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Improvement in Isolation and Identification of Food-Derived Peptides in Human Plasma Based on Precolumn Derivatization of Peptides with Phenyl Isothiocyanate

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For the isolation and detection of food-derived peptides in blood, an approach based on the derivatization of peptides with phenyl isothiocyanate (PITC) was developed. This approach allows hydrophilic peptides to be resolved and specifically detected by reversed-phase (RP) HPLC. For the rapid capturing and clarification of peptides in human plasma, solid-phase extraction by using a mini spin column (5 mm × 5 mm) packed with a strong cation exchanger was used. The clarified peptide fraction was further fractionated by size-exclusion chromatography (SEC). The peptides in the SEC fractions were derivatized with PITC, and the derivatives were resolved by RP-HPLC by using an ammonium acetate buffer or a trifluoroacetic acid system. An automatic peptide sequencer based on Edman degradation with a modified program can directly analyze the resolved derivatives. Some synthetic peptides and food-derived peptides in human plasma were successfully isolated and identified by this approach.

KEYWORDS: Peptide; Pro-Hyp; food; plasma; phenyl isothiocyanate (PITC); hydroxyproline, solid-phase extraction (SPE); reversed-phased HPLC (RP-HPLC)

INTRODUCTION

Numerous pieces of scientific evidence in the past couple decades have revealed that some food-derived peptides exhibit various biological activities in vivo in addition to their established nutritional value as a source of protein (*1*−*8*). These peptides include those with opioid (*9*), hypotensive (*10*), mineral absorption stimulatory (*11*), and immunomodulatory (*12*) activities. The potential biological activities of peptides in food protein hydrolysates have frequently been screened by in vitro assays using cell culture systems, enzymatic reaction systems, etc. Unlike other functional substances, however, some peptides in foods with in vitro activity might be further degraded by peptidases during the process of ingestion, digestion, and absorption; as a result, these peptides might lose their potential biological activity detected using in vitro assays. Alternatively, some of the inactive peptides detected using in vitro assays might be converted to their active form by limited digestion. Therefore, the peptide activities as detected using in vitro assays could not directly express a variety of beneficial activities for humans by oral ingestion. Thus, the potential activity of the peptides should be evaluated through a feeding experiment. The identification of food-derived peptides in blood and target organs is, therefore, critically important for identification of the active and potentially active peptides. Recently, we found that significant amounts (20−60 nmol/mL of plasma) of peptides containing hydroxyproline (Hyp) were present in the peripheral blood of human volunteers after oral ingestion of gelatin hydrolysates (*13*). As the Hyp residue is specially distributed in collagen and no significant amounts of Hyp-containing peptides could be detected in human plasma before oral ingestion, the Hyp-containing peptides present in plasma after oral ingestion can be considered as food-derived peptides. To isolate these food-derived peptides, a deproteinized fraction of human plasma was fractionated by size-exclusion chromatography (SEC) and reversed-phase (RP) HPLC. Although most of these peptides were weakly adsorbed to the RP-HPLC column and resolution of these peptides from nonpeptide components in the plasma was not good, some peptides, such as Pro-Hyp and Pro-Hyp-Gly, with chemotactic activity to fibroblasts, peripheral blood nutrophils, and monocytes, could be identified using Hyp as a maker (*13*). On the other hand, it has been difficult to detect and isolate small amounts of food-derived peptides from animal and human blood after oral ingestion that do not have any marker amino acids or modified amino acids. Therefore, this study aimed to improve resolution and detection of food-derived peptides in a complex matrix such as human plasma for the identification of the food-derived peptides.

