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Comparison of Quantity and Structures of Hydroxyproline-Containing Peptides in Human Blood after Oral Ingestion of Gelatin Hydrolysates from Different Sources

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We compared quantity and structures of food-derived gelatin hydrolysates in human blood from three sources of type I collagen in a single blind crossover study. Five healthy male volunteers ingested type I gelatin hydrolysates from fish scale, fish skin, or porcine skin after 12 h of fasting. Amounts of free form Hyp and Hyp-containing peptide were measured over a 24-h period. Hyp-containing peptides comprised approximately 30% of all detected Hyp. The total area under the concentration−time curve of the fish scale group was significantly higher than that of the porcine skin group. Pro-Hyp was a major constituent of Hyp-containing peptides. Ala-Hyp, Leu-Hyp, Ile-Hyp, Phe-Hyp, and Pro-Hyp-Gly were detected only with fish scale or fish skin gelatin hydrolysates. Ala-Hyp-Gly and Ser-Hyp-Gly were detected only with fish scale gelatin hydrolysate. The quantity and structure of Hyp-containing peptides in human blood after oral administration of gelatin hydrolysate depends on the gelatin source.

KEYWORDS: Collagen; gelatin; gelatin hydrolysate; peptide; absorption

INTRODUCTION

Collagen is a major constituent of connective tissues of animals, birds, and fish. Gelatin, a denatured form of collagen, is prepared on an industrial scale from these materials (*1*). Gelatin is also used as folk medicine to improve blood circulation, arrest bleeding (*2*), and improve joint condition by reducing pain (*3*). Some animal experiments and preclinical human trials have also suggested that oral ingestion of gelatin hydrolysate might have beneficial effects (*4*). Recently, Wu et al. demonstrated the safety of oral ingestion of a high dose (1.66 g/kg of body weight) of collagen hydrolysate in an animal model (*5*). Type II collagen is the major protein in articular cartilage. A dietary supplement of type II collagen was given to alleviate rheumatoid arthritis (*6*). The intake of collagen was quite small in that study, so it is difficult to understand how the type II collagen was supplied to joint cartilage. The mechanism of alleviation of rheumatoid arthritis by dietary collagen is unknown.

Type I collagen is the major matrix protein of skin. We reported that daily ingestion of a type I collagen hydrolysate mixture (including 5 g of fish type I collagen hydrolysate) improved skin properties (*7*). Therefore, it has been hypothesized that supplementation with collagen hydrolysate promotes collagen synthesis in the skin. However, it is difficult to grasp how collagen could be absorbed and transported to the dermis because an average molecular weight of collagen is about 300000. Further experiments are necessary to obtain more detailed information about the mechanisms of improvement of skin properties. We hypothesized that a signal is transmitted to skin fibroblasts after oral administration of collagen hydrolysate.

Oesser et al. determined the bioavailability of collagen hydrolysate after oral administration in mice using ^{14}C-labeled hydrolysate (*8*). The distribution of labeled amino acids in skin was confirmed, and 58% of the peak value of labeled amino acids remained 192 h after administration. Iwai et al. identified a small peptide (Pro-Hyp) in the blood of healthy human volunteers who ingested porcine skin gelatin hydrolysates (*9*). It was found that some small peptides such as carnosine (beta-Ala-L-His) were absorbed from the small intestine (*10*). It is well-known that the abundance of the oligopeptide transporter (PEPT-1) in the brush-border membrane of the intestinal epithelium is the principal mechanism for regulation of transport of products of protein digestion (dipeptides and tripeptides) (*11*). It may be possible for Hyp-containing di- or tripeptides to be absorbed transcellularly, at least partly, via this peptide transporter. In an in vitro study using a cell culture system, some Hyp-containing peptides (Pro-Hyp-Gly, Pro-Hyp) had chemotactic activity for fibroblasts, peripheral blood neutrophils (*12*, *13*), and monocytes (*14*). These facts suggest that oral intake of collagen or gelatin hydrolysate, possibly generated by degradation of extracellular matrix, might be associated with wound healing and inflammation. Regarding gelatin hydrolysate,

