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Identification of Food-Derived Collagen Peptides in Human Blood after Oral Ingestion of Gelatin Hydrolysates

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In the present study, we identified several food-derived collagen peptides in human blood after oral ingestion of some gelatin hydrolysates. Healthy human volunteers ingested the gelatin hydrolysates (9.4−23 g) from porcine skin, chicken feet, and cartilage after 12 h of fasting. Negligible amounts of the peptide form of hydroxyproline (Hyp) were observed in human blood before the ingestion. After the oral ingestion, the peptide form of Hyp significantly increased and reached a maximum level (20−60 nmol/mL of plasma) after 1−2 h and then decreased to half of the maximum level at 4 h after the ingestion. Major constituents of food-derived collagen peptides in human serum and plasma were identified as Pro-Hyp. In addition, small but significant amounts of Ala-Hyp, Ala-Hyp-Gly, Pro-Hyp-Gly, Leu-Hyp, Ile-Hyp, and Phe-Hyp were contained.

KEYWORDS: Collagen; gelatin hydrolysates; Pro-Hyp; fibroblast; peptide; food; gelatin; skin; osteoporosis

INTRODUCTION

Collagen is one of major constituents of connective tissues of animal, bird, fish, and so on. Gelatin, a denatured form of collagen, has been prepared in industrial scale from these materials (*1*). The gelatin-based products have a long history as food ingredients. Gelatin has been also used as folk medicine in Asia to improve blood circulation and arrest bleeding (*2*). In Western countries, the first known description of the beneficial effect of gelatin ingestion can be found in 1175. St. Hildegard described that eating gelatin improved joint condition by reducing pain (*3*). Recently, Koyama et al. reported that oral ingestion of gelatin can increase bone mineral density by an animal experiment (*4*).

To increase solubility of gelatin, some enzymatic hydrolysates of gelatin have been prepared. Some animal experiments and preclinical human trials have also suggested that oral ingestion of the gelatin hydrolysate might have beneficial effects as well as gelatin (*3*, *5*, *6*). Very recently, Wu et al. demonstrated the safety of oral ingestion of a high dose (1.66 g/kg of body weight) of gelatin hydrolysate in an animal model (*6*). However, the efficacy of gelatin-based products has not been confirmed by a well-designed human trial.

On the basis of an *in vitro* study using a cell culture system, some collagen-derived peptides have been demonstrated to have some biological activities. For example, Pro-Hyp-Gly, Pro-Hyp, and so on have chemotactic activity to fibroblast, peripheral blood neutrophil (*7*, *8*), and monocytes (*9*). Asp-Gly-Glu-Ala stimulates osteoblast-related gene expression of bone marrow cells (*10*). Furthermore, Ala-Hyp and Gly-Pro-Val are potential inhibitors of angiotensin-converting enzyme (*11*, *12*), and Gly-Pro-Hyp is suggested to be involved in platelet aggregation (*13*). These facts suggest that collagen peptide possibly generated by degradation of the extracellular matrix might be associated to wound healing and inflammation process. On the other hand, the occurrence of food-derived collagen peptide in serum (*14*) and urine (*15*) from a human who ingested gelatin was reported. Therefore, it could be assumed that food-derived collagen peptides in blood may be involved in some biological activities suggested by the animal and human experiments. However, there are little data about the structure of food-derived collagen peptide in human blood. Then, the mechanism for suggested effects by oral administration of gelatin-based products still remains to be solved. Thus, the objective of this study is to identify food-derived collagen peptides in human blood after oral ingestion of gelatin hydrolysates from several raw materials.

MATERIALS AND METHODS

Gelatin Hydrolysates. Enzymatic hydrolysate of porcine skin gelatin (SCP-5000) was a kind gift from Nitta Gelatin (Osaka, Japan). Enzymatic hydrolysates of chicken feet gelatin (C-LAP) and chicken cartilage (C-mucolla) were prepared by Nippon Meat Packers (Osaka, Japan). All preparations were of food grade and able to be obtained