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Table 1. List of Synthetic Peptides Used in the Present Study

peptide[a]	isolelectric point[b]	molecular weight	peptide[a]	isolelectric point[b]	molecular weight
EL	3.30	260.29	PG	5.97	172.18
GGYR	11.04	451.48	PhP[c]	5.97	259.30
GH	7.80	212.21	PL	5.97	228.29
GL	5.97	188.23	QL	5.97	259.31
GP	5.97	172.18	TLLLPHHA	7.95	901.08
HH	7.95	292.30			

[a] Peptide sequences are presented by one-letter abbreviations for amino acids: E, glutamic acid; L, leucine; G, glycine; Y, tyrosine; R, arginine; H, histidine; P, proline; Q, glutamine; T, threonine; A, alanine. [b] The isoelectric point was calculated by using The Online Bio Database service of Fujitsu Kyushu System Engineering Ltd. [c] PhP represents Pro-Hyp.

To facilitate the specific detection and isolation of food-derived peptides, we developed an approach based on the precolumn derivatizaton of peptides with phenyl isothiocyanate (PITC). Isolated derivatives can be successfully analyzed by a peptide sequencer based on Edman degradation.

MATERIALS AND METHODS

Reagents. Acetonitrile (HPLC grade), a standard mixture of amino acids (type H), trifluoroacetic acid (TFA), PITC, piperidine, and *p*,*p*′-dichlorodiphenyltrichloroethane were purchased from Wako Pure Chemical Industries (Osaka, Japan). Triethlylamine (TEA; sequence grade) was obtained from Pierce (Rockford, IL). Pro-Hyp was purchased from Bachem (Bubendorf, Germany). 9-Fluorenylmethyloxycarbonyl (FMOC) amino acids and resins coupled with FMOC amino acids for peptide synthesis were purchased from Shimadzu (Kyoto, Japan). All other reagents were of analytical grade or better.

Synthesis of Peptides. With the exception of Pro-Hyp, peptides were synthesized by the FMOC strategy by using a PSSM-8 peptide synthesizer (Shimadzu). The synthesized peptides as shown in **Table 1** were stored at −20 °C until use.

Preparation of a Human Plasma Sample. Human studies were performed according to the Helsinki Declaration under the supervision of medical doctors. The experimental protocol was the same as described previously (*13*) and approved by the experimental ethical committees of the Nippon Meat Packers (Osaka, Japan). A healthy volunteer aged 37 years with a body mass of 67 kg fasted overnight for 12 h before the experiment and was administered chicken type II gelatin hydrolysates (C-mucolla, Nippon Meat Packers) at 11 g/60 kg of body mass. Before and after 2 h of the oral ingestion, approximately 10 mL of venous blood was collected from the cubital vein. Blood was centrifuged at 3000 rpm for 15 min, and the plasma was stored at −80 °C until use. The plasma prepared from the volunteer before the ingestion of the gelatin hydrolysate was mixed with synthetic peptides to give a final concentration of 20 nmol/mL of plasma. Three volumes of ethanol was added to the plasma in the absence and presence of the synthetic peptides. The precipitate was removed by centrifugation at 13000 rpm for 10 min. Eight milliliters of the ethanol-soluble fraction was dried under vacuum, dissolved in 350 μL of 10 mM HCl containing 50% methanol, and used for the following experiments.

Solid-Phase Extraction (SPE) of Peptides. As shown in **Figure 1**, a strong cation exchanger (AG 50W-X8, Bio-Rad Laboratories, Hercules, CA) was washed with 50% methanol, packed into a spin column (5 mm × 5 mm i.d., AB1150, Atto, Tokyo, Japan), and placed in a 1.5 mL centrifugation tube. The column was successively washed with 200 μL of 50% methanol (twice) and equilibrated with 200 μL of 10 mM HCl containing 50% methanol (twice). Elution was performed by centrifugation at 12000 rpm for 3 min. The ethanol-soluble fraction dissolved in the equilibrium solution was loaded onto the spin column. The column was successively washed with 200 μL of the equilibrium solution (twice) and 2.0 M ammonium hydroxide containing 50% methanol (once). Bound peptides were eluted with 200 μL of 7.5 M ammonium hydroxide containing 50% methanol (seven times).

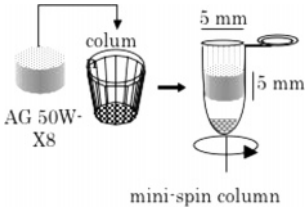

Figure 1. Schematic drawing of the mini spin column. A strong cation exchanger (AG 50W-X8) was washed with 50% methanol and packed into a spin column in a 1.5 mL centrifuge tube.

