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Efficient Absorption of X-Hydroxyproline (Hyp)-Gly after Oral Administration of a Novel Gelatin Hydrolysate Prepared Using Ginger Protease

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ABSTRACT

Recent studies have reported that oral intake of gelatin hydrolysate has various beneficial effects, such as reduction of joint pain and lowering of blood sugar levels. In this paper, we produced novel gelatin hydrolysate using cysteine-type ginger protease having unique substrate specificity with preferential peptide cleavage with Pro at the P_2 position. Substantial amounts of X-hydroxyproline (Hyp)-Gly-type tripeptides were generated up to 2.5% (w/w) concomitantly with Gly-Pro-Y-type tripeptides (5%; w/w) using ginger powder. The *in vivo* absorption of the ginger-degraded gelatin hydrolysate was estimated using mice. The plasma levels of collagen-derived oligopeptides, especially X-Hyp-Gly, were significantly high (e.g., 2.3-fold for Glu-Hyp-Gly, $p<0.05$) compared with that of the control gelatin hydrolysate, which was prepared using gastrointestinal proteases and did not contain detectable X-Hyp-Gly. This study demonstrated that orally administered X-Hyp-Gly was effectively absorbed into blood probably due to high protease resistance of this type of tripeptides.

KEYWORDS: *collagen, gelatin hydrolysate, hydroxyproline, ginger, absorption, LC–MS*

INTRODUCTION

Collagens are the most abundant proteins in the body. The structural characteristics of the collagen family include a repeating Gly-X-Y sequence (X and Y = any amino acid) and a high content of Pro (approximately 200 residues/1000 amino acid residues) in the primary amino acid sequence. Almost all Pro residues lying in the Y position are post-translationally modified to 4-hydroxyproline (4-Hyp) (approximately 100 residues/1000 amino acid residues), which stabilizes the collagen triple helix.[1] Heat-denatured collagen, referred to as gelatin, is extracted from skin, bone, and fish scales. Gelatin hydrolysate prepared by partial hydrolysis of the extracted gelatin is widely used as supplemental food for its recently reported beneficial effects, such as reducing joint pain,[2, 3] increasing bone density,[4, 5] lowering blood pressure,[6, 7] and lowering blood sugar levels.[8, 9] Previous studies have also reported the bioactivities of collagen-derived oligopeptides constituting gelatin hydrolysate prepared by various combinations of enzymatic proteolysis.[10] For example, numerous Gly-X-Y-type tripeptides can be generated by collagenase digestion of gelatin.[11, 12] A study reported a yield of 4–9% (w/w) for Gly-Pro-Hyp by *Streptomyces* collagenase and a competitive inhibitory effect of the tripeptide on dipeptidylpeptidase-IV (DPP-IV).[12] In addition, other studies reported angiotensin I-converting enzyme (ACE) inhibitory effects of Gly-Pro-Leu, Gly-Pro-Met, and Gly-Pro-Val,[13, 14] and an anti-platelet effect of Gly-Pro-Arg.[15]

From more than 50 years ago, peptide-bound Hyp has been known to appear in blood at a significantly high concentration compared with other food-derived peptides.[16] Sato et al. recently identified various kinds of Hyp-containing oligopeptides in human blood after oral ingestion of gelatin hydrolysate.[17-19] The collagen-derived dipeptides and tripeptides are transported into intestinal cells via peptide transporters.[20-22] Pro-Hyp is the most abundant collagen-derived

peptide in blood and was demonstrated to have various physiological functions, such as stimulation of the growth of skin fibroblasts,[23, 24] improvement of skin barrier dysfunction,[25] and enhancement of hyaluronic acid synthesis.[24] Another major blood constituent of collagen-derived peptides, Hyp-Gly, was reported to also stimulate fibroblast growth and improve the skin condition.[19, 25] X-Hyp-Gly-type tripeptides, including Ala-Hyp-Gly, Glu-Hyp-Gly, Pro-Hyp-Gly, and Ser-Hyp-Gly, are also detected in blood at high concentrations after the ingestion of gelatin hydrolysate,[17, 26] suggesting that they have biological activities. However, there have been only a few studies relevant to the function of X-Hyp-Gly,[27, 28] and to date, none have reported the efficient production of this type of tripeptides from collagen/gelatin.

Ginger protease (also known as "zingibain") was first reported by Ichikawa et al. in 1973.[29] They identified two isoforms of the protease from ginger rhizome (*Zingiber officinale*), named GP I and GP II, which are papain family cysteine proteases. Other studies have suggested that there are three forms of ginger protease.[30, 31] Ginger protease has high protease activity toward various proteins, such as gelatin and casein,[32, 33] and is the only reported plant collagenase with the ability to hydrolyze native collagen.[33, 34] Ginger protease has been studied for some industrial applications, including meat tenderization[35, 36] and milk clotting.[37, 38] Herein, to produce novel gelatin hydrolysate, we focused on ginger protease's unique substrate specificity with preferential peptide cleavage with Pro at the P_2 position.[34, 39] Kim et al. investigated the activities and cleavage sites of ginger protease using fluorescent Pro-containing peptide substrates and showed that GP2 (virtually identical to GP II) and GP3 preferably cleaved peptide substrates with Pro at the P_2 position.[34] However, the substrate specificity assay was not conducted for Hyp-containing peptides. If ginger protease recognizes Hyp at the P_2 position, collagen/gelatin that contain many Pro and Hyp residues may be good substrates. The potential recognition of

Hyp by ginger protease was expected to lead to efficient production of X-Hyp-Gly-type tripeptides in addition to Gly-Pro-Y-type tripeptides arising from the specificity for P_2 Pro.

In the present study, we first examined the effects of a reducing agent and reaction pH on oligopeptide generation by gelatin digestion using ginger powder containing ginger protease. Then, oral administration experiments of the ginger-degraded gelatin hydrolysate were performed using mice to estimate the absorption of collagen-derived oligopeptides compared with the control gelatin hydrolysate prepared using gastrointestinal proteases, including pepsin, trypsin, and chymotrypsin. The plasma concentrations of Hyp and 21 collagen-derived oligopeptides were comprehensively measured by liquid chromatography–mass spectrometry (LC–MS).

MATERIALS AND METHODS

Chemicals. Pepsin, trypsin, chymotrypsin, pepsin–agarose, Dulbecco's phosphate buffered saline (DPBS) with calcium chloride and magnesium chloride, and *trans*-4-hydroxy-L-proline were purchased from Sigma-Aldrich (St. Louis, MO). SILAC DMEM medium, dialyzed FBS, $^{13}C_6$-Lys, and $^{13}C_6{}^{15}N_4$-Arg were purchased from Thermo Scientific (Hudson, NH). Dithiothreitol (DTT), Gly-Gly, and L-ascorbic acid phosphate magnesium salt *n*-hydrate were purchased from Wako Chemicals (Osaka, Japan). Sequencing grade trypsin and sequencing grade chymotrypsin were purchased from Promega (Madison, WI). Vivaspin 20-10K was purchased from GE Healthcare (Piscataway, NJ), and $^{13}C_5{}^{15}N_1$-Pro was purchased from Cambridge Isotope Laboratories (Andover, MA). Gly-Ala, Gly-Pro, Pro-Ala, Pro-Hyp, Hyp-Gly, Gly-Ala-Hyp, Gly-Pro-Ala, Gly-Pro-Arg, and Gly-Pro-Hyp were purchased from Bachem (Bubendorf, Switzerland), acetyl-Ile-Ser-Val-Pro-Gly-Pro-Met-Gly-Pro-Ser-Gly-Pro-Arg-Gly-Leu-Hyp-Gly-

Pro-Hyp-Gly-Cys was custom synthesized by Sawady Technology (Tokyo, Japan), and other peptides were custom synthesized by AnyGen (Kwangju, Korea). Ginger rhizomes were purchased from a local supermarket. Pepsin-solubilized skin collagen was prepared from bovine skin as described previously.[40]

Ethics Statement. All animal studies were approved by the Experimental Ethical Committee of Nippi Research Institute of Biomatrix (approval number: 130723).

