% CH_3CN solution containing 0.1% TFA spiked with known concentrations of Ala-Phe, Tyr-Pro, and Trp-Tyr were used for calibration. Under the replicate column-switching conditions described above, their correlation was linear with the coefficient of correlation (r) of greater than 0.9998 over the concentration range of 0.1–10 μg/mL: a typical calibration graph for Ala-Phe, $y = 102.6x - 0.772$ ($r = 0.9998$)]; Tyr-Pro, $y = 388.0x - 16.5$ ($r = 0.9999$); and Trp-Tyr, $y = 1097.7x + 258.7$ ($r = 0.9998$), where y is the peak height (μV s) and x is the peptide concentration (μg/mL).

The limit of detection was found to be 0.03 μg/mL for Ala-Phe, 0.004 μg/mL for Tyr-Pro, and 0.02 μg/mL for Trp-Tyr using the column-switching system and a signal-to-noise ratio of 3.

Validation Performance. Under the established switching HPLC conditions, we determined the accuracy and precision by replicate analysis of 5.0 mg/mL of tryptic casein hydrolysate spiked with 0 and 4.0 μg/mL of Ala-Phe, 10.0 μg/mL of Tyr-Pro, and 0.1 μg/mL of Trp-Tyr. Four replicates of each spiked concentration were analyzed on each of three separate days. As shown in **Figure 3** and **Table 2**, we could determine the peptide levels in tryptic casein hydrolysate with the concentration of 1.88 ± 0.18 μg/mL (0.377 ± 0.037 mg/(g casein hydrolysate)) for Ala-Phe, 12.5 ± 1.3 μg/mL (2.50 ± 0.26 mg/(g casein hydrolysate)) for Tyr-Pro, and 0.482 ± 0.040 μg/mL (0.096 ± 0.008 mg/(g casein hydrolysate)) for Trp-Tyr in one run-in assay. Average within-run C.V. values for Ala-Phe, Tyr-Pro, and Trp-Tyr were 6.2%, 1.3%, and 9.0%, respectively, and between-day C.V. values for Ala-Phe, Tyr-Pro, and Trp-Tyr were 9.8%, 9.3%, and 8.6%, respectively. The recoveries of peptides from spiked casein hydrolysates were 96.6 ± 7.3% for Ala-Phe, 110 ± 11% for Tyr-Pro, and 96.6 ± 55.1% for Trp-Tyr.

In our previous reports (*13–15*), we proposed a fluorimetric column-switching HPLC method for determining an extremely small amount of absorbed ACE inhibitory peptides in blood. In this study, we improved the technique for routine monitoring of the levels of bioactive small peptides in food products. By a combination of two switching valves and a 2 mL PEEK loop,

Table 2. Validation data for Ala-Phe, Tyr-Pro, and Trp-Tyr[a]

	spiked (μg/mL)	found (μg/mL) day 1	found (μg/mL) day 2	found (μg/mL) day 3	average recovery (%)	average within-run C.V. (%)	between-run C.V. (%)
Ala-Phe	0	1.68 ± 0.09	2.04 ± 0.21	1.93 ± 0.06		6.2	9.8
	4.0	5.63 ± 0.49	5.58 ± 0.44	6.04 ± 0.21	96.6 ± 7.3	6.7	4.4
Tyr-Pro	0	13.9 ± 0.2	12.1 ± 0.1	11.4 ± 0.3		1.3	9.3
	10.0	25.6 ± 0.04	23.7 ± 0.4	21.1 ± 0.9	110 ± 11	2.1	8.7
Trp-Tyr	0	0.470 ± 0.052	0.527 ± 0.042	0.450 ± 0.036		9.0	8.6
	0.1	0.620 ± 0.014	0.573 ± 0.012	0.550 ± 0.014	96.6 ± 55.1	1.6	4.6

[a] The determination of each peptide in spiked or non-spiked tryptic casein hydrolysate was made with the proposed column-switching HPLC system for four replicates of each separate day. The non-spiked tryptic casein hydrolysate was prepared at a concentration of 5.0 mg/mL. Details on HPLC separation conditions are described in the text. Data represent the mean ± SD ($n = 4$).

we successfully resolved the puzzles of the separation of Ala-Phe and Tyr-Pro because their retention times were close (within 5 min) on the preseparation column. A rapid (<70 min) and reproducible (<10% C.V.) column-switching HPLC technique for the determination of ACE inhibitory small peptides (Ala-Phe, Tyr-Pro, and Trp-Tyr) in tryptic casein hydrolysate in one run-in assay was developed. The method may be also applicable for the successive determination of peptides in other crude protein hydrolysates by, for example, accommodating column species and/or mobile phase–solvent systems to desired peptides without any change in the proposed separation technique.

LITERATURE CITED

(1) Matsui, T.; Matsumoto, K. Antihypertensive peptides from nature resources. In *Lead Molecules from Natural Products: Discovery and New Trends*; Khan, M. T. H., Ather, A., Eds.; Elsevier: Amsterdam, The Netherlands, 2006; Chapter 15, pp 259–276.