Size-Exclusion Chromatography. Peptide fractions eluted from the AG 50W-X8 spin column were combined, dried under vacuum, and dissolved in 250 μL of 30% acetonitrile in the presence of 0.1% TFA. In some cases, the sample solution was clarified by passing it through another spin column packed with Sephadex G-25 fine grade (Amersham Biosciences, Piscataway, NJ); the column was equilibrated with 30% (v/v) acetonitrile containing 0.1% TFA. Two hundred microliters of the clarified sample was loaded onto a Superdex Peptide 10/30 HR (Amersham Biosciences) column equilibrated with 30% (v/v) acetonitrile in the presence of 0.1% TFA at 0.5 mL/min. Fractions were collected every 1 min.

Derivatization of Peptides with PITC. Aliquots of the peptide fractions were dried in glass tubes (50 mm × 6 mm i.d.) under vacuum. The peptides in the tubes were derivatized with PITC by the method of Bidlingmeyer et al. (*14*) with a slight modification (*15*, *16*). Ten microliters of an alkaline solution containing methanol, TEA, and water in the ratio 7:2:1 was added to the tube and then dried under vacuum in a reaction vial with a resealable enclosure (Waters, Milford, MA). The dried sample was reacted with 20 μL of a derivatizing solution containing methanol, TEA, water, and PITC in the ratio 7:1:1:1 for 20 min at 25 °C. The excess reagent was removed by vacuum. The resultant phenylthiocarbamyl (PTC) peptides were dissolved in 5 mM sodium phosphate buffer, pH 7.4, containing 10% (v/v) acetonitrile, and the solution was filtered through a 0.45 μm filter (Column Guard LCR4, Millipore, Bedford, MA). The PTC-peptides were separated by a Supersphere RP-18 (e) column (250 mm × 4 mm i.d., Merck, Darmstadt, Germany) equilibrated with 0.15 M ammonium acetate buffer, pH 6.0, containing 5% (v/v) acetonitrile (solvent A) at 0.8 mL/min. Elution was performed by binary gradient elution with 60% (v/v) acetonitrile (solvent B) as described previously (*15*, *16*). The gradient profile was as follows: 0−5 min, 0% B; 5.1−25 min, 10−47.5% B; 25−30 min, 47.5−100% B; 30−37 min, 100% B; 37.1−40 min, 0% B. The column was maintained at 45 °C. Absorbance at 254 nm was monitored. In some cases, the PTC-peptides were separated by Inertsil ODS-3 columns (GL Sciences, Tokyo, Japan) of two different sizes, 250 mm × 2.1 mm i.d. and 250 mm × 4.6 mm i.d., by another binary gradient elution at 0.2 and 0.8 mL/min, respectively. The solvents contained 10% (v/v) acetonitrile with 0.01% TFA (solution A) and 80% (v/v) acetonitrile with 0.1% TFA (solvent B). The gradient profile was as follows: 0−30 min, 0−50% B; 30.1−40 min, 50−100% B. The column was maintained at 45 °C. The absorbance at 254 nm was also monitored.

Amino Acid Analysis. Peptide hydrolysis was carried out by using vapor HCl at 150 °C by the method of Bidlingmeyer et al. (*14*). The resultant amino acids were derivatized with PITC, and the PTC-amino acids were resolved by using a Supersphere RP-18 (e) column (250 mm × 4 mm i.d.) under the same conditions used for the PTC-peptides as described above. The Hyp-containing peptide content was estimated by subtracting the free Hyp from the total Hyp in the HCl hydrolysate (*13*).