Preparation of Ginger Powder. Ginger rhizomes were cut into small pieces after peeling and then frozen at −30°C. The ginger dices were homogenized in five volumes (w/v) of chilled acetone by a Polytron homogenizer CH-6010 (Kinematica, Kriens-Luzern, Switzerland). The homogenate was filtered, and the residue was rinsed with five volumes of chilled acetone. After air drying, the ginger powder was stored at 4°C.

Gelatin Digestion Using Ginger Powder. Pepsin-solubilized bovine skin collagen was denatured at 60°C for 30 min, and the gelatin solution was lyophilized using a Virtis Genesis 25EL freeze dryer (SP Industries, Gardiner, NY). Then, 50 mg/mL gelatin solution containing 0 or 2 mM DTT was prepared in 100 mM sodium acetate buffer (pH 3.6–5.6) or 100 mM sodium phosphate buffer (pH 6.0–6.4). Gelatin hydrolysis by ginger powder (1/10 of gelatin; w/w) was performed at 50°C for 16 h with shaking. After the reaction, the digest was diluted in 10 mM ammonium acetate for LC–MS analysis of collagen-derived oligopeptides.

Preparation of Gelatin Hydrolysates. Three kinds of gelatin hydrolysates were prepared using ginger powder or gastrointestinal proteases, including pepsin, trypsin, and chymotrypsin. Gelatin solution (50 mg/mL, pH 4.0 adjusted by HCl) containing 2 mM DTT was digested with ginger powder (1/50 or 1/10 of gelatin; w/w) at 50°C for 16 h with shaking. After the reaction, the ginger powder was removed by filtration. Another gelatin solution (50 mg/mL, pH 2.5

adjusted by HCl) was digested with pepsin (1/50 of gelatin; w/w) at 37°C for 24 h. Subsequently, digestion with trypsin and chymotrypsin (1/50 of gelatin, respectively; w/w) were performed at 37°C for 16 h following pH adjustment to 8.0 by NaOH. The three resulting peptide solutions were acidified with HCl (final 0.03 M), filtered through a 0.8-μm filter, and subjected to ultrafiltration using Vivaspin 20-10K devices to remove the proteases. The flow-through fraction was lyophilized by freeze drying. The lyophilized gelatin hydrolysates were washed with acetone to remove DTT and redried using a centrifugal evaporator CVE-3100 (EYELA, Tokyo, Japan). The ginger-degraded high molecular weight gelatin hydrolysate (G-HMW-GH), ginger-degraded low molecular weight gelatin hydrolysate (G-LMW-GH), and pepsin and trypsin/chymotrypsin-degraded gelatin hydrolysate (PTC-GH) were used for the oral administration experiments.

Size Exclusion Chromatography. The average molecular weight of gelatin hydrolysates was determined by size exclusion chromatography using a Superdex Peptide HR 10/30 column (GE Healthcare). The samples were run at a flow rate of 750 μL/min for 40 min with isocratic 20% acetonitrile/100 mM ammonium bicarbonate using an Alliance 2690 separation module equipped with a 2487 dual absorbance detector (Waters, Milford, MA). The elution was monitored at 220 nm. Gly-Gly, Gly-Pro-Hyp, $(Gly\text{-}Pro\text{-}Hyp)_2$, $(Gly\text{-}Pro\text{-}Hyp)_3$, $(Gly\text{-}Pro\text{-}Hyp)_4$, and acetyl-Ile-Ser-Val-Pro-Gly-Pro-Met-Gly-Pro-Ser-Gly-Pro-Arg-Gly-Leu-Hyp-Gly-Pro-Hyp-Gly-Cys were used as molecular weight standards at 132 Da, 285 Da, 553 Da, 820 Da, 1087 Da, and 2003 Da (monomer)/4002 Da (disulfide-bonded dimer), respectively. Millennium 32 software version 4.01 (Waters) was used for the data acquisition and calculation of the weight-average molecular weight.

Preparation of Protease Digest of Stable Isotope-Labeled Collagen (SI-Collagen). Protease digest of SI-collagen (SI-digest) was prepared as described previously.[26] Briefly, SI-

collagen was first prepared in the culture of human embryonic lung fibroblasts with SILAC DMEM medium supplemented with 0.5% dialyzed FBS, 100 mg/L $^{13}C_6$-Lys, 100 mg/L $^{13}C_6{}^{15}N_4$-Arg, 200 mg/L $^{13}C_5{}^{15}N_1$-Pro, and 200 μM L-ascorbic acid phosphate, and purified from the culture medium by pepsin–agarose digestion and subsequent salt precipitation.[40, 41] After denaturation of the purified SI-collagen at 60°C for 30 min, digestion with sequencing grade trypsin and sequencing grade chymotrypsin (1:50 enzyme/substrate ratio, respectively) was performed in 100 mM ammonium bicarbonate/1 mM $CaCl_2$ at 37°C for 16 h. The solution was dried using the centrifugal evaporator following deactivation of the enzymes at 100°C for 5 min. The peptide mixture was further digested with freshly prepared mouse plasma, which was dialyzed against DPBS at 4°C overnight after collection from a male ICR mouse (8 weeks of age; Japan SLC, Shizuoka, Japan) fed a collagen-free diet, AIN-93M (Oriental Yeast, Tokyo, Japan), at 37°C for 24 h. After ethanol deproteinization followed by drying the ethanol-soluble fraction using the centrifugal evaporator, the SI-digest solution was reconstituted in distilled water and stored at −20°C.

Oral Administration of Gelatin Hydrolysates. Male ICR mice at 6 months of age (Japan SLC) were used for the oral administration experiments. Their normal diet was replaced with the collagen-free diet, AIN-93M, at a day before the procedure. The mice were given 20 mg of PTC-GH, G-HMW-GH, or G-LMW-GH dissolved in 500 μL of water using gastric sonde. Blood was collected from the tail vein before (0 h) and 0.5, 1, 2, 4, and 6 h after the administration. Plasma was prepared by centrifugation of the blood at 10000*g* for 10 min at 4°C and stored at −80°C until analysis. SI-digest was mixed into the plasma samples as an internal standard mixture of Hyp and collagen-derived oligopeptides. Three volumes of ethanol were added to the samples followed by centrifugation at 10000*g* for 10 min at 4°C, and the ethanol-soluble fraction was

dried using the centrifugal evaporator and reconstituted in 10 mM ammonium acetate for LC–MS analysis. The plasma concentrations of Hyp and collagen-derived oligopeptides were quantified using external calibration curves corrected with stable isotope-labeled internal standards of those analytes from SI-digest as reported previously.[26] The area under the plasma concentration–time curve ($AUC_{0-6\,h}$) was calculated using the trapezoidal rule.

Protease Resistance Assay of Collagen-Derived Oligopeptides. Mouse plasma was prepared from male ICR mice at 8 weeks of age (Japan SLC) fed the collagen-free diet, AIN-93M. Whole blood was collected from the heart in the presence of heparin and immediately centrifuged at 10000*g* for 10 min at 4°C. Synthetic Pro-Hyp, Gly-Ala-Hyp, Gly-Pro-Ala, and Ala-Hyp-Gly were mixed with the freshly prepared plasma at a concentration of 0.2 mg/mL and incubated at 37°C. Time-course samples were collected at 0, 0.5, 1, 2, 3, and 6 h of the incubation. The reactants were deproteinized by ethanol precipitation, and the ethanol-soluble fraction was diluted with 10 mM ammonium acetate for quantification of the respective peptides by LC–MS analysis.