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Table 1. Characteristics of Gelatin Hydrolysates

	fish scale gelatin hydrolysate	fish skin gelatin hydrolysate	porcine skin gelatin hydrolysate
protein	97%	90%	94%
fat	0.1%	0%	0%
ash	0.3%	3.0%	2.0%
carbohydrate	2.6%	7.0%	2.0%
collagen/protein	100%	100%	100%
average molecular weight	5000	5000	5000

Iwai et al. also reported that the structure of food-derived peptides in human blood differed between the type I and type II collagens (*9*). This result indicated that the structure and amount of food-derived peptides in human blood is different according to the collagen type and collagen source. Therefore, the biological activity of orally administered collagen, gelatin, or their hydrolysates is thought to be different according to collagen type or collagen source. However, there are few data available for comparison of the structures and amounts of Hyp-containing peptides in human blood. Thus, this study compared quantity and structures of Hyp-containing peptides in human blood with respect to three kinds of type I collagen.

MATERIALS AND METHODS

Gelatin Hydrolysates. Enzymatic hydrolysate of fish scale gelatin and porcine skin gelatin were a kind gift from Nitta Gelatin (Osaka, Japan). Enzymatic hydrolysate of fish skin gelatin was purchased as a food ingredient in a market. All preparations were of food grade and can be obtained commercially. Animal skin and fish scale consist predominantly of type I collagen. Thus, these products are referred to as type I gelatin hydrolysates in the following sections. Characteristics of these gelatin hydrolysates are listed in **Table 1**. Average molecular weight of these gelatin hydrolysates were 5000, and free form of Hyp was not contained.

Chemicals. Amino acid standard mixture (type H), acetonitrile (HPLC grade), and trifluoroacetic acid (TFA) were purchased from Wako Pure Chemical Industries (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 Institute (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. This study was designed as a single blind crossover study. The subjects took three kinds of gelatin hydrolysate with a 1-week washout period, respectively. This study was performed according to the Helsinki Declaration and was approved by the local Ethical Committee. Five healthy male volunteers with no incidence of gelatin allergy (33.0 ± 5.6 years old and 69.8 ± 7.4 kg of body weight) participated in this study. Subjects did not take in any food or beverages except for water in the 12 h period prior to the experiment. On the morning of the experiment, subjects were fasting and each subject orally took the fish scale, fish skin, or porcine skin gelatin hydrolysate concentrates (0.385 g/kg of body weight) in water (20% w/v). Three hours after ingestion of the gelatin hydrolysate preparation, the subjects were served collagen-free lunch, consisting of only rice ball with salt. At the supper, the subjects were served vegetarian food which does not including any meats or fish. Approximately 10 mL of venous blood was collected from the cubital vein before and 0.5, 1, 2, 4, 7, and 24 h after the ingestion. Until the 24 h blood draw, the subjects were fasting from their last supper. Plasma was prepared and stored at −80 °C until use.

Isolation of Small Peptide Fraction from Blood. The plasma was 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 Sephadex G-15 (Amersham Biosciences, Piscataway, NJ) that was pre-equilibrated with the same solution. The column was spun at 12000 rpm for 3 min and the effluent 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 reverse-phase (RP)-HPLC using 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.

Other Analytical Procedures. Amino acid analysis was performed by the method of Bidlingmeyer et al. (*15*) with slight modifications. Resultant phenylthiocarbamyl amino acids were separated by a Supersphare RP-18 (e) column (250 × 4 mm, Merck, Darmstadt, Germany) at 0.8 mL/min with a binary gradient elution as described previously (*16*). The amount of the peptide form of Hyp was estimated by subtracting free Hyp from total Hyp in the HCl hydrolysate of the ethanol-soluble fraction of plasma. The peptide sequence was determined by the automatic Edman degradation method using a protein sequencer, PPSQ-21 (Shimadzu, Kyoto, Japan).