Sequence Analysis of Peptides. Sequence analysis of the PTC-peptides was carried out by using a peptide sequencer (PPSQ-21, Shimadzu) based on Edman degradation; the program was changed to start from the cleavage step as described previously (*16*, *17*). In the case of PTC-peptides isolated by using an ammonium acetate buffer system, the sample was dried under vacuum and redried with 10 μL of a mixture of methanol, TEA, and water in the ratio 7:2:1 to remove ammonia. The sample was then dissolved in 50 μL of 30% acetonitrile and applied to the peptide sequencer.

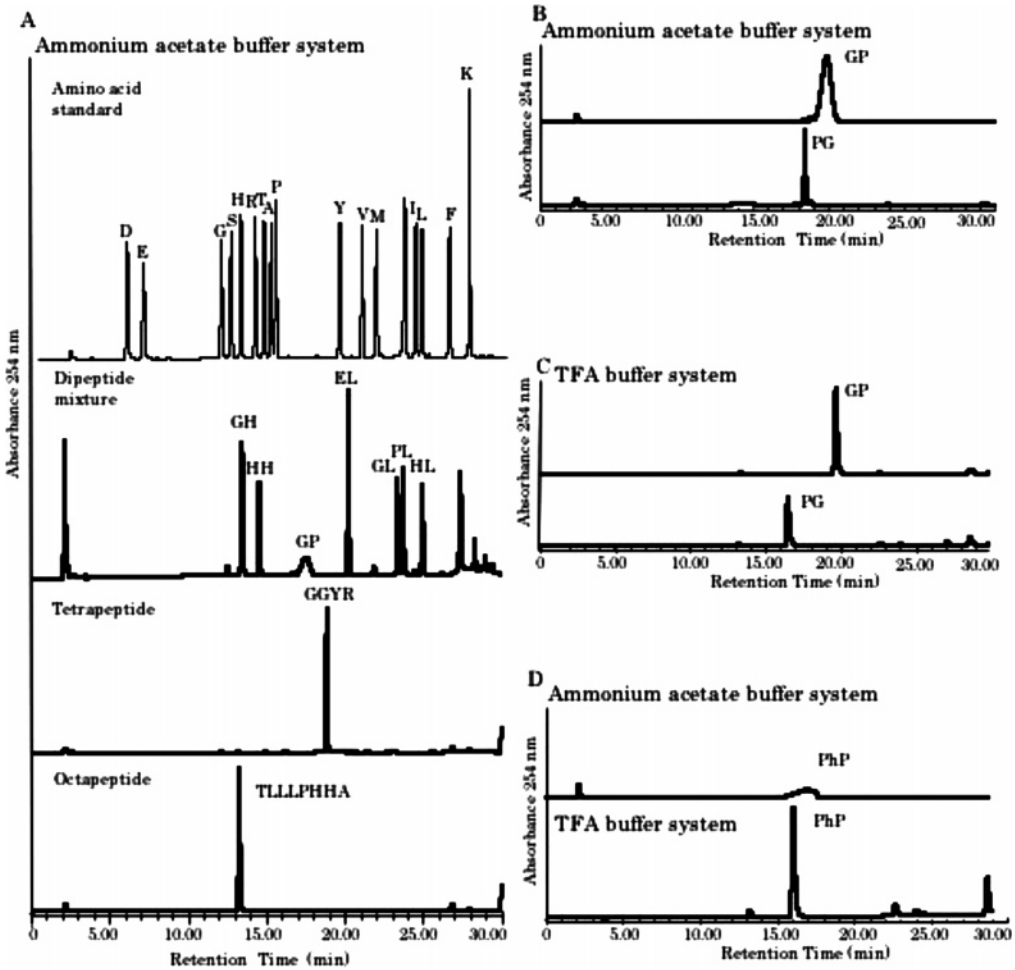

Figure 2. Resolution of PTC-peptides and PTC-amino acids. Five hundred picomoles of PTC-amino acids and di-, tetra-, and octapeptides was resolved by RP-HPLC by using an ammonium acetate buffer system, pH 6.0. PTC-Gly-Pro (**A**), PTC-Pro-Gly (**B**, **C**), and PTC-Pro-Hyp (**D**) were resolved by RP-HPLC by using an ammonium acetate buffer system and a TFA system. PhP means Pro-Hyp. For the TFA system, a 250 mm × 4.6 mm i.d. column was used.