LC–MS Analysis. Gelatin hydrolysates and plasma samples were analyzed by LC–MS using a hybrid triple quadrupole/linear ion trap 3200 QTRAP mass spectrometer (AB Sciex, Foster City, CA) coupled to an Agilent 1200 Series HPLC system (Agilent Technologies, Palo Alto, CA). The samples were loaded onto an Ascentis Express F5 HPLC column (5 μm particle size, L × I.D. 250 mm × 4.6 mm; Supelco, Bellefonte, PA) at a flow rate of 400 μL/min and separated by a binary gradient as follows: 100% solvent A (10 mM ammonium acetate) for 7.5 min, linear gradient of 0–75% solvent B (100% acetonitrile) for 12.5 min, 75% solvent B for 5 min, and 100% solvent A for 5 min. Analyst 1.6.2 (AB Sciex) was used to perform the data acquisition and analysis, and quantification of Hyp and collagen-derived oligopeptides was performed in

multiple reaction monitoring (MRM) mode. The MRM transitions of collagen-derived oligopeptides for the gelatin digestion experiments and the oral administration experiments are shown in **Tables S1 and S2 in the Supporting Information**, respectively. Capillary voltage was 3 kV, declustering potential was 15 V, heater gas temperature was 700°C, curtain gas was 15 psi, nebulizer gas was 80 psi, and heater gas was 80 psi.

Statistical Analysis. Data are expressed as the mean ± standard deviation (SD). The $AUC_{0–6\ h}$ of ginger-degraded gelatin hydrolysates (G-HMW-GH and G-LMW-GH) were compared with that of the control gelatin hydrolysate (PTC-GH) using one-way ANOVA followed by Dunnett's multiple comparison test with InStat version 3.0b for Macintosh (GraphPad Software, San Diego, CA).

RESULTS

Generation of Oligopeptides from Gelatin Using Ginger Powder. Ginger powder was prepared by homogenizing ginger rhizome in acetone followed by filtration and air drying. When stored at 4°C, the protease activity of ginger powder was maintained at 6 months after the preparation (data not shown). Ginger powder was directly added into denatured bovine skin collagen solution for gelatin digestion without purification steps of ginger protease. First, we evaluated the oligopeptides generated in the presence or absence of 2 mM DTT using LC–MS (Fig. 1; detailed data are shown in **Table S3 in the Supporting Information**). A number of oligopeptides were generated by the direct digestion using ginger powder (1/10 of gelatin; w/w) at pH 4.8 and 50°C. The most abundant peptides were Gly-Pro-Y-type tripeptides generated by the activity of ginger protease toward Pro at the P_2 position (Fig. 1A). In addition, significant amounts of X-Hyp-Gly-type tripeptides were also detected (Fig. 1B), which indicates that ginger

protease can also recognize Hyp at the P_2 position. The total amounts of generated X-Hyp-Gly and Gly-Pro-Y both increased by the addition of DTT, and the degree of increase was more significant for X-Hyp-Gly (3.7-fold) than that for Gly-Pro-Y (1.4-fold). Ala-Pro-Gly and Leu-Pro-Gly, which are derived from partial prolyl 4-hydroxylation, were also generated from the gelatin digestion using ginger powder (Fig. 1C). Gly-Pro-Hyp, Pro-Hyp-Gly, and Pro-Hyp were not detected, indicating that ginger protease can not cleave Gly-Pro and Hyp-Gly bonds. Thus, we also monitored Gly-Pro-Hyp-Gly and found that the tetrapeptide was produced in substantial amounts (Fig. 1C). Generation of these peptides was also increased by the DTT addition (1.9-fold for X-Pro-Gly and 3.5-fold for Gly-Pro-Hyp-Gly). Almost no dipeptides were detected, except for Gly-Pro and some others. The generation of a small but non-negligible amount of Gly-Pro suggests that ginger powder contains a low level of prolyl endopeptidase activity as reported previously.[39] The enhancement effect of DTT on peptide generation did not increase at concentrations more than 2 mM (data not shown).

Next, the effect of reaction pH (3.6–6.4) on the peptide generation using ginger powder in the presence of DTT was examined as shown in Fig. 2 (detailed data are shown in **Table S4 in the Supporting Information**). Interestingly, the peptide patterns that varied with the reaction pH depended on the peptide types. The highest generation of Gly-Pro-Y-type tripeptides was observed at pH 4.0 (56.6 mg/g gelatin in total), but large amounts of the tripeptides were also generated at other pHs (Fig. 2A). For example, at pH 6.4, the total amount of Gly-Pro-Y decreased to 31.8 mg/g, but it was still approximately 60% of that at pH 4.0. In contrast, the maximum generation of X-Hyp-Gly-type tripeptides was observed at pH 4.0–4.4 (26.8 mg/g in total), while this type of peptides significantly decreased with pH (Fig. 2B). The total amount of X-Hyp-Gly was only 1.8 mg/g at pH 6.4 (approximately 7% of the maximum level). These

inconsistent peptide generation patterns between Gly-Pro-Y and X-Hyp-Gly are likely related to the substrate specificity of ginger protease isoforms. The generation of Gly-Pro-Y seemed to peak at both pH 4.0 and pH 5.6–6.0, roughly consistent with a previous study reporting that proteolytic activity of ginger protease showed two peaks at pH 5.0 and pH 5.6 with BSA as substrate.[32] In contrast, X-Hyp-Gly peaked only at pH 4.0–4.4. These results suggest that one isoform showing maximum activity at pH 4.0–4.4 recognizes both Pro and Hyp at the P_2 position, and another isoform showing maximum activity at pH 5.6–6.0 preferentially recognizes Pro at the P_2 position. To evaluate the substrate specificity of ginger protease isoforms in detail, separation and purification of the enzymes are needed. The pH dependency on generation of Ala-Pro-Gly and Leu-Pro-Gly was similar to that of Gly-Pro-Y, and that of Gly-Pro-Hyp-Gly was similar to that of X-Hyp-Gly (Fig. 2C). Thus, the pH dependency apparently depended on if the P_2 position amino acid is Pro or Hyp.

Absorption of Orally Administered Gelatin Hydrolysates Prepared by Ginger Powder. The X-Hyp-Gly-type tripeptides efficiently generated from gelatin using ginger powder are known to appear in blood after oral ingestion of gelatin hydrolysate.[17, 26] Thus, we performed oral administration experiments of the ginger-degraded gelatin hydrolysate in anticipation of increases in the blood levels of X-Hyp-Gly. As shown in Table 1, we prepared two gelatin hydrolysates, abbreviated as G-HMW-GH and G-LMW-GH, using different amounts of ginger powder (1/50 or 1/10 of gelatin; w/w) under the optimized reaction condition defined in Figs. 1 and 2 (pH 4.0 with 2 mM DTT). PTC-GH was prepared using pepsin, trypsin, and chymotrypsin as the control gelatin hydrolysate with an average molecular weight matched to G-HMW-GH. The molecular weight distributions of the three gelatin hydrolysates analyzed by size exclusion chromatography are shown in Fig. 3A. The average molecular weights of PTC-GH, G-HMW-

GH, and G-LMW-GH were determined to be 917 Da, 963 Da, and 590 Da, respectively. The peak corresponding to the Gly-Pro-Hyp (285 Da) tripeptide (Fig. 3B) was significantly higher in G-LMW-GH compared with the others, and the molecular weight distribution pattern of G-HMW-GH was very similar to that of PTC-GH. While Gly-Pro-Y was contained in PTC-GH to some extent, no X-Hyp-Gly was detected in the control gelatin hydrolysate (Table 1). The total content of X-Hyp-Gly in G-HMW-GH and G-LMW-GH was 4.6 mg/g and 25.7 mg/g, respectively. The reaction conditions for G-LMW-GH corresponded to those for the above experiment (pH 4.0 in Fig. 2) and the generation of X-Hyp-Gly was equally well.