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Table 1. Characteristics of Gelatin Hydrolysates

	porcine type I gelatin hydrolysate	chicken type I gelatin hydrolysate	chicken type II gelatin hydrolysate
origin	porcine skin	chicken feet	chicken cartilage
protein	94%	92%	64%
fat	0%	0%	0%
ash	2%	2%	10%
carbohydrate	3%	6%	26%
collagen/protein	100%	100%	68%
average molecular weight	5000	12 498	12 048

commercially. Animal skin and avian feet predominantly consist of type I collagen, and cartilage consists of type II collagen. Thus, these products are referred to type I (SCP-5000 and C-LAP) and type II (C-mucolla) gelatin hydrolysates in the following sections, respectively. Characteristics of these gelatin hydrolysates are listed in **Table 1**. These data were obtained from the suppliers. For SCP-5000, a 10 g preparation, which contained 9.4 g of gelatin hydrolysate, was used for experiment 1. For C-LAP and C-mucolla, 25 g preparations, which contained 23 and 11 g of gelatin hydrolysates, were used in experiment 2, respectively.

Chemicals. Amino acid standard mixture (type H), acetonitrile (HPLC-grade), and trifluoroacetic acid (TFA) were purchased from Wako Chemicals (Osaka, Japan). Hydroxyproline (Hyp) and hydroxylysine (Hyl) were purchased from Nacalai Tesque (Kyoto, Japan) and Calbiochem (La Jolla, CA), respectively. Gly-Gly, Glu-Glu, Arg-Gly-Asp-Ser, Gly-Gly-Thr-Arg-AcHO·H_2O, Thr-Lys-Pro-Arg, and Pyr-His-Pro-NH_2 were purchased from Peptide Institue (Osaka, Japan), and Pro-Hyp was from Bachem (Bubendort, Germany). These synthetic peptides were used for molecular weight calibration for gel-filtration chromatography. All other reagents were of analytical grade or better.

Human Study Design. The experimental protocol was submitted to and approved by experimental ethical committees of the Department of Food Sciences and Nutritional Health of Kyoto Prefectural University and Nippon Meat Packers. Human studies were performed according to the Helsinki Declaration under the control of medical doctors. The potential risk of the ingestion of gelatin and the objective of the present study were informed to the volunteers. In experiment 1, five healthy volunteers with no incidence of gelatin allergy (aged from 21 to 39 years old and body weight from 45 to 80 kg) were fasted for 12 h before the experiment and given the same amount of the porcine type I gelatin hydrolysates (9.4 g of gelatin hydrolysate in 100 mL solution). After the oral ingestion, approximately 10 mL of venous blood was collected from the cubital vein before and after 15, 30, 60, and 180 min from the ingestion. Serum was prepared and stored at −80 °C until used.

In experiment 2, seven healthy male subjects aged from 25 to 37 years old and body weight from 47 to 67 kg were fasted for 12 h before the experiment and given the chicken type I or II gelatin hydrolysates at 23 or 11 g per 60 kg of body weight, respectively. After the oral ingestion, approximately 10 mL of venous blood was collected from the cubital vein at suitable intervals. Plasma was prepared and stored at −80 °C until used. The doses were determined on the basis of the results from previous animal and preclinical trials (*3*−*6*). No obvious adverse effects were observed in both experiments.

Isolation of Collagen Peptides from Blood. The serum and plasma were deproteinized by addition of three volumes of ethanol. A total of 5 mL of the ethanol-soluble fraction was dried under vacuum and dissolved in 200 μL of 30% acetonitrile in water in the presence of 0.1% TFA. For clarification, the sample was applied to a spin column (15 × 5 mm i.d., AB1150, Atto, Tokyo, Japan) packed with a Sephadex G-15 (Amersham Biosciences, Piscataway, NJ), which was pre-eqilibrated with the same solution. The effluent from the spin column by spin at 12000 rpm for 3 min was subjected to gel-filtration chromatography using a Superdex Peptide HR 10/30 (Amersham Biosciences). Elution was performed with 30% acetonitrile in water in the presence of 0.1% TFA over 1 h at 0.5 mL/min. Fractions were collected every 1 min. Peptide fractions obtained by the gel-filtration HPLC were further fractionated by reversed-phase (RP)-HPLC using two different columns, Cosmosil MS-5C18 (250 × 4.6 mm i.d., Nacarai tesque) or Inertsil ODS-3 (250 × 4.6 mm i.d., GL Science, Tokyo, Japan). Elution was performed with 0.1% TFA for 15 min followed by a linear gradient to 50% acetonitrile in the presence of 0.1% TFA over 15 min at 1 mL/min. Absorbance at 214 nm was monitored. The columns were maintained at 40 °C.

