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Dose-dependent changes in the levels of free and peptide forms of hydroxyproline in human plasma after collagen hydrolysate ingestion

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ABSTRACT

The presence of hydroxyproline (Hyp)-containing peptides in human blood after collagen hydrolysate ingestion is believed to exert beneficial effects on human health. To estimate the effective beneficial dose of these peptides, we examined the relationship between ingested dose and food-derived Hyp levels in human plasma. Healthy volunteers ($n = 4$) ingested 30.8, 153.8 and 384.6 mg per kg body weight of collagen hydrolysate. The average plasma concentration of Hyp-containing peptides was dose-dependent, reaching maximum levels of 6.43, 20.17 and 32.84 nmol/ml following ingestion of 30.8, 153.8 and 384.6-mg doses of collagen hydrolysate, respectively. Ingesting over 153.8 mg of collagen hydrolysate significantly increased the average concentrations of the free and peptide forms of Hyp in plasma. The Hyp absorption limit was not reached with ingestion of as much as 384.6 mg of collagen hydrolysate. These finding suggest that ingestion of less than 30.8 mg of collagen hydrolysate is not effective for health benefits.

1. Introduction

Collagen is a major proteinaceous component of the extracellular matrix and contains a specific amino acid, hydroxyproline (Hyp). Genetically, 28 types of collagen have been identified and are classified by type and Roman numeral (Veit et al., 2011). The most abundant collagen in mammalian tissues such as the dermis, blood vessels and tendons is type I. The heat-denatured form of collagen, referred to as gelatin, is often used in foods, pharmaceuticals, photographic films and cosmetics. Gelatin is also used in folk medicine in Asia to improve blood circulation and arrest bleeding (Yao, Zhang, & Zhou, 1989). The oldest description of beneficial health effects associated with gelatin ingestion was written in 1175 by St. Hildegard, who indicated that gelatin ingestion improves joint conditions by reducing pain (Moskowitz, 2000). Other human studies have demonstrated that daily ingestion of gelatin leads to improvement of brittle nails and increases in hair diameter (Rosenberg, Oster, Kallos, & Burroughs, 1957; Scala, Hollies, & Sucher, 1976).

In order to increase the solubility of gelatin, partially hydrolysed gelatin products have been developed and are referred to as collagen hydrolysates. Ingestion of collagen hydrolysate has been shown to moderate joint pain in athletes and subjects with knee osteoarthritis, as effectively as gelatin (Clark et al., 2008; Deal & Moskowitz, 1999). Both human and animal studies have reported improvements in skin condition associated with collagen hydrolysate ingestion. Matsumoto, Ohara, Itoh, Nakamura, and Takahashi (2006) suggested that a daily ingestion of collagen hydrolysate increases the moisture content of the epidermis of women during winter. Zhuang, Hou, Zhao, Zhang, and Li (2009) and Tanaka, Koyama, and Nomura (2009) reported that collagen hydrolysate ingestion suppresses UV-induced skin damage in mice. In addition to these studies, Saito, Kiyose, Higuchi, Uchida, and Suzuki (2009) reported that levels of total lipids and triglycerides in rat plasma decrease after ingestion of collagen hydrolysate.

A number of food-derived peptides have been identified in human blood following ingestion of collagen hydrolysate, including Pro-Hyp, Pro-Hyp-Gly, Ala-Hyp, Ala-Hyp-Gly, Ser-Hyp, Ser-Hyp-Gly, Leu-Hyp, Ile-Hyp, Phe-Hyp and Hyp-Gly, of which Pro-Hyp and Hyp-Gly are the major peptides found (Iwai et al., 2005; Ohara, Matsumoto, Ito, Iwai, & Sato, 2007; Shigemura et al., 2011). A previous study involving a murine chondrocytic cell line demonstrated that Pro-Hyp inhibits mineralization of chondrocytes and modulates expression of the Runx1 and osteocalcin genes (Nakatani, Mano, Sampei, Shimizu, & Wada, 2009). Ohara, Iida, Ito, Takeuchi, and Nomura (2010) reported that Pro-Hyp stimulates hyaluronic acid production in cultured synovial cells.

