Quantification of hydroxyprolyl-glycine (Hyp-Gly) in human blood after ingestion of collagen hydrolysate

Fumihito Sugihara,¹,* Naoki Inoue,¹ Masanori Kuwamori,² and Makoto Taniguchi²

Collagen is denatured to gelatin by heating, and the collagen hydrolysate (CH) formed by the hydrolysis of this gelatin in the presence of enzymes is used in foods and cosmetics. It has been found that, after oral ingestion of CH, not only amino acids but also di- and tri-peptides were assimilated in human peripheral blood and that the peptides remained in the blood for a relatively long time (1–3). After the ingestion, Pro-Hyp, Pro-Hyp-Gly, Ala-Hyp, Ala-Hyp-Gly, Ser-Hyp-Gly, Leu-Hyp, Ile-Hyp, and Phe-Hyp have been identified as CH-derived peptides in the blood (4). Of these peptides, Pro-Hyp has been reported to stimulate cell proliferation, cell growth and hyaluronic acid synthesis in situ and in vitro (5–7). Pro-Hyp also exerted chondroprotective effects in the articular cartilage and synovium cells (5–7). Pro-Hyp-Gly was transported across the rat small intestinal tract into the blood using the in situ vascular perfusion techniques (9). The objective of the present study was to quantify Hyp-Gly in the blood of subjects by liquid chromatography–tandem mass spectrometry (LC–MS/MS) after oral ingestion of CH.

Collagen hydrolysate was prepared from porcine skin gelatin by enzymatic hydrolysis. This can be obtained commercially (Collapep PU, Nitta Gelatin Inc.), and consisted of peptides with the average molecular weight of 1200 Da. containing a small amount of Pro-Hyp and Hyp-Gly. The human study was carried out according to a protocol described by Iwai et al. (1). This study was performed according to the Helsinki Declaration and was approved by the Ethical Committee of Nitta Gelatin Inc. Five male volunteers (mean age, 39.8±7.9; mean body weight, 68.6±8.8) ingested 8 g of the CH dissolved in 100 ml of water, and the venous blood samples were collected before and after the ingestion of CH. The blood samples were collected with the collaboration of Dr. Shigehiro Arita at Arita Clinic (Osaka, Japan). The plasma prepared from the samples was then deproteinized by adding three volumes of ethanol and ethanol-soluble fraction was stored at −80°C until analysis. The amounts of (a) free Hyp and (b) total Hyp in the plasma samples were measured for the CH at the measurement time, and the amount of Hyp-containing peptide (Hyp-peptide) [(b)−(a)] was calculated. Measurements were carried out by high-performance liquid chromatography (HPLC; TSK-GEI ODS-80TsQ, Tosoh, Tokyo, Japan), and the phenylisothiocyanate (PITC; Wako Pure Chemical Industries, Osaka, Japan) labeling method was used for the N-terminus (4). After the plasma samples were diluted with 50 mM aqueous ammonium bicarbonate solution, Pro-Hyp and Hyp-Gly were detected and measured by liquid chromatography–tandem mass spectrometry (LC–MS/MS). The measurement conditions were as follows: HPLC/NANOSPACE SI-2 (Shiseido Co., Ltd., Tokyo, Japan); column/HyperSil GOLD PFP (2.1×150 mm, Thermo Fisher Scientific Inc., MA, USA); elution condition, (i) 0.2% formic acid and 2 mM aqueous solution of ammonium acetate and (ii) methanol in gradient condition; injection volume, 5 μl; column temperature, 40°C. A tandem mass spectrometer (TSQ Vantage, Thermo Fisher Scientific) was used. The ionization method was positive electrospray ionization (ESI) at 1500 V. The selective reaction monitoring (SRM) conditions were as follows: Pro-Hyp plus charge ESI SRM ms/ms, 229.1→70.3 at 41 eV; Hyp-Gly plus charge ESI SRM ms/ms, 189.1→85.9 at 16 eV.

As shown in Fig. 1, the concentration of Hyp-peptide increased to maximum level (32.3 nmol/ml) 1 h after the CH ingestion. The concentration decreased half of the maximum 4 h after the ingestion. Hyp-Gly and Pro-Hyp were identified in all subjects by LC–MS/MS. The ratio of Hyp-Gly to Pro-Hyp in human plasma 1 h after the ingestion was distributed ranging from 6.3% to 22.1% (Table 1). As shown in Fig. 2, the concentration of Hyp-Gly increased to maximum
level (4.2 nmol/ml) 1 h after the ingestion. The concentration decreased one-quarter of the maximum 4 h after the ingestion.

In the present study, Hyp-Gly, which has not been detected in the previous studies,(1-4) is quantified in the blood of all the five subjects by LC–MS/MS. Hyp-Gly can be detected by using the LC–MS/MS system, because CH used contained a small part of Hyp-Gly. Very recently, Shigemura et al. also identified Hyp-Gly as a novel food-derived collagen peptide in human peripheral blood by pre-column derivatization with phenyl isothiocyanate. The ratio of Hyp-Gly to Pro-Hyp depended on subjects and ranged from 0.00 to 5.04 (10). As Hyp-Gly inhibited the differentiation of mouse osteoclasts in vitro and in vivo (Mano, H., Nakatani, S., Sekiguchi, Y., Shimizu, J., Sugihara, F., Haketa, Y., and Wada, M., Abstr. 27th Annu. Meet. Jpn. Soc. Bone Miner. Res., p. 227, 2009), Hyp-Gly may play active roles in the bone tissue.

We thank Professor Kenji Sato, Faculty of Life and Environmental Sciences, Kyoto Prefectural University, for quantifying Hyp peptides in human plasma.

References


