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 GlyPro-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.
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-AcH0H0, Thr-Lys-Pro-Arg, and Pyr-His-Pro-NH2 were purchased from Peptide Institute (Osaka, Japan), and Pro-Hyp was from Bachem (Bubendorf, 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. Plasma 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-equilibrated 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 Supersphere 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-SC18 (250 × 4.6 mm i.d., Nacarai

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**Table 1. Characteristics of Gelatin Hydrolysates**

<table>
<thead>
<tr>
<th></th>
<th>porcine type I gelatin hydrolysate</th>
<th>chicken type I gelatin hydrolysate</th>
<th>chicken type II gelatin hydrolysate</th>
</tr>
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<tbody>
<tr>
<td>origin</td>
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<td>chicken feet</td>
<td>chicken cartilage</td>
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<tr>
<td>protein</td>
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<td>92%</td>
<td>64%</td>
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<td>2%</td>
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<td>100%</td>
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<tr>
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**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.
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 (O 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
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 hydrolysates. 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 shown 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 oligopeptides 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-NH₂ (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
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 indigestibility 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 lymphocytes 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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LITERATURE CITED


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