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On the other hand, we demonstrated that Pro-Hyp and Hyp-Gly stimulate the growth of mouse skin fibroblasts cultured on collagen gel (Shigemura et al., 2009, 2011). Minaguchi et al. (2012) reported that Pro-Hyp reduces the size of lipid droplets in mouse 3T3-L1 preadipocytes. These studies suggest that food-derived Hyp-containing peptides in human blood have significant roles in promoting beneficial effects pertaining to the skin, joints and plasma lipids. However, the effective dose of collagen hydrolysates for these beneficial effects has not been determined. Therefore, in the present study, we determined the relative levels of the free and peptide forms of Hyp in human plasma after ingestion of different doses of collagen hydrolysate in order to estimate the effective dose for promoting health benefits.

2. Materials and methods

2.1. Collagen hydrolysate

Collagen hydrolysate was prepared from cod (*Gadus macrocephalus*) skin gelatin by enzymatic hydrolysis and was a kind gift from Yaizu Suisankagaku Industry (Shizuoka, Japan). The preparation used in this study consisted mainly of peptides with a molecular weight of 1–5 kDa (Tables 1 and 2).

2.2. Chemicals

The amino acid standard mixture (Type H), hydroxyproline, acetonitrile (HPLC-grade) and phenyl isothiocyanate (PITC) used in the study were purchased from Wako Chemicals (Osaka, Japan). All other reagents were of analytical grade or better.

2.3. Human study

The human study was carried out according to a previously described protocol (Iwai et al., 2005). The study was performed according to the Helsinki Declaration under the supervision of medical doctors and was approved by the experimental ethical committee of Yaizu Suisankagaku Industry. Previous human studies have reported no negative effects associated with collagen hydrolysate ingestion (Iwai et al., 2005; Matsumoto et al., 2006; Ohara et al., 2007), and the safety of high-dose collagen hydrolysate ingestion has been demonstrated in animal experiments (Wu, Fujioka, Sugimoto, Mu, & Ishimi, 2004). The volunteers were informed of the objectives of the study and the potential risks of ingestion of collagen hydrolysate, such as diarrhoea and abdominal pain. Before the experiment, four healthy volunteers (two males and two females, average age 27 years) fasted for 12 h before ingesting 30.8, 153.8 and 384.6 mg per kg (2, 10 and 25 g per 65 kg) body weight of collagen hydrolysate dissolved in 100 ml of water. All four volunteers ingested three different doses of collagen hydrolysate with a week-long washout period between ingestions. Approximately 10 ml of venous blood was collected from the cubital vein of each subject before and 15, 30, 60, 120, 240 and 360 min after ingestion. Plasma prepared from each venous blood sample was then deproteinised by adding three volumes of ethanol, after which the ethanol-soluble fraction was removed and stored at −80 °C prior to analysis.

Table 1
Characteristics of collagen hydrolysate.

Components	%
Protein	97.00
Fat	0.10
Carbohydrate	0.00
Ash	0.10
Water	2.80

Table 2
Molecular weight distribution.

MW	%
−1000	4.13
1000–5000	60.03169
5000–	35.83437

2.4. Estimation of Hyp-containing peptide levels in human plasma

Amino acid analysis was performed according to the method of Bidlingmeyer, Cohen, and Tarvin (1984) with slight modifications (Iwai et al., 2005; Sato et al., 1992). Amino acids in the ethanol-soluble fraction of plasma were derivatised with PITC, and the resulting phenyl thiocarbamyl (PTC)-amino acids were resolved on a LiChro CART 250-4.0 column (Kanto Kagaku, Tokyo, Japan) using an LC-20 series HPLC system (Shimadzu, Kyoto, Japan). Binary gradient elution was performed with 150 mM ammonium acetate containing 5% acetonitrile (pH 6.0) (solvent A) and 60% acetonitrile (solvent B) as the mobile phases at a flow rate of 0.5 ml/min. The column was equilibrated with 100% solvent A prior to the analysis. The gradient profile was as follows: 0-1 min, 0% B; 1.0-1.01 min, 0-10% B; 1.01–20 min, 10–47.5% B; 20–25 min, 47.5–100% B; 25–37 min, 100% B; 37–37.1 min, 100–0% B; and 37.1–50 min, 0% B. The column was maintained at 45 °C and the absorbance of the eluate was monitored at 254 nm. The levels of Hyp-containing peptides in the ethanol-soluble fraction were estimated by subtracting the concentration of free Hyp from that of total Hyp in the HCl hydrolysate, as described previously (Iwai et al., 2005).