(2) Kawasaki, T.; Seki, E.; Osajima, K.; Yoshida, M.; Asada, K.; Matsui, T.; Osajima, Y. Antihypertensive effect of Valyl-Tyrosine, a short chain peptide derived from sardine muscle hydrolyzate, on mild hypertensive subjects. *J. Hum. Hypertens.* **2000**, *14*, 519–523.

(3) Hata, Y.; Yamamoto, M.; Ohni, M.; Nakajima, K.; Nakamura, Y.; Takano, T. A placebo-controlled study of the effect of sour milk on blood pressure in hypertensive subjects. *Am. J. Clin. Nutr.* **1996**, *64*, 767–771.

(4) Matsui, T.; Matsufuji, H.; Seki, E.; Osajima, K.; Nakashima, M.; Osajima, Y. Inhibition of angiotensin I-converting enzyme by Bacillus licheniformis alkaline protease hydrolyzates derived from sardine muscle. *Biosci., Biotechnol., Biochem.* **1993**, *57*, 922–925.

(5) Matsui, T.; Yukiyoshi, A.; Doi, S.; Sugimoto, H.; Yamada, H.; Matsumoto, K. Gastrointestinal enzyme production of bioactive peptides from royal jelly protein and their antihypertensive ability in SHR. *J. Nutr. Biochem.* **2002**, *13*, 80–86.

(6) Ma, M.-S.; Bae, I. Y.; Lee, H. G.; Yang, C.-B. Purification and identification of angiotensin I-converting enzyme inhibitory peptide from buckwheat (Fagopyrum esculentum Moench). *Food Chem.* **2006**, *96*, 36–42.

(7) Abubakar, A.; Saito, T.; Kitazawa, H.; Kawai, Y.; Itoh, T. Structural analysis of new antihypertensive peptide from cheese whey protein by proteinase K digestion. *J. Dairy Sci.* **1998**, *81*, 3131–3138.

(8) van Platerink, C. J.; Janssen, H.-G. M.; Horsten, R.; Haverkamp, J. Quantification of ACE inhibiting peptides in human plasma using high performance liquid chromatography-mass spectrometry. *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.* **2006**, *830*, 151–157.

(9) McMahon, G. P.; Kelly, M. T. Determination of aspirin and salicylic acid in human plasma by column-switching liquid chromatography using on-line solid-phase extraction. *Anal. Chem.* **1998**, *70*, 409–414.

(10) Uno, T.; Shimizu, M.; Sugawara, K.; Tateishi, T. Sensitive determination of itraconazole and its active metabolite in human plasma by column-switching high-performance liquid chromatography with ultraviolet detection. *Ther. Drug Monit.* **2006**, *28*, 526–531.

(11) Ban, E.; Meang, J.-E.; Woo, J. S.; Kim, C.-K. Sensitive column-switching high-performance liquid chromatography method for determination of propiverine in human plasma. *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.* **2006**, *831*, 230–235.

(12) Li, Y.; Gao, J.-P.; Xu, X.; Dai, L. Simultaneous determination of baicalin, rhein and berberine in rat plasma by column-switching high-performance liquid chromatography. *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.* **2006**, *838*, 50–55.

(13) Matsui, T.; Tamaya, K.; Kawasaki, T.; Osajima, Y. Determination of angiotensin metabolites in human plasma by fluorimetric high-performance liquid chromatography using a heart-cut column-switching technique. *J. Chromatogr., B* **1999**, *729*, 89–95.

(14) Ueno, T.; Tanaka, M.; Matsui, T.; Matsumoto, K. Determination of antihypertensive small peptides, Val-Tyr and Ile-Val-Tyr, by fluorimetric high-performance liquid chromatography combined with a double heart-cut column-switching technique. *Anal. Sci.* **2005**, *21*, 997–1000.

(15) Matsui, T.; Tamaya, K.; Seki, E.; Osajima, Y.; Matsumoto, K.; Kawasaki, T. Val-Tyr as a natural antihypertensive dipeptide can be absorbed into human circulatory blood system. *Clin. Exp. Pharmacol. Physiol.* **2002**, *29*, 204–208.

(16) Meredith, D.; Temple, C. S.; Guha, N.; Sword, C. J.; Richard Boyd, C. A.; Collier, I. D.; Morgan, K. M.; Bailey, P. D. Modified amino acids and peptides as substrates for the intestinal peptide transporter PepT1. *Eur. J. Biochem.* **2000**, *267*, 3723–3728.

(17) Matsui, T.; Li, C.-H.; Osajima, Y. Preparation and characterization of novel bioactive peptides responsible for angiotensin I-converting enzyme inhibition from wheat germ. *J. Pept. Sci.* **1999**, *5*, 289–297.

(18) Yamamoto, N.; Takano, T. Antihypertensive peptides derived from mild proteins. *Nahrung* **1999**, *43*, 159–164.

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