RESULTS

Separation of Authentic PTC-Peptides by RP-HPLC. Authentic peptides as listed in **Table 1** were derivatized with PITC, and the resultant PTC-peptides were resolved by RP-HPLC under two different conditions. As shown in **Figure 2A**, most of the PTC-peptides including di-, tetra-, and octapeptides can be resolved by RP-HPLC by using an ammonium acetate buffer system, which has been optimized for resolution of PTC-amino acids (*15*, *16*). However, PTC-Gly-Pro and PTC-Pro-Hyp were resolved in broad peaks by the ammonium acetate buffer system, while PTC-Pro-Gly was resolved in a sharp symmetric peak (**Figure 2B**−**D**). To improve the resolution of PTC-Pro-Hyp and PTC-Gly-Pro, couples of RP-HPLC columns with different natures of the stationary phase, including wide porous silica (Vydac ODS-218TP, 5 μm, 250 mm × 4.6 mm i.d., The Separations Group, Hesperia, CA), nonporous silica (TSKgel Octadecyl-NPR, 2.5 μm, 35 mm × 4.6 mm i.d., Tosoh, Tokyo, Japan), polymer-coated porous silica (YMC-Pack Polymer C18, 6 μm, 250 mm × 4.6 mm i.d., YMC, Kyoto, Japan), and porous polymers (Shodex Asahipak ODP-50 4E, 5 μm, 250 mm × 4.6 mm i.d., Showa Denko, Tokyo, Japan), were used to separate these PTC-peptides containing the prolyl residue. However, no significant improvement of peak shape was obtained in all cases (data not shown). On the other hand, the resolution was drastically improved by using the TFA buffer system with the conventional porous silica RP-HPLC column (Inertsil ODS-3) as shown in **Figure 2C,D**.

Peptide Clarification by SPE. Twenty nanomoles of authentic dipeptides was loaded onto a mini spin column and successively eluted with 2.0 and 7.5 M ammonium hydroxide containing 50% methanol. As shown in **Figure 3**, all peptides including acidic peptides, such as Glu-Leu (**Table 1**, pI 3.3), were adsorbed to the resin and were not eluted with 10 mM HCl and 2.0 M ammonium hydroxide containing 50% methanol. All peptides, including the basic ones (**Table 1**, Gly-Gly-Tyr-Arg, pI 11.04), adsorbed to the resin were eluted with 7.5 M ammonium hydroxide containing 50% methanol and recovered in only fractions 3 and 4. The recovery of the peptides on the basis of peak area was estimated to be greater than 70%. On the basis of these data, 8 mL of the ethanol-soluble fraction of human plasma added with and without the authentic peptides at 20 nmol/mL was loaded onto the mini spin column. The peptides were eluted in the same manner, and fractions 3 and 4 were collected and dried under vacuum. In the nonadsorbed fraction, with the exception of taurine, no significant amounts of amino acids were observed (data not shown).

Separation of PTC-Peptides and PTC-Amino Acids. When 8 mL of the ethanol-soluble fraction of human plasma was directly dried without SPE pretreatment, a yellow turbid and viscous solution was obtained that could not be clarified by