2.5. Statistical analysis

Differences between means were evaluated by analysis of variance followed by Fisher's protected least significant difference (PLSD) method ($P < 0.05$) using StatView software, version 5.0 (Abacus Concepts, Berkeley, CA, USA).

3. Results

3.1. The concentrations of free and peptide-incorporated Hyp in human plasma

The average changes in the concentrations of free and peptide-incorporated Hyp over time in the plasma of human volunteers following collagen hydrolysate ingestion are shown in Figs. 1 and 2, respectively. The concentrations of both forms of Hyp began to increase at 0.5 h and reached a maximum level within 2 h after hydrolysate ingestion. The concentration of Hyp-containing peptides in plasma increased in a dose-dependent manner, reaching maximum levels of 6.43, 20.17 and 32.84 nmol/ml after ingestion of 30.8, 153.8, and 384.6 mg doses, respectively, of collagen hydrolysate. Although the level of free Hyp decreased to two-thirds of its maximum 6 h after ingestion, the level was still significantly higher than before ingestion. At 4–6 h after ingestion, the level of Hyp-containing peptides returned to the pre-ingestion level (Fig. 2).

Ingestion of 153.8 and 384.6 mg doses of collagen hydrolysate significantly increased the levels of free and peptide-incorporated Hyp in plasma. However, ingestion of a 30.8 mg dose of collagen hydrolysate had no significant enhancing effect on the plasma level of either form of Hyp. Regarding the maximum amount of total Hyp (free + peptide-incorporated) in plasma, one-fourth was in the form of Hyp-containing peptides. Table 3 shows the maximum

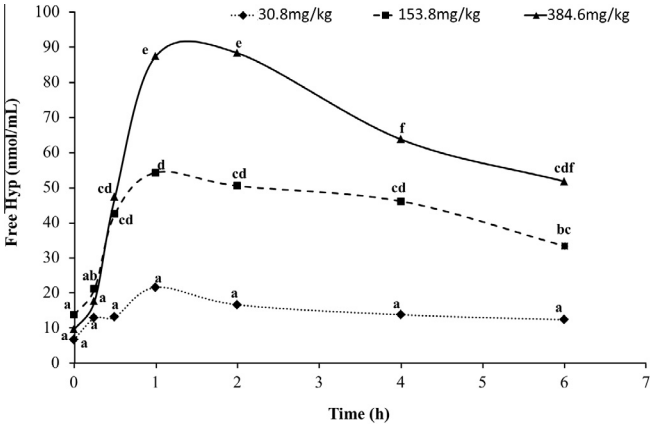

Fig. 1. Plasma level of free hydroxyproline after ingestion of 30.8 mg (▲), 153.8 mg (■) and 384.6 mg (♦) of collagen hydrolysate/kg body weight. Data are shown as the mean ± SD; $n = 4$. Different letters adjacent to data points indicate significant differences ($P < 0.05$).

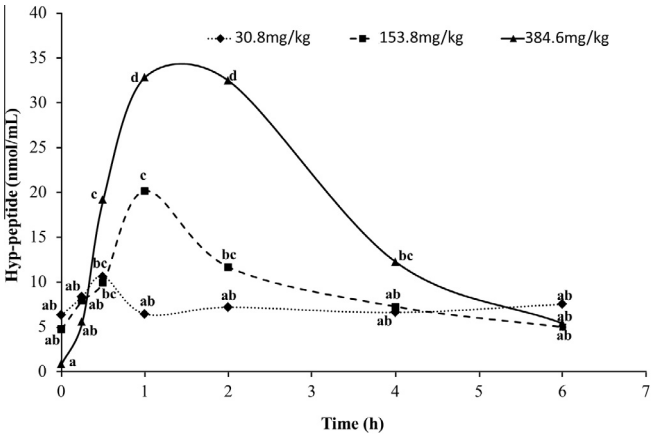

Fig. 2. Plasma level of the peptide form of hydroxyproline after ingestion of 30.8 mg (▲), 153.8 mg (■) and 384.6 mg (♦) of collagen hydrolysate/kg body weight. Each time point shows the average peptide-incorporated hydroxyproline concentrations of 4 subjects. Data are shown as the mean ± SD; $n = 4$. Different letters adjacent to data points indicate significant differences ($P < 0.05$).