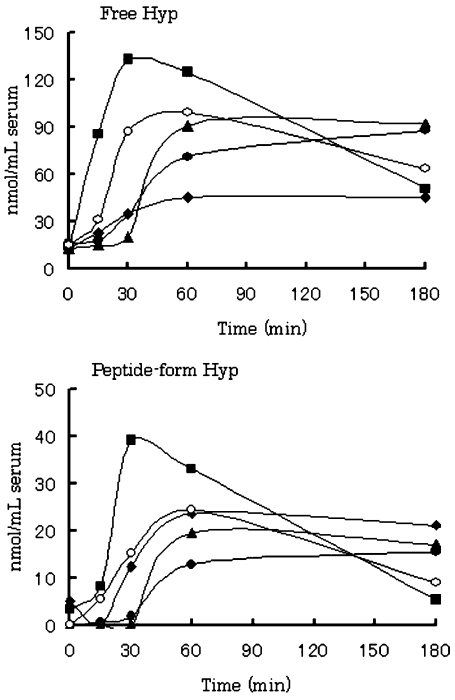

Figure 1. Serum levels of hydroxyproline in free (top) and peptide forms (bottom) after oral ingestion of the porcine type I gelatin hydrolysate (9.4 g). Data from all subjects are shown. Subjects, ○, 45 kg female 21 years old; ●, 45 kg female 23 years; ■, 55 kg female 23 years; ▲, 80 kg male 39 years; ◆, 65 kg male 25 years.

Digestion of Pro-Hyp by Human Serum Peptidase. Synthetic Pro-Hyp was mixed with human serum to give 200 nmol/mL, and the reaction mixture was sterilized by passing through a 0.25 μm pore-size filter (Millex, Millpore, Billerica, MA) and incubated at 37 °C for a suitable interval. Free Hyp liberated from Pro-Hyp by the reaction was determined.

Other Analytical Procedures. Amino acid analysis was performed by the method of Bidlingmeyer et al. (*16*) with slight modifications (*17*). Resultant phenylthiocarbamyl amino acids were separated by a Supersphare RP-18 (e) column (250 × 4 mm, Merck) at 0.8 mL/min according to binary gradient elution as described previously (*17*). The peptide form of Hyp was estimated by subtracting free Hyp from total Hyp in the HCl hydrolysate of the ethanol-soluble fraction of serum and plasma. The peptide sequence was determined by the automatic Edoman degradation method using a PPSQ-21 (Shimadzu, Kyoto, Japan).

Statistical Analyses. One-way ANOVA and multiple-comparison test of Fisher's PLSD were used to evaluate the increase of free and peptide forms of Hyp in human plasma. These analyses were performed using StatView version 4 (Abacus Concept, Berkeley, CA).

RESULTS

Collagen Peptide Level in Serum or Plasma. *Experiment 1.* To simulate ingestion of a bottled or canned beverage containing gelatin hydrolysate, the subjects were given the same dose of the porcine type I gelatin hydrolysate solution (9.4 g/100 mL). As shown in **Figure 1**, only negligible amounts of free and peptide forms of Hyp were observed before the ingestion. After the oral ingestion, free and peptide forms of Hyp in the serum significantly increased and reached a maximum level after 30−60 min. The ratio of the peptide form of Hyp to the free one was approximately 1:3.

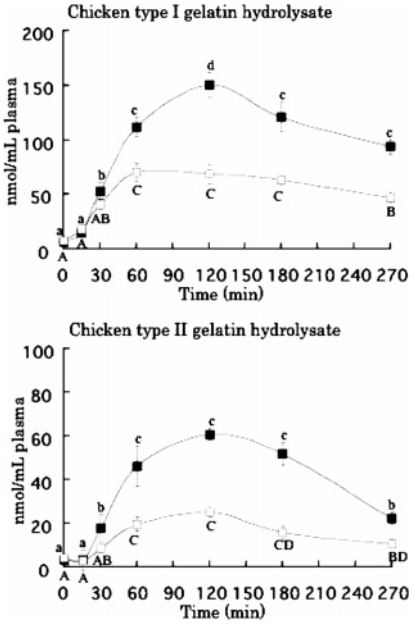

Figure 2. Plasma levels of hydroxyproline in free and peptide forms after oral ingestion of the chicken type I (top) and II (bottom) gelatin hydrolysates (23 or 11 g of gelatin hydrolysate/60 kg of body weight, respectively). (■) Free Hyp, (□) peptide form of Hyp. Data were shown as mean ± SE, $n = 7$. Different letters indicate significant difference ($p < 0.05$).