The three kinds of gelatin hydrolysates were orally administered to ICR mice, and plasma samples were obtained until 6 h after the administration. Figure 4 shows the plasma concentration–time curves of Hyp and 21 collagen-derived oligopeptides, which were simultaneously quantified by LC–MS. To ensure accurate quantification of the plasma samples, we used a recently developed internal standard mixture, named SI-digest, in which Hyp and collagen-derived oligopeptides are stable isotopically labeled.[26] In all the three groups, most peptides increased in the plasma after the administration except for Gly-Pro, Gly-Pro-Val, and Gly-Pro-Hyp-Gly. Maximum plasma concentrations were observed at 0.5 or 1 h after the administration for each peptide, and the concentrations gradually decreased with time and returned to the basal level at 6 h. The maximum concentration of each peptide is summarized in **Table S5 in the Supporting Information**. Although PTC-GH did not contain detectable X-Hyp-Gly, increases of this type of tripeptides were also observed after the administration of the control gelatin hydrolysate. The X-Hyp-Gly-type tripeptides in the PTC-GH group were probably derived from other large peptides through serial digestion by various proteases in the body. As expected, the plasma levels of X-Hyp-Gly were significantly higher in the G-LMW-

GH group compared with the PTC-GH group. In addition, the administration of G-LMW-GH resulted in high plasma concentrations for Hyp and almost all of the other oligopeptides more than expected. Similarly, the G-HMW-GH group, although to a lesser extent, was found to show high plasma levels of Hyp and almost all of the analyzed peptides compared with the PTC-GH group. The $AUC_{0–6\ h}$ of Hyp and each oligopeptide are shown in Table 2. In all the groups, the major oligopeptide in blood was Pro-Hyp (5.43–9.18 nmol/mL·h) followed by Hyp-Gly (1.19–2.57 nmol/mL·h). The plasma concentrations of the collagen-derived oligopeptides in the mouse samples were considerably lower compared with those in the human samples as described in our previous report (Pro-Hyp, 163.0 nmol/mL·h; Hyp-Gly, 17.5 nmol/mL·h),[26] possibly due to differences in interspecific protease activity and/or different administration methods (forced vs voluntarily). Upon comparison of the $AUC_{0–6\ h}$ of the G-LMW-GH and PTC-GH groups, significantly increased levels of a number of oligopeptides were observed in the G-LMW-GH group. It was noteworthy that five out of seven of the X-Hyp-Gly-type tripeptides showed a significant difference ($p<0.05$) ranging from 1.6-fold (Leu-Hyp-Gly and Phe-Hyp-Gly) to 2.3-fold (Glu-Hyp-Gly), while only two out of the other 14 oligopeptides did (Pro-Hyp and Hyp-Gly). In contrast, although the total content of Gly-Pro-Y-type tripeptides in G-LMW-GH was approximately 2.5-fold higher than that in PTC-GH (Table 1), significantly increased plasma levels in the G-LMW-GH group were not observed for the Gly-Pro-Y peptides. These trends were not significant but still observed for the G-HMW-GH administration despite its similar molecular weight distribution to PTC-GH.

Protease Resistance of X-Hyp-Gly. We assumed that X-Hyp-Gly-type tripeptides have high protease resistance, and thus, that the high content of these peptides in administered ginger-degraded gelatin hydrolysates directly resulted in their high plasma concentrations. To confirm

this hypothesis, the protease resistance of synthetic peptides was examined using mouse plasma (Fig. 5). We found that Ala-Hyp-Gly remained at 91.3% in mouse plasma after a 6-h incubation at 37°C as did Pro-Hyp (93.3%), which was reported to be stable in human serum in previous studies.[17, 19] In contrast, other types of tripeptides, Gly-Pro-Ala and Gly-Ala-Hyp, were dramatically decreased to 12.6% and 0.2%, respectively, by the plasma digestion for 6 h. These data suggest that Hyp located in the middle position primarily contributes to the protease resistance, and other X-Hyp-Gly-type tripeptides are likely to be also stable in blood.

DISCUSSION

Herein, we demonstrated that ginger powder enables simple and efficient production of gelatin hydrolysate enriched with Gly-Pro-Y and X-Hyp-Gly-type tripeptides. To the best of our knowledge, this is the first report showing that ginger protease recognizes Hyp at the P_2 position, as demonstrated by the substantial generation of X-Hyp-Gly. Although it seems that the protease activity toward P_2 Hyp was less than P_2 Pro, >2.5% (w/w) of X-Hyp-Gly-type tripeptides were generated from gelatin using ginger powder.

In the oral administration experiments using mice, collagen-derived dipeptides and tripeptides were more efficiently absorbed by use of the ginger-degraded gelatin hydrolysates, G-HMW-GH and G-LMW-GH, compared with the control gelatin hydrolysate, PTC-GH (Fig. 4 and Table 2). A previous study reported that the plasma level of peptide-form Hyp was increased 2.6-fold by using gelatin hydrolysate with a smaller average molecular weight (1300 Da) compared with a larger one (5000 Da).[42] The high absorption of collagen-derived oligopeptides in the G-LMW-GH group is likely partly due to its low average molecular weight (590 Da) relative to G-HMW-

GH (963 Da) and PTC-GH (917 Da). However, the absorption of G-HMW-GH was also high compared with PTC-GH despite their similar molecular weight distributions. In the mice administered the ginger-degraded gelatin hydrolysates, the increasing levels of collagen-derived oligopeptides in blood were particularly high for X-Hyp-Gly-type tripeptides, which indicates that at least part of the orally administered X-Hyp-Gly can be transported into intestinal cells via peptide transporters in an intact form. This is supported by the results in Fig. 5 showing the high protease resistance of Ala-Hyp-Gly. Almost all the other oligopeptides also showed high plasma concentrations after the administration of the ginger-degraded gelatin hydrolysates, especially G-LMW-GH. The high levels of dipeptides may have resulted from partial cleavage of X-Hyp-Gly or Gly-Pro-Y. In addition, although Pro-Hyp and Pro-Hyp-Gly were not contained in the ginger-degraded gelatin hydrolysate (Fig. 1), the $AUC_{0–6h}$ of these peptides was significantly high in the G-LMW-GH group compared with the control group ($p<0.05$). We postulate that the increasing absorption of Pro-Hyp and Pro-Hyp-Gly was a result of partial gastrointestinal digestion of Gly-Pro-Hyp-Gly, which was present in the ginger-degraded gelatin hydrolysate in considerable amounts (Fig. 1C).

A recent study described peroral and intraperitoneal administration experiments using collagenase-digested gelatin hydrolysate.[43] Gly-Pro-Hyp was detected in blood at a concentration second only to Pro-Hyp after the administrations because of the Gly-X-Y-rich peptide composition of the gelatin hydrolysate. In contrast, the plasma concentration of Hyp-Gly was near the basal level after the administrations and was approximately 1/1000 of that of Gly-Pro-Hyp because the Hyp-Gly bond was cleaved by collagenase. Accordingly, we argue that the gelatin hydrolysate prepared using ginger powder combines the advantages of having both the Gly-Pro-Y and X-Hyp-Gly-type tripeptides for the absorption of collagen-derived oligopeptides,

and the particular increases in the plasma levels of X-Hyp-Gly are expected to lead to some beneficial effects.

While the major blood constituents of the collagen-derived peptides, Pro-Hyp and Hyp-Gly, were reported to have various physiological functions,[19, 23-25] almost no reports exist on the identification of the bioactivity of the X-Hyp-Gly-type tripeptides. However, various functions are suggested by their tripeptide structure. For example, peptide substrates in which Pro, Hyp, or Ala is the penultimate N-terminal residue possess inhibitory activity toward DPP-IV,[44] and thus, the Gly-Pro-Y and X-Hyp-Gly-rich gelatin hydrolysate may have significant antidiabetic effects. In addition, a study reported ACE inhibitory activity, which leads to antihypertensive effects, for Ala-Hyp-Gly, Glu-Hyp-Gly, Pro-Hyp-Gly, and Ser-Hyp-Gly.[28] Further studies are warranted to explore the benefits of X-Hyp-Gly-type tripeptides as food-derived oligopeptides in the blood.