concentrations of free and peptide-incorporated forms of Hyp in each subject after ingestion. The maximum level and the time required to reach that level varied between subjects. Only one subject (No. 3) reached a limit in the absorption of Hyp-peptides, which occurred with ingestion of the 153.8 mg dose of collagen hydrolysate.

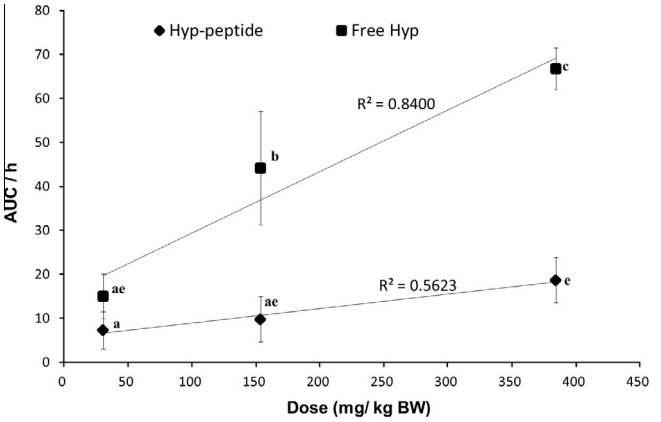

Fig. 3. Relationship between the Hyp area under the curve (AUC)/h and collagen hydrolysate dose. The AUC/h of free Hyp (■) and peptide-bound Hyp (♦) were calculated by the linear trapezoidal method from the data of 4 subjects after ingestion of collagen hydrolysate (30.8, 153.8 and 384.6 mg/kg body weight [BW]). Data are shown as the mean ± SD; $n = 4$. Different letters adjacent to data points indicate significant differences ($P < 0.05$).

3.2. Total area under the concentration–time curve (AUC/h)

As shown in Fig. 3, the area under the concentration–time curves (AUC/h) was calculated from the levels of free and peptide-incorporated forms of Hyp using the trapezoidal rule. There was no significant difference in AUC/h values between the free and peptide forms of Hyp with ingestion of the 30.8 mg collagen hydrolysate dose. In contrast, the AUC/h of free Hyp was 3-fold higher than that of Hyp-containing peptides following ingestion of 153.8 and 384.6 mg doses of collagen hydrolysate. The coefficient of determination for the relationship between the dose of collagen hydrolysate and the AUC/h calculated from the levels of free and peptide-incorporated forms of Hyp were 0.8400 and 0.5623 for the 153.8 and 384.6 mg doses, respectively.

4. Discussion

In the present study, we examined changes in the levels of food-derived, free and peptide-incorporated forms of Hyp in human plasma after ingestion of different doses of collagen hydrolysate. The results showed that ingestion of more than 153.8 mg (per kg body weight) of collagen hydrolysate leads to a significant increase in the levels of both Hyp forms in plasma. The results also suggest that ingestion of a dose of only 30.8 mg of collagen hydrolysate might not be effective in promoting beneficial effects on skin, joints and blood lipid concentrations. No limit in the amount of

Table 3
Maximum food-derived Hyp levels in human-plasma and time reached at maximum level.

		Ingestion dose		
	Subject No.	30.8 (mg/kg)	153.8 (mg/kg)	384.6 (mg/kg)
Free Hyp nmol/mL (h)	1	10.95 (1)	34.00 (1)	102.72 (1)
	2	21.25 (1)	49.47 (1)	81.06 (1)
	3	31.07 (1)	74.59 (1)	115.12 (2)
	4	22.89 (1)	68.43 (1)	87.95 (2)
	Average	21.53	56.87	96.72
Hyp-peptides nmol/mL (h)	1	18.45 (2)	19.85 (1)	24.70 (2)
	2	20.29 (0.25)	19.91 (2)	54.56 (2)
	3	8.53 (0.5)	30.42 (1)	29.07 (1)
	4	6.94 (2)	12.45 (1)	56.03 (1)
	Average	13.55	20.66	41.09

A number in parentheses indicates the time (h) reached at maximum level after the ingestion.