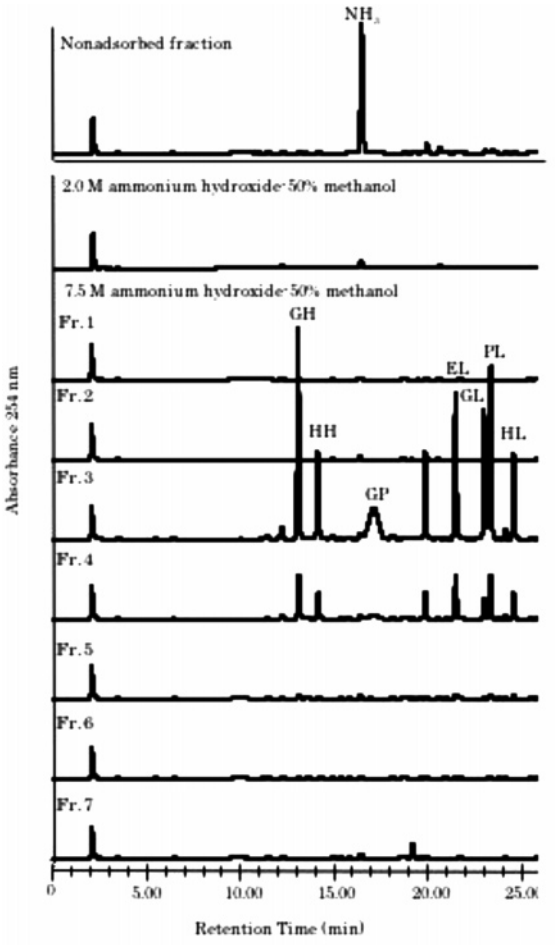

Figure 3. Elution of dipeptides from a mini spin column packed with a strong cation exchanger (AG 50WX-8W). Twenty nanomoles of the authentic peptides was loaded onto a mini spin column and successively eluted with 200 μL of 2.0 and 7.5 M ammonium hydroxide containing 50% methanol. Aliquots of fractions were derivatized with PITC and resolved by RP-HPLC by using an ammonium acetate buffer system.

ultrafiltration due to rapid clotting. The crude preparation was then clarified by passing it through a mini spin column packed with Sephadex-G 25 (Amersham Biosciences). On injection of the sample without SPE pretreatment into the SEC column, large peaks were observed near the column void volume and exclusion limit. Amino acid analysis revealed that no significant amounts of peptides were present in these large peaks (data not shown). On the other hand, SPE pretreatment dramatically reduced these nonpeptide peaks without reducing the small-molecular-weight peptide and amino acid peaks (indicated with bars in **Figure 4**). Peptides and amino acids in SEC fractions 29, 32, and 36 were derivatized with PITC and resolved by RP-HPLC by using the ammonium acetate buffer system. As shown in **Figure 5**, good resolution of the added peptides and endogenous amino acids was obtained without serious interference. Fractions 29, 32, and 36 consisted predominantly of added peptides, added peptides and bulky amino acids such as Val, Leu, and Ile, and other amino acids, respectively.

Isolation of a Food-Derived Collagen Peptide from Human Plasma. Peripheral blood was collected from a human volunteer before and after 2 h of oral ingestion of chicken type II gelatin hydrolysates. While no significant amount of the Hyp-containing peptide was observed before ingestion, a 22.7 nmol/mL concentration of the Hyp-containing peptides was present in the plasma after ingestion. These data agree with those from the

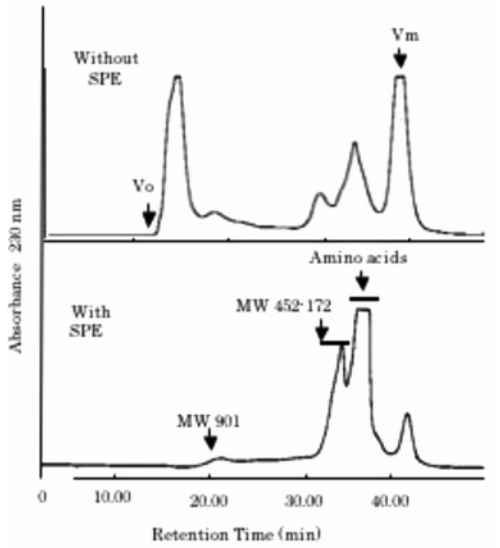

Figure 4. Effect of SPE treatment on the chromatogram of the SEC mode. Eight milliliters of the ethanol-soluble fraction with and without SPE pretreatment was loaded onto a Superdex Peptide 10/30 HR column. Key: V_o, void volume; V_m, exclusion limit.

previous study (*13*). Two milliliters of the plasma after ingestion was deproteinized, clarified, and fractionated by SEC in the same manner as described above. Aliquots of SEC fractions 30 and 31 were combined and reacted with PITC. As shown in **Figure 6**, a large symmetric peak was eluted at 8.55 min by using the TFA system. Sequence analysis revealed that this peak consisted of PTC-Pro-Hyp.