Experiment 2. The subjects were given 23 g of the chicken type I gelatin hydrolysate or 11 g of the chicken type II gelatin hydrolysate per body weight of 60 kg. As shown in **Figure 2**, only negligible amounts of the peptide form of Hyp were observed before the ingestion. The peptide form of Hyp increased significantly and reached maximum levels (60 and 25 nmol/mL of plasma, respectively) after 60 and 120 min from the ingestion, respectively. The ratio of the peptide form of Hyp to the free one was also approximately 1:3. For one subject, blood was collected after 12 and 24 h from the ingestion of the chicken type I or type II gelatin hydrolysates. The peptide form of Hyp level returned to the initial level after 12 h.

Identification of Food-Derived Collagen Peptides in Blood. As shown in **Figure 3A**, the porcine type I gelatin hydrolysate consists of peptides with molecular weight from 1000 to 20 000 Da based on the elution volume from a gel-filtration column (Superdex peptide). Only negligible amounts of peptide less than 500 Da were contained.

Elution patterns of the peptide and amino acid in the ethanol-soluble fraction of the serum from a subject (○ in **Figure 1**) are shown in parts **B** and **C** of **Figure 3**. Before the ingestion, high molecular weight (>5000 Da) peptide and free amino acid were observed. However, only negligible amounts of Hyp were detected in all fractions. After 60 min from the ingestion, Hyp was observed in the low molecular weight peptide and amino acid fractions. On the other hand, no significant change was observed in the high molecular weight peptide fraction. The low molecular weight peptide fraction was further fractioned with the RP-HPLC by using two different columns. All peaks obtained were subjected to sequence analysis. As shown in **Figure 4**, peaks yielding peptide sequence are marked with arrows. Inertsil ODS-3 column, which has higher hydrophobicity than Cosmosil MS-5C18, showed better resolution for hydrophilic peptides, such as Pro-Hyp. On the other hand, better resolution of hydrophobic peptides, such as Ile-Hyp, Leu-Hyp, Phe-Hyp, and Phe-Leu, were obtained by the Cosmosil MS-5C18 column. The high molecular weight peptide fraction was also subjected to the RP-HPLC analysis. No peptide with a