The peptide generation from gelatin using ginger powder depended on reaction pH and reducing conditions, and Gly-Pro-Y and X-Hyp-Gly were differently generated with the reaction conditions (Figs. 1 and 2). These results suggest the possibility that substrate preference for Pro or Hyp at the P_2 position differs between ginger protease isoforms. Ichikawa et al. reported that the protease activity of GP II was activated more efficiently by the addition of reducing reagents to 1.5-fold higher than that of GP I.[29] Thus, given the different substrate specificity of the ginger protease isoforms, peptide bond cleavage with Hyp at the P_2 position may be mainly catalyzed by GP II (Fig. 1A and B). However, the generation of Gly-Pro-Y or X-Hyp-Gly requires a tandem -Gly-Pro-Y-Gly-Pro-Y- or -X-Hyp-Gly-X-Hyp-Gly- sequence, respectively, and cleavage of two peptide bonds. Thus, the substrate specificity cannot be determined from only the peptide generation patterns. To clarify the detailed substrate specificity of the ginger protease

isoforms for Pro and Hyp at the P_2 position, enzymatic kinetic analysis is required along with separation and purification of the isoforms.

ABBREVIATIONS USED

Hyp, hydroxyproline; DPP-IV, dipeptidylpeptidase-IV; ACE, angiotensin I-converting enzyme; LC–MS, liquid chromatography–mass spectrometry; DPBS, Dulbecco's phosphate buffered saline; DTT, dithiothreitol; G-HMW-GH, ginger-degraded high molecular weight gelatin hydrolysate; G-LMW-GH, ginger-degraded low molecular weight gelatin hydrolysate; PTC-GH, pepsin and trypsin/chymotrypsin-degraded gelatin hydrolysate; SI-collagen, stable isotope-labeled collagen; AUC, area under the plasma concentration–time curve; MRM, multiple reaction monitoring; SD, standard deviation

ACKNOWLEDGMENT

We would like to thank Takashi Midorikawa (Nippi) for providing bovine skin collagen.

SUPPORTING INFORMATION AVAILABLE

Table S1: MRM transitions of collagen-derived oligopeptides for gelatin digestion experiments. **Table S2**: MRM transitions of Hyp and collagen-derived oligopeptides for oral administration experiments. **Table S3**: Peptide generation by gelatin digestion using ginger powder with or without DTT (mg/g collagen). **Table S4**: Peptide generation by gelatin digestion using ginger powder with varying pH (mg/g collagen). **Table S5**: Maximum plasma concentration of Hyp and collagen-derived oligopeptides after oral administration of gelatin hydrolysates. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Berg, R. A.; Prockop, D. J., The thermal transition of a non-hydroxylated form of collagen. Evidence for a role for hydroxyproline in stabilizing the triple-helix of collagen. *Biochem Biophys Res Commun* **1973**, *52*, 115-20.

2. Clark, K. L.; Sebastianelli, W.; Flechsenhar, K. R.; Aukermann, D. F.; Meza, F.; Millard, R. L.; Deitch, J. R.; Sherbondy, P. S.; Albert, A., 24-Week study on the use of collagen hydrolysate as a dietary supplement in athletes with activity-related joint pain. *Curr Med Res Opin* **2008**, *24*, 1485-96.

3. Schauss, A. G.; Stenehjem, J.; Park, J.; Endres, J. R.; Clewell, A., Effect of the novel low molecular weight hydrolyzed chicken sternal cartilage extract, BioCell Collagen, on improving osteoarthritis-related symptoms: a randomized, double-blind, placebo-controlled trial. *J Agric Food Chem* **2012**, *60*, 4096-101.

4. Wu, J.; Fujioka, M.; Sugimoto, K.; Mu, G.; Ishimi, Y., Assessment of effectiveness of oral administration of collagen peptide on bone metabolism in growing and mature rats. *J Bone Miner Metab* **2004**, *22*, 547-53.

5. Nomura, Y.; Oohashi, K.; Watanabe, M.; Kasugai, S., Increase in bone mineral density through oral administration of shark gelatin to ovariectomized rats. *Nutrition* **2005**, *21*, 1120-6.

6. Saiga, A.; Iwai, K.; Hayakawa, T.; Takahata, Y.; Kitamura, S.; Nishimura, T.; Morimatsu, F., Angiotensin I-converting enzyme-inhibitory peptides obtained from chicken collagen hydrolysate. *J Agric Food Chem* **2008**, *56*, 9586-91.

7. Saiga-Egusa, A.; Iwai, K.; Hayakawa, T.; Takahata, Y.; Morimatsu, F., Antihypertensive effects and endothelial progenitor cell activation by intake of chicken collagen hydrolysate in pre- and mild-hypertension. *Biosci Biotechnol Biochem* **2009**, *73*, 422-4.

8. Huang, S. L.; Hung, C. C.; Jao, C. L.; Tung, Y. S.; Hsu, K. C., Porcine skin gelatin hydrolysate as a dipeptidyl peptidase IV inhibitor improves glycemic control in streptozotocin-induced diabetic rats. *J Funct Foods* **2014**, *11*, 235-242.

9. Hsieh, C. H.; Wang, T. Y.; Hung, C. C.; Chen, M. C.; Hsu, K. C., Improvement of glycemic control in streptozotocin-induced diabetic rats by Atlantic salmon skin gelatin hydrolysate as the dipeptidyl-peptidase IV inhibitor. *Food Funct* **2015**, *6*, 1887-92.

10. Gómez-Guillén, M. C.; Giménez, B.; López-Caballero, M. E.; Montero, M. P., Functional and bioactive properties of collagen and gelatin from alternative sources: A review. *Food Hydrocolloids* **2011**, *25*, 1813-1827.

11. Fujita, A.; Kawakita, H.; Saito, K.; Sugita, K.; Tamada, M.; Sugo, T., Production of tripeptide from gelatin using collagenase-immobilized porous hollow-fiber membrane. *Biotechnol Prog* **2003**, *19*, 1365-7.

12. Hatanaka, T.; Kawakami, K.; Uraji, M., Inhibitory effect of collagen-derived tripeptides on dipeptidylpeptidase-IV activity. *J Enzyme Inhib Med Chem* **2014**, *29*, 823-8.

13. Kim, S. K.; Byun, H. G.; Park, P. J.; Shahidi, F., Angiotensin I converting enzyme inhibitory peptides purified from bovine skin gelatin hydrolysate. *J Agric Food Chem* **2001**, *49*, 2992-7.

14. Byun, H. G.; Kim, S. K., Purification and characterization of angiotensin I converting enzyme (ACE) inhibitory peptides from Alaska pollack (Theragra chalcogramma) skin. *Process Biochemistry* **2001**, *36*, 1155-1162.

15. Nonaka, I.; Katsuda, S.; Ohmori, T.; Shigehisa, T.; Nakagami, T.; Maruyama, S., In vitro and in vivo anti-platelet effects of enzymatic hydrolysates of collagen and collagen-related peptides. *Biosci Biotechnol Biochem* **1997**, *61*, 772-5.

16. Prockop, D. J.; Keiser, H. R.; Sjoerdsma, A., Gastrointestinal absorption and renal excretion of hydroxyproline peptides. *Lancet* **1962**, *2*, 527-8.

17. Iwai, K.; Hasegawa, T.; Taguchi, Y.; Morimatsu, F.; Sato, K.; Nakamura, Y.; Higashi, A.; Kido, Y.; Nakabo, Y.; Ohtsuki, K., Identification of food-derived collagen peptides in human blood after oral ingestion of gelatin hydrolysates. *J Agric Food Chem* **2005**, *53*, 6531-6.

18. Ohara, H.; Matsumoto, H.; Ito, K.; Iwai, K.; Sato, K., Comparison of quantity and structures of hydroxyproline-containing peptides in human blood after oral ingestion of gelatin hydrolysates from different sources. *J Agric Food Chem* **2007**, *55*, 1532-5.

19. Shigemura, Y.; Akaba, S.; Kawashima, E.; Park, E. Y.; Nakamura, Y.; Sato, K., Identification of a novel food-derived collagen peptide, hydroxyprolyl-glycine, in human peripheral blood by pre-column derivatisation with phenyl isothiocyanate. *Food Chem* **2011**, *129*, 1019-24.