DISCUSSION

A few food-derived peptides have been isolated and identified from human blood. In early studies, the fraction of human plasma deproteinized by perchloric acid was directly resolved by RP-HPLC. Two casein-derived peptides were identified from the plasma of subjects who ingested milk or yogurt (*18*, *19*). However, as shown in **Figure 5A**, many interference peaks appeared on direct injection of deproteinized human plasma. Thus, we concluded that it is difficult to detect and isolate relatively small amounts of food-derived peptides by this approach. In our previous study, some food-derived peptides were isolated from human plasma after oral ingestion of the gelatin hydrolysates by using SEC and RP-HPLC in a series (*13*). However, even when SEC was performed before RP-HPLC, many interference peaks were still observed by monitoring the absorbance at 214 nm. In addition, the food-derived peptides identified in the previous study included short-chain and hydrophilic peptides such as Pro-Hyp. These peptides were weakly adsorbed onto the RP-HPLC column even in the absence of organic solvents, making it difficult to isolate them by conventional RP-HPLC.

To improve the resolution of hydrophilic peptides in the water-soluble fraction of an enzyme-modified cheese, peptides were derivatized with 9-FMOC (*20*). Recently, Matsui et al. have used naphthalene-2,3-dialdehyde (NDA) to detect and resolve trace levels of food-derived antihypertensive peptides (Val-Tyr and Ile-Val-Tyr) in animal and human plasmas (*21*−*25*). These derivatives can be resolved by RP-HPLC and detected with high sensitivity. However, these techniques have not been used for the sequence analysis of peptides. On the other hand, we have demonstrated that peptides derivatized with PITC, namely, PTC-peptides, can be directly analyzed by the

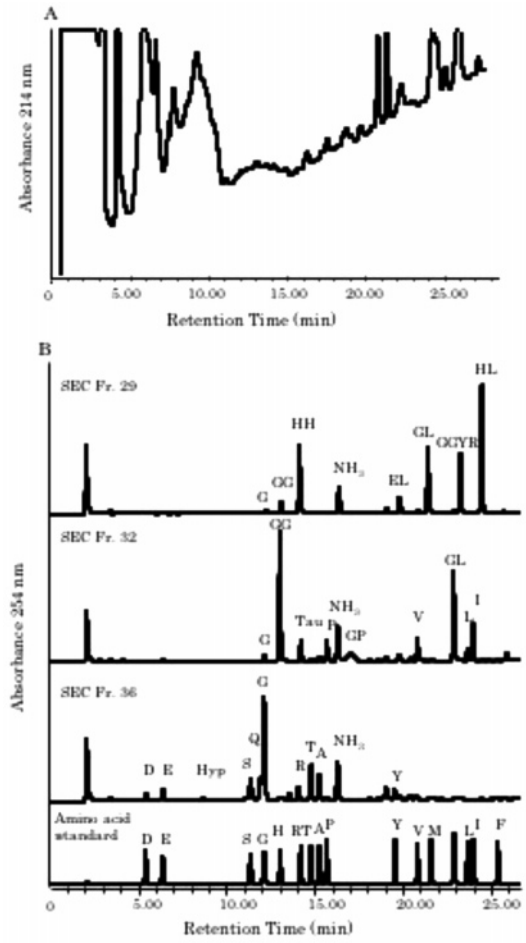

Figure 5. Resolution of peptides by RP-HPLC. (**A**) Eight milliliters of the ethanol-soluble fraction with the authentic peptides was directly loaded onto the RP-HPLC column and eluted with a linear gradient of acetonitrile in the presence of 0.1% TFA. Absorbance at 214 nm was monitored. (**B**) The same sample was clarified by SPE and fractioned by SEC. PTC-peptides and PTC-amino acids in SEC fractions 29, 32, and 36 were resolved by an ammonium acetate buffer system. Absorbance at 254 nm was monitored.