Pharmacokinetic Analysis. Analysis of blood concentration−time data was done with a noncompartment model using WinNonlin Professional (version 3.1, Pharsight Co., Mountain View, CA). The total area under the concentration−time curve [AUC(o→obs)] was calculated by the trapezoidal rule based on the plasma concentrations up to the time of final measurement using WinNonlin Professional program.

Statistical Analyses. Statistical analyses were carried out using SPSS version 10.02 for Windows with the data expressed as mean ± S.D. For statistical evaluation, the increase and [AUC_{0-24h}] of free form Hyp and Hyp-containing peptides in human plasma were analyzed by one-way ANOVA with a multiple-comparison test of Dunnet and a Tukey post hoc test.

RESULTS

Free Form Hyp and Hyp-Containing Peptide Levels in Plasma. The amount of free form Hyp in plasma after administration of gelatin hydrolysate is shown in **Figure 1A**. Only negligible amounts of free form Hyp were observed before the administration of gelatin hydrolysate. In all subjects, free form Hyp in the plasma increased significantly after oral ingestion and reached a maximum 1 to 2 h after administration. The amount of free form Hyp in the fish scale group was significantly higher than that in the fish skin group 2 and 7 h after administration. The amount of free form Hyp in the porcine skin group was also significantly higher than that in the fish skin group 2, 4, and 7 h after administration.

The amount of Hyp-containing peptide in plasma after administration of gelatin hydrolysate is shown in **Figure 1B**. Hyp-containing peptide was present in negligible amounts before administration. In almost all subjects, Hyp-containing peptides increased significantly after administration and reached maximum levels 2 h afterward. The amount of Hyp-containing peptide in the fish scale group was significantly higher than that in both the fish skin and porcine skin groups 1 and 2 h after administration. The amount of Hyp-containing peptide in the porcine group was significantly higher than that in the fish skin group 2 h after administration. The fish scale group had a significantly higher amount of Hyp-containing peptide than the fish skin group 4 h after administration. In all subjects, the Hyp-containing peptide level returned to the initial zero level 7 h after administration.

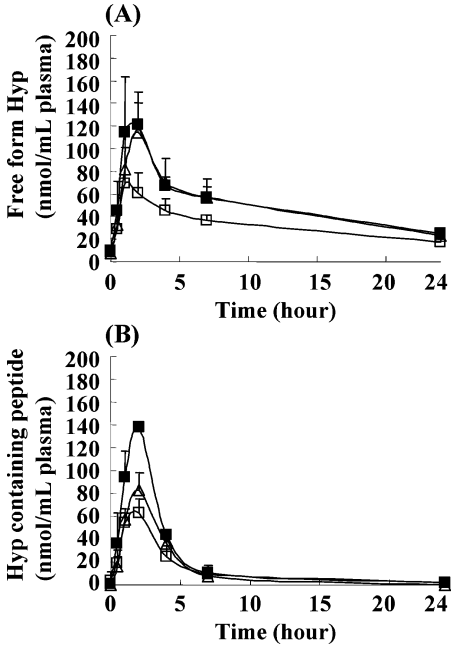

Figure 1. Amount of free form Hyp and Hyp-containing peptide in plasma after oral ingestion of fish scale (■), fish skin (□), and porcine skin (Δ) gelatin hydrolysates. (A) Amount of free form Hyp in plasma (nmol/mL). (B) Amount of Hyp-containing peptide in plasma (nmol/mL). Data are presented as the mean ± S.D., $n = 5$. Statistical evaluation was analyzed by one-way ANOVA with a multiple-comparison test of Dunnet and a Tukey post hoc test.