20. Aito-Inoue, M.; Lackeyram, D.; Fan, M. Z.; Sato, K.; Mine, Y., Transport of a tripeptide, Gly-Pro-Hyp, across the porcine intestinal brush-border membrane. *J Pept Sci* **2007**, *13*, 468-74.

21. Liu, C.; Sugita, K.; Nihei, K.; Yoneyama, K.; Tanaka, H., Absorption of hydroxyproline-containing peptides in vascularly perfused rat small intestine in situ. *Biosci Biotechnol Biochem* **2009**, *73*, 1741-7.

22. Watanabe-Kamiyama, M.; Shimizu, M.; Kamiyama, S.; Taguchi, Y.; Sone, H.; Morimatsu, F.; Shirakawa, H.; Furukawa, Y.; Komai, M., Absorption and effectiveness of orally administered low molecular weight collagen hydrolysate in rats. *J Agric Food Chem* **2010**, *58*, 835-41.

23. Shigemura, Y.; Iwai, K.; Morimatsu, F.; Iwamoto, T.; Mori, T.; Oda, C.; Taira, T.; Park, E. Y.; Nakamura, Y.; Sato, K., Effect of Prolyl-hydroxyproline (Pro-Hyp), a food-derived

collagen peptide in human blood, on growth of fibroblasts from mouse skin. *J Agric Food Chem* **2009**, *57*, 444-9.

24. Ohara, H.; Ichikawa, S.; Matsumoto, H.; Akiyama, M.; Fujimoto, N.; Kobayashi, T.; Tajima, S., Collagen-derived dipeptide, proline-hydroxyproline, stimulates cell proliferation and hyaluronic acid synthesis in cultured human dermal fibroblasts. *J Dermatol* **2010**, *37*, 330-8.

25. Shimizu, J.; Asami, N.; Kataoka, A.; Sugihara, F.; Inoue, N.; Kimira, Y.; Wada, M.; Mano, H., Oral collagen-derived dipeptides, prolyl-hydroxyproline and hydroxyprolyl-glycine, ameliorate skin barrier dysfunction and alter gene expression profiles in the skin. *Biochem Biophys Res Commun* **2015**, *456*, 626-30.

26. Taga, Y.; Kusubata, M.; Ogawa-Goto, K.; Hattori, S., Highly accurate quantification of hydroxyproline-containing peptides in blood using a protease digest of stable isotope-labeled collagen. *J Agric Food Chem* **2014**, *62*, 12096-102.

27. Laskin, D. L.; Kimura, T.; Sakakibara, S.; Riley, D. J.; Berg, R. A., Chemotactic activity of collagen-like polypeptides for human peripheral blood neutrophils. *J Leukoc Biol* **1986**, *39*, 255-66.

28. Iwai, K.; Zhang, Y.; Kouguchi, T.; Saiga-Egusa, A.; Shimizu, M.; Ohmori, T.; Takahata, Y.; Morimatsu, F., Blood concentration of food-derived peptides following oral intake of chicken collagen hydrolysate and its angiotensin-converting enzyme inhibitory activity in healthy volunteers. *Nippon Shokuhin Kagaku Kogaku Kaishi* **2009**, *56*, 326-330.

29. Ichikawa, Y.; Sasa, H.; Michi, K., Purification of ginger protease. *J Jpn Soc Food Nutr* **1973**, *26*, 377-383.

30. Ohtsuki, K.; Taguchi, K.; Sato, K.; Kawabata, M., Purification of ginger proteases by DEAE-Sepharose and isoelectric focusing. *Biochim Biophys Acta* **1995**, *1243*, 181-4.

474 31. Choi, K. H.; Laursen, R. A., Amino-acid sequence and glycan structures of cysteine
475 proteases with proline specificity from ginger rhizome Zingiber officinale. *Eur J Biochem* **2000**,
476 *267*, 1516-26.
477 32. Thompson, E. H.; Wolf, I. D.; Allen, C. E., Ginger rhizome: a new source of proteolytic
478 enzyme. *J Food Sci* **1973**, *38*, 652-655.
479 33. Hashimoto, A.; Takeuti, Y.; Kawahara, Y.; Yasumoto, K., Proteinase and collagenase
480 activities in ginger rhizome. *J Jpn Soc Nutr Food Sci* **1991**, *44*, 127-132.
481 34. Kim, M.; Hamilton, S. E.; Guddat, L. W.; Overall, C. M., Plant collagenase: unique
482 collagenolytic activity of cysteine proteases from ginger. *Biochim Biophys Acta* **2007**, *1770*,
483 1627-35.
484 35. Naveena, B. M.; Mendiratta, S. K.; Anjaneyulu, A. S., Tenderization of buffalo meat
485 using plant proteases from Cucumis trigonus Roxb (Kachri) and Zingiber officinale roscoe
486 (Ginger rhizome). *Meat Sci* **2004**, *68*, 363-9.
487 36. Ha, M.; Bekhit, A. E. D.; Carne, A.; Hopkins, D. L., Characterisation of commercial
488 papain, bromelain, actinidin and zingibain protease preparations and their activities toward meat
489 proteins. *Food Chem* **2012**, *134*, 95-105.
490 37. Su, H. P.; Huang, M. J.; Wang, H. T., Characterization of ginger proteases and their
491 potential as a rennin replacement. *J Sci Food Agric* **2009**, *89*, 1178-1185.
492 38. Hashim, M. M.; Mingsheng, D.; Iqbal, M. F.; Xiaohong, C., Ginger rhizome as a
493 potential source of milk coagulating cysteine protease. *Phytochemistry* **2011**, *72*, 458-464.
494 39. Choi, K. H.; Laursen, R. A.; Allen, K. N., The 2.1 A structure of a cysteine protease with
495 proline specificity from ginger rhizome, Zingiber officinale. *Biochemistry* **1999**, *38*, 11624-33.

40. Taga, Y.; Kusubata, M.; Ogawa-Goto, K.; Hattori, S., Stable isotope-labeled collagen: a novel and versatile tool for quantitative collagen analyses using mass spectrometry. *J Proteome Res* **2014**, *13*, 3671-8.

41. Taga, Y.; Kusubata, M.; Ogawa-Goto, K.; Hattori, S., Developmental stage-dependent regulation of prolyl 3-hydroxylation in tendon type I collagen. *J Biol Chem* **2016**, *291*, 837-47.

42. Ichikawa, S.; Ohara, H.; Ito, K.; Oba, C.; Matsumoto, H.; Takeuchi, Y.; Kanegae, M., Influence on quantity of hydroxyproline-containing peptides in human blood after oral ingestion by the different average molecular weight collagen peptides. *Jpn J Med Pharm Sci* **2009**, *62*, 801-807.

43. Yamamoto, S.; Hayasaka, F.; Deguchi, K.; Okudera, T.; Furusawa, T.; Sakai, Y., Absorption and plasma kinetics of collagen tripeptide after peroral or intraperitoneal administration in rats. *Biosci Biotechnol Biochem* **2015**, *79*, 2026-33.

44. Puschel, G.; Mentlein, R.; Heymann, E., Isolation and characterization of dipeptidyl peptidase IV from human placenta. *Eur J Biochem* **1982**, *126*, 359-65.

FIGURE CAPTIONS

Figure 1. Effect of DTT on peptide generation from gelatin using ginger powder. Bovine skin gelatin was digested by 1/10 (w/w) of ginger powder with 0 or 2 mM DTT at pH 4.8 and 50°C for 16 h with shaking. The generated (A) Gly-Pro-Y-type tripeptides, (B) X-Hyp-Gly-type tripeptides, and (C) other oligopeptides were measured by LC–MS in MRM mode. The data represent the mean ± SD of three separate experiments. Grayed peptides were under the detection limit.