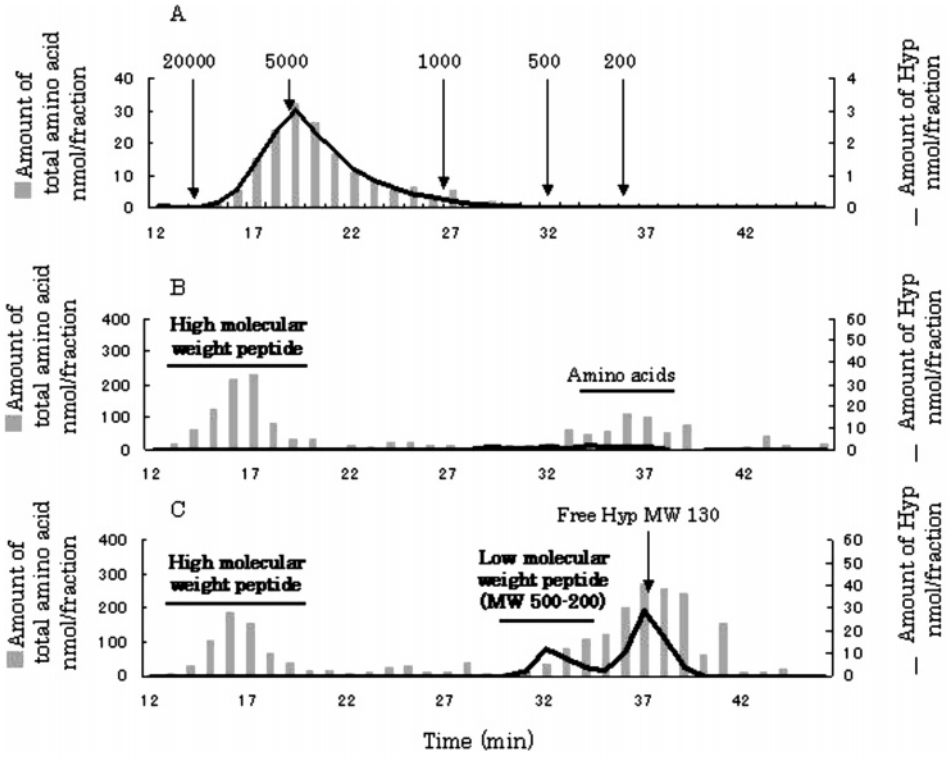

Figure 3. Fractionation of peptides in the porcine type I gelatin hydrolysate and ethanol-soluble fraction of serum by gel-filtration chromatography. (A) porcine type I gelatin hydrolysate. (B) Ethanol-soluble fraction of serum from the subject (○ in **Figure 1**) before the ingestion. (C) Ethanol-soluble fraction of serum collected after 60 min from the ingestion of the porcine type I gelatin hydrolysate. Arrows indicate the elution position of molecular weight marker peptides.

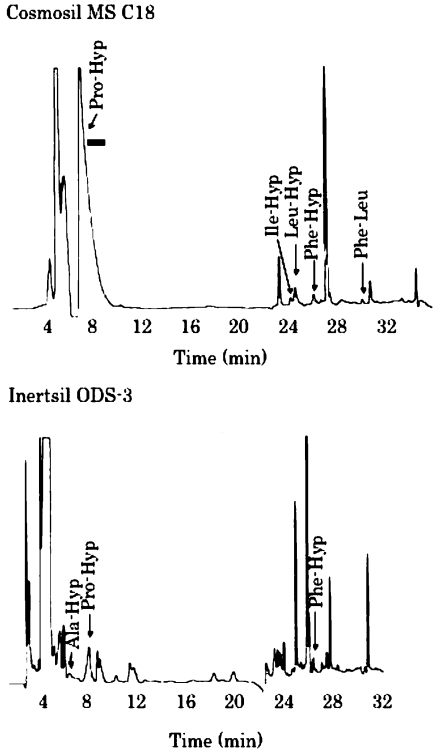

Figure 4. Isolation of food-derived collagen peptides in serum by reversed-phase mode HPLC by using the two different columns. Collagen peptide fraction obtained by the gel-filtration chromatography as shown with a bar in **Figure 3** were further fractionated. Each peak was subject to amino acid composition and sequence analyses. Peptide peaks are indicated by arrows. Pro-Hyp was eluted in a tailing peak indicated with a bar from the Cosmosil MS C18 column. Absorbance at 214 nm was monitored.

Table 2. Summary of Structure and Recovery of Food-Derived Collagen Peptide in Human Serum or Plasma after Oral Ingestion of Gelatin Hydrolysates

sequence	porcine type I	chicken type I	chicken type II
Ala-Hyp-Gly	nd[a]	>3%	>4%
Pro-Hyp	95%	92%	>70%
Pro-Hyp-Gly	nd	>3%	>19%
Ile-Hyp	>1%	>1%	>1%
Leu-Hyp	>3%	>1%	>5%
Phe-Hyp	>1%	>1%	>1%

[a] Not detected.

collagen sequence was found in the high molecular weight peptide fraction, while some fibrin fragments were isolated.

The low molecular weight peptide fractions were also prepared from the plasma after the ingestion of types I and II gelatin hydrolysates. As shown in **Figure 6**, Ala-Pro-Hyp, Pro-Hyp, Pro-Hyp-Gly, Ile-Hyp, Leu-Hyp, and Phe-Hyp were identified.

The collagen peptide composition in human serum and plasma at a maximum level was semiquantitatively estimated on the basis of the Hyp content in the each peptide peak. As shown in **Table 2**, from the subjects who ingested type I gelatin hydrolysates, Pro-Hyp was a major constituent and the other peptides accounted for few percents. As shown in **Figure 5**, the Pro-Hyp peak was also detected in the serum obtained after 30 and 180 min from the ingestion of the porcine type I gelatin hydrolysate. The peak area of Pro-Hyp varied with the content of the peptide form of Hyp as shown in **Figure 1**. Together with these facts, Pro-Hyp can be concluded to be a major constituent in human blood after the ingestion of the type I gelatin hydrolysates. On the other hand, a significant amount (19%) of Pro-Hyp-Gly was observed with Pro-Hyp (70%) in the plasma from a subject who ingested type II gelatin hydrolysates.