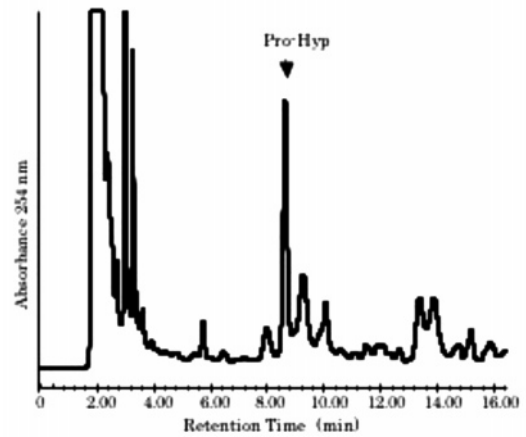

Figure 6. Isolation of food-derived collagen peptides (Pro-Hyp) in the plasma of a volunteer after oral ingestion of type II gelatin hydrolysates. Aliquots of SEC fractions 30 and 31 were reacted with PITC and resolved by the TFA system. The peak marked with an arrow was identified as Pro-Hyp. A 250 mm × 2.1 mm i.d. column was used.

peptide sequencer based on Edman degradation; the sequencer program was changed to start from the cleavage reaction (*16*, *17*). Thus, PITC was used for the precolumn derivatization of food-derived peptides in human plasma in the present study. As endogenous amino acids in blood are also derivatized with PITC and resolved by RP-HPLC, the fractionation of amino acids and peptides by SEC would be effective for the isolation of food-derived peptides in plasma (**Figure 5B**). As shown in **Figure 2A**, most PTC-peptides, including di-, tri-, tetra-, and octapeptides, are resolved by RP-HPLC by using an ammonium acetate buffer system. However, we found that some PTC-peptides containing the prolyl residue, such as PTC-Pro-Hyp and PTC-Pro-Gly, were eluted in a broad peak by using an ammonium acetate buffer system with a pH greater than 4. The resolution of these peptides was improved by adding TFA in the eluents to give a concentration of 0.01−0.1% TFA. On the other hand, it has been suggested that PTC-amino acids could be degraded in an acidic condition (*26*). We confirmed that PTC-Pro-Hyp isolated by the TFA system and stored at −20 °C in a solution for greater than 1 month can be used for sequence analysis. Together with these facts, we conclude that the precolumn derivatization of peptides with PITC followed by RP-HPLC separation can be used for the isolation and identification of food-derived peptides in plasma.

For the clarification, a mini spin column packed with a strong cation exchanger was used in this study. Peptides and amino acids in several milliliters of plasma from an individual who has ingested 11 g of gelatin hydrolysate can be captured and recovered in a small volume (200−400 μL). This pretreatment allows the use of relatively large volumes of plasma samples (several milliliters) for subsequent HPLC analysis.

By coupling the SPE, SEC, and prelabel RP-HPLC techniques, authentic peptides added to human plasma at a concentration of 20 nmol/mL can be isolated without serious interference with amino acids and other plasma components. If necessary, subfractionation by using conventional RP-HPLC before the prelabel RP-HPLC could be added to purify food-derived peptides.

The food-derived Hyp-Pro in human plasma can be successfully isolated as a major symmetric peak and sequenced by the present approach; this peptide was eluted in the shoulder peak of the nonpeptide component by conventional RP-HPLC without SPE pretreatment and derivatization. These facts confirm that the present approach would be a useful tool for the identification of food-derived peptides in human plasma.

Further studies on the isolation and identification of food-derived peptides in plasma from rats and humans who have ingested soy protein hydrolysate, sugar-modified fish meat, and so on are now in progress. Structural information on food-derived peptides in human and animal plasma would facilitate the design of an in vitro experiment based on enzyme reaction, cell culture systems, etc., for the investigation of the transportation, metabolism, and potential biological activity of food-derived peptides.

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