Figure 2. Effect of reaction pH on peptide generation from gelatin using ginger powder. Bovine skin gelatin was digested by 1/10 (w/w) of ginger powder with 2 mM DTT at 50°C for 16 h with shaking at pH 3.6, 4.0, 4.4, 4.8, 5.2, 5.6, 6.0, and 6.4. The generated (A) Gly-Pro-Y-type tripeptides, (B) X-Hyp-Gly-type tripeptides, and (C) other oligopeptides were measured by LC–MS in MRM mode. The data represent the mean ± SD of three separate experiments.

Figure 3. Molecular weight distributions of gelatin hydrolysates. (A) Gelatin hydrolysates prepared using pepsin and trypsin/chymotrypsin (PTC-GH, *green*) or ginger powder (G-HMW-GH, *blue*; G-LMW-GH, *red*) were subjected to size exclusion chromatography using a Superdex Peptide HR 10/30 column. (B) Chromatogram of the molecular weight standard mixture including Gly-Gly (132 Da), Gly-Pro-Hyp (285 Da), $(Gly\text{-}Pro\text{-}Hyp)_2$ (553 Da), $(Gly\text{-}Pro\text{-}Hyp)_3$ (820 Da), $(Gly\text{-}Pro\text{-}Hyp)_4$ (1087 Da), and acetyl-Ile-Ser-Val-Pro-Gly-Pro-Met-Gly-Pro-Ser-Gly-Pro-Arg-Gly-Leu-Hyp-Gly-Pro-Hyp-Gly-Cys (2003 Da/4002 Da).

532 **Figure 4.** Plasma concentrations of Hyp and collagen-derived oligopeptides after oral
533 administration of gelatin hydrolysates. Three types of gelatin hydrolysates (PTC-GH, *green*; G-
534 HMW-GH, *blue*; G-LMW-GH, *red*) were orally administered to ICR mice, and plasma samples
535 were obtained before (0 h) and 0.5, 1, 2, 4, and 6 h after the administration. After ethanol
536 deproteinization, the plasma concentrations of Hyp and collagen-derived oligopeptides were
537 quantified by LC–MS in MRM mode with SI-digest used as an internal standard mixture of those
538 analytes. The data represent the mean ± SD (n = 6).
539
540 **Figure 5.** Stability of collagen-derived oligopeptides in mouse plasma. Synthetic Pro-Hyp, Gly-
541 Ala-Hyp, Gly-Pro-Ala, and Ala-Hyp-Gly were incubated with mouse plasma at 37°C for 0, 0.5,
542 1, 2, 3, and 6 h. Each peptide was measured at each time point by LC–MS in MRM mode after
543 ethanol deproteinization. The data represent the mean ± SD (n = 3).
544

TABLES

Table 1. Characteristics of gelatin hydrolysates

	PTC-GH	G-HMW-GH	G-LMW-GH
Reaction enzyme	Pepsin and trypsin/chymotrypsin	Ginger protease (ginger powder)	Ginger protease (ginger powder)
Gelatin : ginger powder (w/w)	–	50 : 1	10 : 1
Average molecular weight (Da)	917	963	590
Gly-Pro-Y content (mg/g)	22.3	37.0	55.0
X-Hyp-Gly content (mg/g)	N.D.	4.6	25.7

N.D., not detected.

Table 2. $AUC_{0–6h}$ of Hyp and collagen-derived oligopeptides after oral administration of gelatin hydrolysates

	PTC-GH (nmol/mL·h)	G-HMW-GH (nmol/mL·h)	G-LMW-GH (nmol/mL·h)
Hyp	231.62 ± 53.66	286.09 ± 28.98	302.89 ± 33.48*
Gly-Pro	0.53 ± 0.05	0.61 ± 0.18	0.55 ± 0.14
Pro-Ala	0.10 ± 0.02	0.11 ± 0.03	0.12 ± 0.03
Ala-Hyp	0.50 ± 0.05	0.57 ± 0.12	0.62 ± 0.22
Glu-Hyp	1.02 ± 0.19	1.02 ± 0.22	1.30 ± 0.33
Ile-Hyp	0.23 ± 0.03	0.28 ± 0.06	0.29 ± 0.10
Leu-Hyp	0.79 ± 0.09	0.89 ± 0.16	1.03 ± 0.36
Phe-Hyp	0.59 ± 0.12	0.78 ± 0.14	0.86 ± 0.35
Pro-Hyp	5.43 ± 1.36	7.07 ± 1.71	9.18 ± 3.52*
Ser-Hyp	0.39 ± 0.04	0.43 ± 0.07	0.49 ± 0.14
Hyp-Gly	1.19 ± 0.23	1.55 ± 0.58	2.57 ± 0.69**
Gly-Pro-Ala	0.05 ± 0.01	0.06 ± 0.03	0.06 ± 0.01
Gly-Pro-Hyp	0.81 ± 0.19	0.95 ± 0.27	1.17 ± 0.35
Gly-Pro-Val	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.01
Ala-Hyp-Gly	0.53 ± 0.12	0.66 ± 0.15	1.02 ± 0.51*
Glu-Hyp-Gly	0.66 ± 0.19	0.75 ± 0.23	1.51 ± 0.78*
Leu-Hyp-Gly	0.05 ± 0.01	0.08 ± 0.05	0.09 ± 0.03
Phe-Hyp-Gly	0.11 ± 0.01	0.16 ± 0.05	0.18 ± 0.04*
Pro-Hyp-Gly	0.94 ± 0.11	1.43 ± 0.54	1.70 ± 0.30**
Ser-Hyp-Gly	0.62 ± 0.14	0.89 ± 0.26	1.17 ± 0.54
Thr-Hyp-Gly	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.01**
Gly-Pro-Hyp-Gly	0.35 ± 0.07	0.46 ± 0.18	0.50 ± 0.14

*p<0.05 and **p<0.01 compared to the control gelatin hydrolysate, PTC-GH (ANOVA/Dunnet's test).

FIGURE GRAPHICS

Figure 1

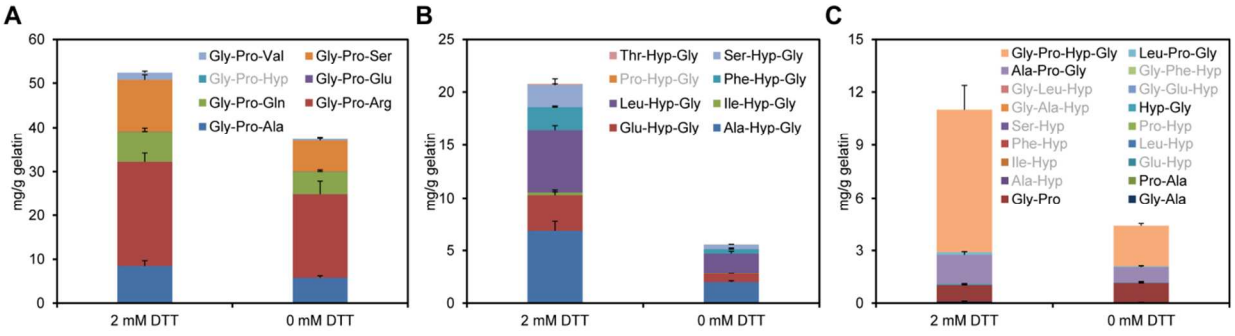
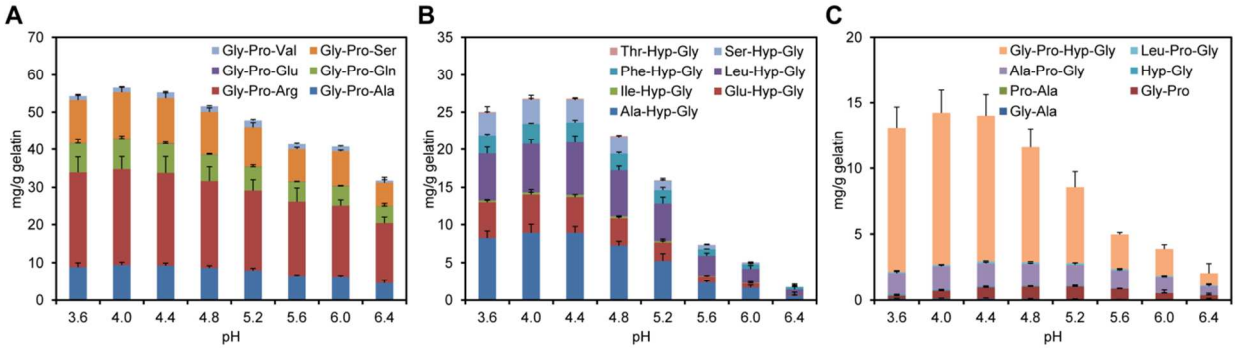