Table 2. AUC_{0-24h} of Free Form Hyp and Hyp-Containing Peptide in Human Plasma after the Ingestion of Gelatin Hydrolysates

	AUC_{0-24h} (hr nmol/mL)[a]	
gelatin hydrolysates	free form Hyp	peptide form Hyp
fish scale	1238 ± 116^b	522 ± 62^b
fish skin	799 ± 99^c	315 ± 67^c
porcine skin	1194 ± 305^b	345 ± 66^c

[a] Values are the mean ± S.D., $n = 5$; those with different superscript letters differ, $P < 0.05$. The AUC_{0-24h} is calculated by the trapezoidal rule using WinNonlin Professional program. Statistical evaluation was analyzed by one-way ANOVA with a multiple-comparison test of Dunnet and a Tukey post hoc test.

Comparison of the Total Areas under the Concentration Time Curves. The AUC_{0-24h} of both free form Hyp and Hyp-containing peptide are shown in **Table 2**. The AUC_{0-24h} of Hyp-containing peptides in the fish scale group was significantly higher than that of both the fish skin and porcine skin groups. The fish scale value was approximately 1.5-fold higher than the skin value. The AUC_{0-24h} of the fish skin group was significantly lower than that of the porcine group. In the case of free form Hyp, fish skin gelatin hydrolysate showed a significant decrease as compared with fish scale and porcine skin gelatin hydrolysate. In all groups, the Hyp in Hyp-containing peptide was approximately 20−30% of all detected Hyp.

Identification of Food-Derived Peptides in Blood after Gelatin Hydrolysate Administration. Analysis was carried out with the 1-h postadministration samples of all groups. Ethanol-soluble fractions of plasma were collected and fractionated by a gel-filtration column. The high molecular weight peptide fraction (>MW 500) was not identified. The low molecular weight peptide fraction (MW 200−500) was collected and further fractioned with the RP-HPLC. All peaks of the RP-HPLC were fractionated and subjected to sequence analysis. All of the peptides identified in human plasma are summarized in **Table 3**. The quantities of peptides were semiquantitatively estimated on the basis of the Hyp content in each peptide peak. In the case of the fish scale group, Pro-Hyp (39%) was the major component; however, many other peptides were observed including Ala-Hyp-Gly (16%), Ser-Hyp-Gly (12%), Leu-Hyp (10%), Pro-Hyp-Gly (5%), Phe-Hyp (3%), and Ile-Hyp (2%). In the fish skin group, Pro-Hyp (42%) was also the major component; and the other components were Leu-Hyp (27%), Ala-Hyp (15%), Ile-Hyp (7%), Phe-Hyp (7%), and Pro-Hyp-Gly (3%). The porcine skin group composition was different from those of the fish origin groups. Pro-Hyp (95%) was also the major constituent, but other peptides were scarce. Only three peptides (Ile-Hyp, Leu-Hyp, and Phe-Hyp) were detected but the amounts were just a few percent. The high molecular weight fraction (>MW 5000) was also subjected to RP-HPLC analysis in human blood. But no peptide with a collagen sequence was found in this high molecular weight fraction of human blood.

Table 3. Summary of Structure and Recovery of Food-Derived Collagen Peptide in Human Plasma after Oral Ingestion of Gelatin Hydrolysates

sequence	fish scale	fish skin	porcine skin
Ala-Hyp	15%	15%	nd[a]
Ala-Hyp-Gly	16%	nd	nd
Ser-Hyp-Gly	12%	nd	nd
Pro-Hyp	39%	42%	95%
Pro-Hyp-Gly	5%	3%	nd
Ile-Hyp	2%	7%	>1%
Leu-Hyp	10%	27%	>3%
Phe-Hyp	3%	7%	>1%

[a] Not detected.

DISCUSSION

After protein administration, food-derived peptides are detected in human blood. Matsui et al. reported that the dipeptide Val-Tyr was observed in the plasma 2 h after oral administration (*17*). Park et al. also reported that dietary carnosine (dipeptide, beta-alanyl-L-histidine) is absorbed into human plasma after the consumption of beef (*18*). Iwai et al. identified a small peptide (Pro-Hyp) in blood in humans who ingested porcine skin gelatin hydrolysates (*9*). However, the variation of bioavailability from collagen hydrolysates from different sources was not determined.