In vitro digestion of Pro-Hyp by human serum was carried out to estimate digestibility of Pro-Hyp. Pro-Hyp was degraded slowly. Even after a 24 h reaction at 37 °C, only a quarter of Hyp in Pro-Hyp was liberated (date not shown).

DISCUSSION

In the present study, we isolated and identified some food-derived collagen peptides in human serum and plasma as show in **Table 2**. Among them, Pro-Hyp, which has been demonstrated to be present in urine (*15*), is a major constituent in any case. In the case of the oral ingestion of chicken type II gelatin hydrolysates, a significant amount of Pro-Hyp-Gly was detected in human plasma. This motif is also abundantly present in type I and II collagens. However, only a less amount of Pro-Hyp-Gly was observed in the blood from those who ingested type I gelatin hydrolysates. The chicken type II gelatin hydrolysate preparation contained a significant amount of mucopolysaccharide (**Table 1**), which might affect digestion and absorption of collagen peptides. Another tripeptide, such as Gly-Pro-Hyp, could not be detected in all cases. Some dipeptides consisting of hydrophobic amino acids (Ile, Leu, and Phe) and Hyp are contained in human blood as minor constituents after loading of the gelatin hydrolysates. So far up to now, biological activities of them have not been described.

In vivo and *in vitro* experiments have demonstrated that oligo peptides are frequently degraded in a short time by peptidase in blood. For example, half-lives of Ala-Tyr, Gly-Tyr, Ala-Gln, and Gly-Gln in human blood after intravenous injection have been demonstrated to be 0.8, 2, 2.5, 8.6 min, respectively (*18*, *19*). In addition, N-terminal-blocked peptides such as *N*-acetyl-Ser-Asp-Lys-Pro and Pyr-His-Pro-NH_2 (thyrotropin-releasing hormone, TRH) also decrease to half within 5 and 10 min, respectively (*20*, *21*). However, food-derived collagen peptides in human blood decreased to half of the maximum level after 4 h from the oral ingestion. In addition, more than 75% of Pro-Hyp remained after the *in vitro* reaction with human serum for 24 h. Therefore, Pro-Hyp can be considered as one of the indigestible peptides against peptidase in human blood. Pro-Hyp has been demonstrated to be excreted in urine without degradation (*15*). On the other hand, collagen peptides larger than Pro-Hyp-Gly could not be detected in all cases, while the gelatin hydrolysates used in the present study contained only negligible amounts of collagen peptides of less than 500 Da. These facts strongly suggest that the larger collagen peptides in the gelatin hydrolysates are degraded into tri- and dipeptides and free amino acids in the digestive tract and other organs.

There are few data on the content of food-derived peptides in human blood. Recently, Matsui et al. demonstrated that Val-Tyr, a peptide derived from sardine meat hydrolysates, increases to approximately 2 pmol/mL in plasma after the oral ingestion of 12 mg of Val-Tyr, which could be potentially generated from 8 g of the sardine meat hydrolysate (*22*, *23*). The present study demonstrates the presence of Pro-Hyp in human plasma at 25−60 nmol/mL after oral ingestion of 9.4−23 g of gelatin hydrolysates. The extensively higher level of Pro-Hyp in blood could be partly explained by the abundance of the Pro-Hyp motif in collagen. On the basis of the primary structure of type I collagen subunits, approximately 1.7 g of Pro-Hyp might be potentially liberated from 25 g of gelatin. Nevertheless, a 10 000