Hyp that could be absorbed into the plasma was observed with ingestion of up to 384.6 mg of collagen hydrolysate per kg of body weight. Administration of [^{14}C]-Pro-Hyp in rats results in a wide distribution of radioactivity in the skin and articular cartilage (Kawaguchi, Nanbu, & Kurokawa, 2012). The present results suggest that ingestion of larger doses of collagen hydrolysate enhances the distribution of food-derived Hyp-containing peptides in tissues and the consequent beneficial effects on the skin and joints.

As shown in Fig. 3, the correlation between collagen hydrolysate dose and AUC/h value was higher for free Hyp (R^2 = 0.8400) than the peptide-incorporated form (R^2 = 0.5623). As shown in Table 2, based on its molecular weight, collagen hydrolysate contains very little free Hyp (MW = 131.13 Da). The data indicate that a higher proportion of free Hyp is absorbed into the blood rather than Hyp-containing peptides and/or free Hyp is increased by enzymatic hydrolyzatation of Hyp-containing peptides in blood. Food-derived collagen peptides of more than two or three amino acids have not been detected in human blood (Ichikawa et al., 2010; Iwai et al., 2005; Ohara et al., 2007). Several studies demonstrated that peptide transporter-1 (PEPT1) plays a significant role in the absorption of di- and tri-peptides in enterocytes (Adibi, 1997; Aito-Inoue, Lackeyram, Fan, Sato, & Mine, 2007). These data suggest that digestion of higher molecular weight collagen hydrolysates might produce long-chain Hyp-containing peptides that cannot be absorbed into the blood. More efficient absorption of food-derived collagen peptides into the blood via PEPT1 might be accomplished by altering the molecular weight of collagen hydrolysate so that it can be digested into di- and/or tri-peptides.

Increasing the absorption of Hyp-containing peptides would also enhance the beneficial effect of collagen ingestion on human health. It has been reported that the ingestion of collagen hydrolysate along with fermented milk products increases the plasma levels of various collagen-derived amino acids (e.g., glycine, proline, hydroxyproline and hydroxylysine) (Walrand, Chiotelli, Noirt, Mwewa, & Lassel, 2008). The results of Walrand et al. (2008) suggest that activating the digestion of higher molecular weight collagen hydrolysates by addition of food matrices may lead to more effective digestion into di- and/or tri-peptides.

Clark et al. (2008) and Trc and Bohmova (2011) reported that ingestion of collagen hydrolysate for 24 or 13 weeks, respectively, results in improvement in joint conditions. Moreover, Matsumoto et al. (2006) and Postlethwaite et al. (2008) reported improvement in skin conditions after ingestion of collagen hydrolysate for 6 weeks or 12 months, respectively.

Food-derived Hyp-containing peptides have been detected in human blood after only a single ingestion of collagen hydrolysate (Ichikawa et al., 2010; Iwai et al., 2005; Ohara et al., 2007). However, to obtain a complete practical understanding of how Hyp-containing peptides exert beneficial effects in the human body, changes in the blood concentrations of these peptides following repetitive ingestion of collagen hydrolysate must be examined. As shown in Fig. 2, differences between individuals were observed in terms of food-derived Hyp-containing peptide absorption. It is likely that differences between peptidase activity in individuals affected cleavage of larger collagen hydrolysate molecules into small peptides. This factor could result in individual differences in beneficial effects derived from collagen hydrolysate ingestion. As discussed above, development of readily absorbed forms of collagen hydrolysate and effective repetitive ingestion over a prolonged period may reduce these individual differences. Further studies aimed at determining how levels of food-derived collagen peptides change in human blood after repetitive ingestion and ingestion with food additive matrices are currently in progress in our laboratory.

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