Figure 2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Gly-Pro-Glu
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Gly-Pro-Arg
Gly-Pro-Ala
Thr-Hyp-Gly
Ser-Hyp-Gly
Phe-Hyp-Gly
Leu-Hyp-Gly
Ile-Hyp-Gly
Glu-Hyp-Gly
Ala-Hyp-Gly
Gly-Pro-Hyp-Gly
Leu-Pro-Gly
Ala-Pro-Gly
Hyp-Gly
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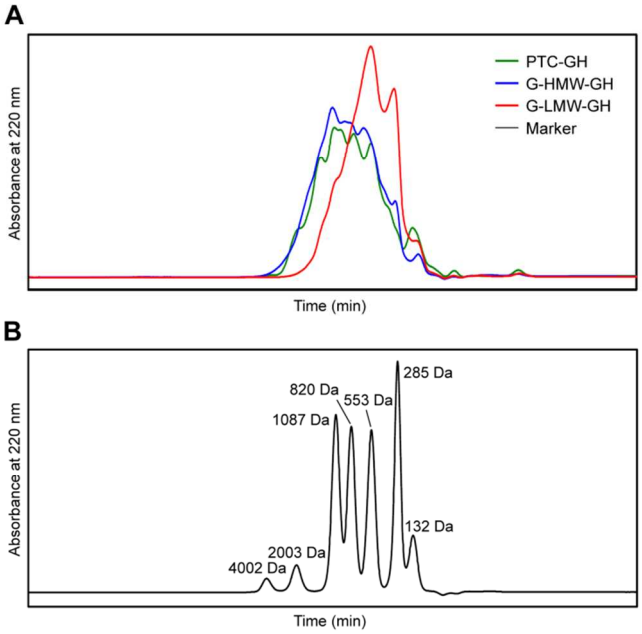
6.0
6.4

Figure 3


A
PTC-GH
G-HMW-GH
G-LMW-GH
Marker
Absorbance at 220 nm
Time (min)
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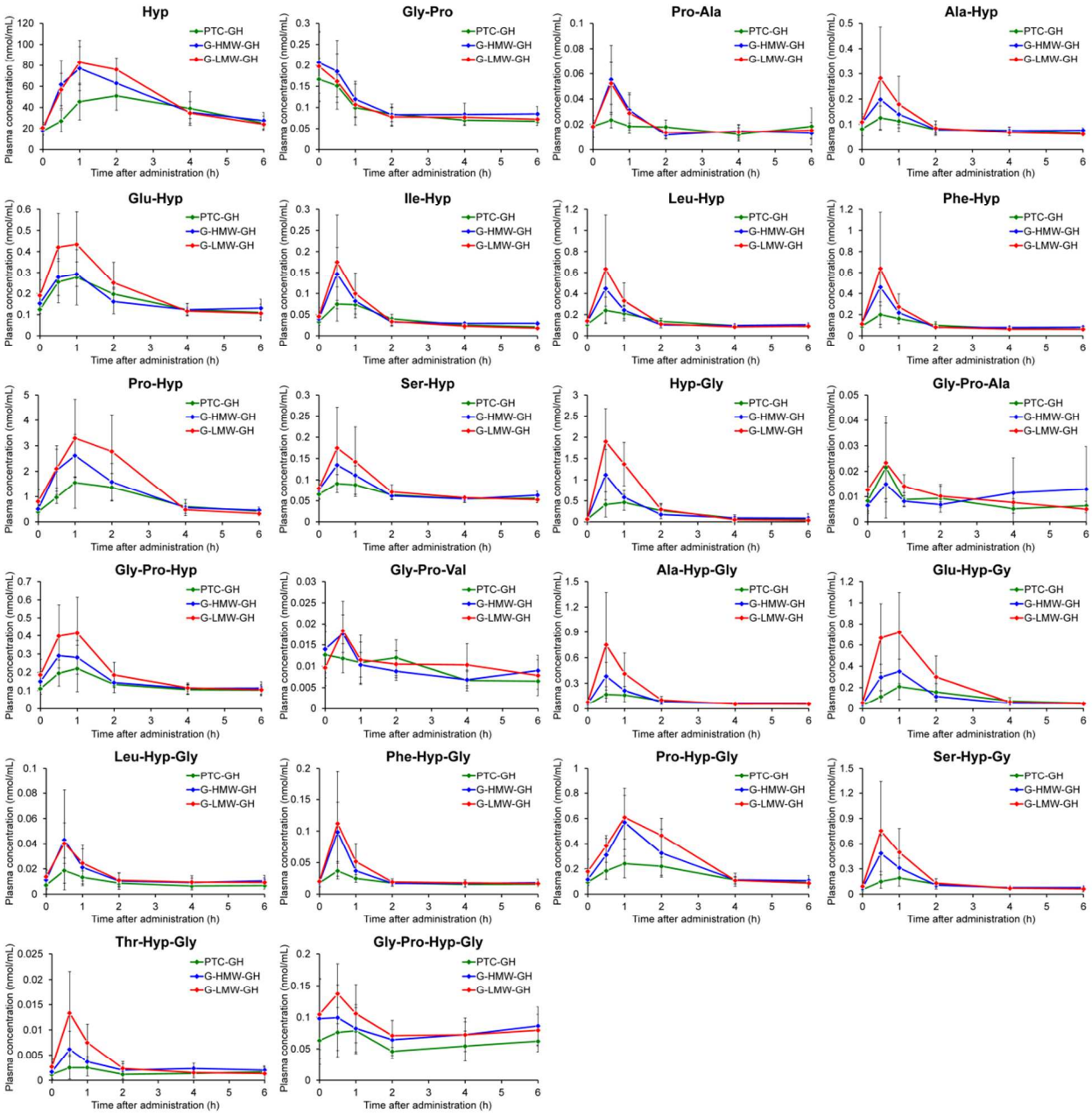
820 Da
553 Da
285 Da
1087 Da
132 Da
2003 Da
4002 Da
Absorbance at 220 nm
Time (min)

Figure 4


Hyp
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Pro-Ala
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Glu-Hyp
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Ser-Hyp
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Gly-Pro-Hyp
Gly-Pro-Val
Ala-Hyp-Gly
Glu-Hyp-Gy
Leu-Hyp-Gly
Phe-Hyp-Gly
Pro-Hyp-Gly
Ser-Hyp-Gy
Thr-Hyp-Gly
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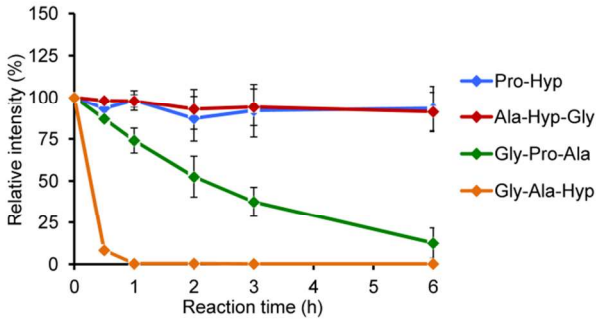
PTC-GH
G-HMW-GH
G-LMW-GH
Plasma concentration (nmol/mL)
Time after administration (h)

Figure 5


150
125
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Relative intensity (%)
0
1
2
3
4
5
6
Reaction time (h)
Pro-Hyp
Ala-Hyp-Gly
Gly-Pro-Ala
Gly-Ala-Hyp

GRAPHICS FOR TABLE OF CONTENTS