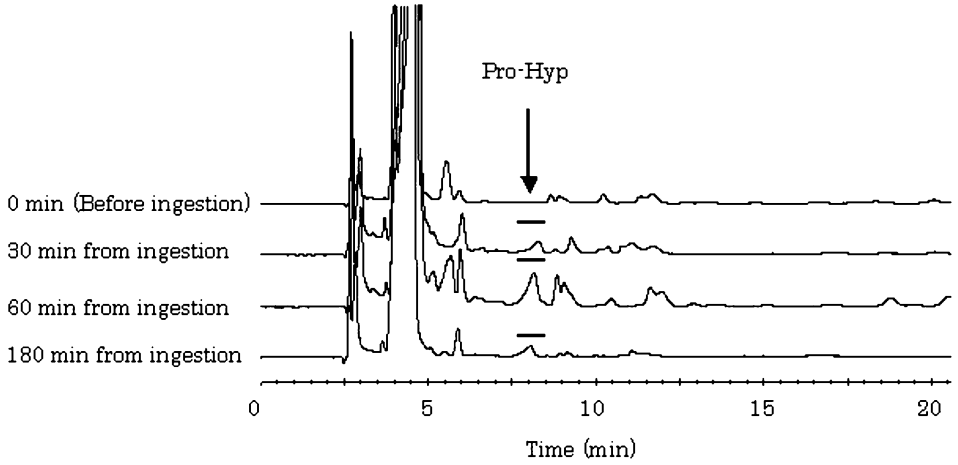

Figure 5. Elution of Pro-Hyp from the reversed-phase HPLC. The low molecular weight peptide fractions of gel-filtration chromatography from the serum obtained before and after the ingestion at 30, 60, and 180 min were analyzed. The elution position of Pro-Hyp is identified with a bar.

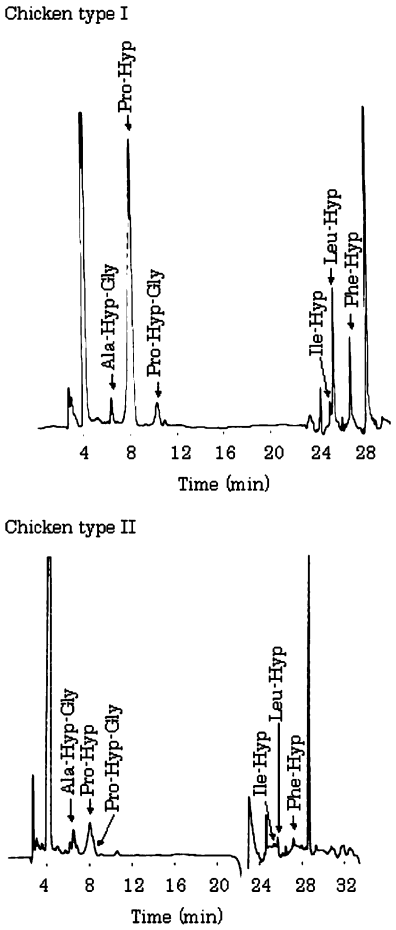

Figure 6. Isolation of food-derived collagen peptides in plasma by the reversed-phase HPLC by using Inertsil ODS-3. The low molecular weight peptide fractions from the plasma after the ingestion of chicken type I or II gelatin hydrolysates at 60 and 120 min were used, respectively. Each peak was subject to amino acid composition and sequence analyses. Peptide peaks are indicated by arrows with a sequence. Absorbance at 214 nm was monitored.

times higher level of food-derived collagen peptides was observed in human blood in comparison with Val-Tyr in the blood level. Thus, we suggest that indigestibly of Pro-Hyp may also account for its higher blood level.

Pro-Hyp has been also observed in the blood of patients suffering from bone metastases of prostatic cancer and osteoarthritis (*24*, *25*). Then, Pro-Hyp in serum has been considered as a bone resorption marker. In addition, some *in vitro* studies demonstrated that Pro-Hyp and Pro-Hyp-Gly have chemotactic activity to human fibroblast and peripheral blood neutrophils and monocytes in the cell culture system (*7*−*9*). Migration of these cells has been demonstrated to play a critical role in the early stage of wound healing. These facts suggest that Pro-Hyp might act as a biological messenger of degradation of the extracellular matrix and trigger the wound healing process by stimulating migration of fibroblast and so on. In addition, collagen-derived oligo peptides have been demonstrated to show other biological activities, such as the inhibition of angiotensin-converting enzyme (*11*, *12*), platelet activation (*13*), and so on. The present data on the food-derived collagen peptides enable us to design an *in vitro* experiment to examine the potential biological effects by ingestion of gelatin-based products. In addition, human trials are in progress to confirm the suggested beneficial effects by oral ingestion of the gelatin hydrolysates.

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