In this study, we compared quantity and structures of food-derived gelatin hydrolysate peptides in human blood from three different sources of type I collagen. In the case of free form Hyp, the AUC_{0-24h} varied in the order of fish scale gelatin hydrolysate (1238 ± 116) ≥ porcine skin gelatin hydrolysate (1194 ± 305) > fish skin gelatin hydrolysate (799 ± 99) (**Table 2**). On the other hand, the AUC_{0-24h} of Hyp-containing peptide varied in the order of fish scale gelatin hydrolysate (522 ± 62) > porcine scale gelatin hydrolysate (345 ± 66) ≥ fish skin gelatin hydrolysate (315 ± 67). In all cases, the ratio of the peptide form of Hyp to free Hyp was approximately 2:7−3:7. These results suggest Hyp is absorbed as both the amino acid (free form) and the peptide form with a profile similar to that reported by Iwai et al. (*9*). Moreover, the amount of Hyp-containing peptide differed according to the gelatin source.

Pro-Hyp is always a major peptide in human plasma after oral ingestion of any gelatin hydrolysates. Our result is similar to the results for the oral ingestion of chicken type I and II collagens (*9*). In the case of fish scale and fish skin gelatin hydrolysates, significant amounts of Ala-Hyp and Leu-Hyp were

detected in human plasma. Moreover, Ala-Hyp-Gly and Ser-Hyp-Gly were detected in human plasma 1 h after oral ingestion of fish scale gelatin hydrolysate. Because these tripeptides were also identified in the plasma 4 h after administration, we think these peptides have a resistance to digestion by the plasma and gastrointestinal peptidases. Iwai et al. also reported that the minor peptides (Ile-Hyp, Leu-Hyp, Phe-Hyp, and Phe-Leu) in human blood differed between the chicken type I and type II collagens (*9*). These results indicate that the structure and amount of food-derived peptides in human blood differs according to the collagen type and collagen source. Therefore, the biological activity of orally administered collagen, gelatin, or their hydrolysates is thought to depend on the collagen type or collagen source.

Saito et al. compared the amino acid sequences of type I collagen from rainbow trout, human, chicken, and frog (*19*). They indicated that the quantity of an amino acid sequence such as Gly-Pro-Hyp differed with the collagen origin. And Ala is the rich amino acid in fish collagen compared to the mammalian collagen. Thus, we expected that a difference of amino acid sequences of collagens would result in a difference in the peptides detected, such as Ala-Hyp, Ala-Hyp-Gly, Ser-Hyp-Gly, and Leu-Hyp, in human plasma after oral administration of fish scale or skin gelatin hydrolysate.

It has been shown in cell culture systems that some Hyp-containing peptides (Pro-Hyp-Gly, Pro-Hyp) have chemotactic activity for fibroblasts, peripheral blood neutrophils (*12*, *13*), and monocytes (*14*). These facts suggest that oral intake of collagen or gelatin hydrolysate possibly generated by degradation of extracellular matrix might be associated with wound healing and inflammation. In the present study, the amount of Hyp-containing peptide differed with the gelatin source. These facts suggest that the biological activity with oral administration of collagen depends on the collagen type or collagen origin.

It is known that Ala-Hyp is an inhibitor of angiotensin-converting enzyme (*20*, 21). In the present study, Ala-Hyp was detected in human plasma only in the case of fish gelatin hydrolysate. This result suggests that the physiological activity of food-derived collagen peptide depends on the collagen origin.

Our results suggest that Hyp-containing peptide is resistant to decomposition and digestion through the gastrointestinal tract. Hyp-containing peptide might have physiological activity after oral administration of gelatin hydrolysate. Further experiments are necessary to obtain more detailed information about the mechanisms behind the effects seen in this study.

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