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Article

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Identification of Collagen-Derived Hydroxyproline (Hyp)-Containing Cyclic Dipeptides with High Oral Bioavailability: Efficient Formation of Cyclo(X-Hyp) from X-Hyp-Gly-Type Tripeptides by Heating

Yuki Taga,* Masashi Kusubata, Kiyoko Ogawa-Goto, and Shunji Hattori

Nippi Research Institute of Biomatrix, 520-11 Kuwabara, Toride, Ibaraki 302-0017, Japan

To whom correspondence should be addressed: Yuki Taga

Nippi Research Institute of Biomatrix, 520-11 Kuwabara, Toride, Ibaraki 302-0017, Japan

Tel: +81-297-71-3046; Fax: +81-297-71-3041

E-mail: y-taga@nippi-inc.co.jp

ABSTRACT

Cyclic dipeptides (2,5-diketopiperazines) are present in a variety of foods and are reported to demonstrate antioxidant, antidepressant, and other beneficial effects. We recently developed a novel collagen hydrolysate characterized by a high content of X-hydroxyproline (Hyp)-Gly-type tripeptides using ginger protease. In the present study, we found that through heating, X-Hyp-Gly can be easily converted into Hyp-containing cyclic dipeptides. After heating for 3 h at 85°C and pH 4.8, Ala-Hyp-Gly was almost completely cyclized to cyclo(Ala-Hyp) in contrast to a slight cyclization of Ala-Hyp. The contents of cyclo(Ala-Hyp) and cyclo(Leu-Hyp) reached 0.5–1% (w/w) each in the ginger-degraded collagen hydrolysate under the heating condition. Oral administration experiments using mice revealed that cyclo(Ala-Hyp) and cyclo(Leu-Hyp) were absorbed into the blood at markedly higher efficiencies compared to collagenous oligopeptides, including Pro-Hyp. The high productivity and oral bioavailability of the collagen-specific cyclic dipeptides suggest significant health benefits of the heat-treated ginger-degraded collagen hydrolysate.

Keywords: *collagen hydrolysate, cyclic dipeptide, hydroxyproline, ginger, oral bioavailability*

INTRODUCTION

Collagen is an extracellular matrix protein abundant in connective tissues, such as skin, bone, and tendon. Collagen consists of repeating Gly-Xaa-Yaa triplets where Xaa and Yaa are frequently Pro and 4-hydroxyproline (4-Hyp), respectively, forming a stable triple-helical structure in the body. Heat-denatured collagen (gelatin) can be extracted from skin, bone, and fish scales. Oral ingestion of enzymatic hydrolysates of gelatin, referred to as collagen hydrolysate or gelatin hydrolysate, has been reported to have various beneficial effects, including reducing joint pain,[1, 2] increasing bone density,[3, 4] modulating lipid metabolism,[5, 6] and lowering blood pressure.[7, 8] Although the mechanism of these effects has not been clarified, the structures of the collagen-derived active ingredients have been determined over the last decade. In 2005, Sato et al. first identified Pro-Hyp as the most abundant collagen-derived peptide in the blood after oral ingestion of collagen hydrolysate.[9] Additionally, after the ingestion, other collagen-derived Hyp-containing dipeptides and tripeptides, including Hyp-Gly and X-Hyp-Gly-type tripeptides, were also found in the blood at significantly high concentrations compared to other food-derived peptides.[9-13] The presence of Hyp within the peptide sequence confers striking protease resistance, leading to the high blood levels of the collagen-derived dipeptides and tripeptides.[9, 11, 13, 14] Recent studies have revealed that collagen-derived oligopeptides, such as Pro-Hyp and Hyp-Gly, have physiological functions, including growth stimulation of skin fibroblasts,[11, 15, 16] promotion of osteoblast and myoblast differentiation,[17, 18] and improvement of skin barrier dysfunction.[19]

2,5-Diketopiperazines (cyclic dipeptides, cyclo(Xaa-Yaa)) are found in a wide variety of foods and beverages, such as bread,[20] chicken essence,[21] beer,[22] coffee,[23] cocoa,[24] and the distillation residue of awamori spirits.[25] Cyclic dipeptides are mainly formed from peptides and proteins

during heating and fermentation in food processing and contribute to the taste of foods, especially bitterness.[26] Beneficial effects have been reported for cyclic dipeptides; for example, cyclo(Ile-Pro), cyclo(Phe-Pro), cyclo(Pro-Val), and cyclo(Leu-Pro) generated during the production of awamori spirits have shown antioxidant activity,[25] and cyclo(Phe-Phe) found in chicken essence has shown antidepressant and antidementia effects.[27] Moreover, cyclic dipeptides exhibit various other beneficial activities, such as antimicrobial,[28-30] antitumor,[31, 32] and antihepatitis activity.[33, 34] Cyclic dipeptides can be produced from dipeptides by heating at high temperature (>100°C) via head-to-tail cyclization with dehydration.[35-37] In addition, thermal formation of cyclic dipeptides also occurs for tripeptides.[36-39] Hayasaka et al. recently reported that collagenous Gly-Pro-Y-type tripeptides, including Gly-Pro-Hyp and Gly-Pro-Ala, are converted into cyclo(Gly-Pro) through heating.[39] The generation rate of cyclo(Gly-Pro) from Gly-Pro-Y was much higher than that of Gly-Pro. However, heating at 95°C for 24 h was needed to cyclize ~80% of the Gly-Pro-Y-type tripeptides.

Several studies have previously reported the presence of Hyp-containing cyclic dipeptides. Cyclo(Phe-Hyp) was found in culture supernatant of *Lactobacillus plantarum*,[28] and cyclo(Ala-Hyp), cyclo(Leu-Hyp), and cyclo(Phe-Hyp) were extracted from cultures of *Alternaria alternata*.[29] These microorganism-derived cyclic dipeptides showed antifungal activity.[28, 29] Cyclo(Ser-Hyp), named JBP485, was identified in a hydrolysate of human placenta and considered to be derived from collagen present in the tissue.[40] Previous studies demonstrated that cyclo(Ser-Hyp) has antihepatitis effects, which were suggested to be associated with anti-inflammatory, antioxidant, and antiapoptotic activities.[33, 34] Moreover, improvement of acute renal failure[41] and promotion of corneal wound healing[42] were also reported for cyclo(Ser-Hyp).

We recently developed a novel collagen hydrolysate using ginger protease that preferentially cleaves peptide bonds with Pro/Hyp at the P_2 position.[13] This collagen hydrolysate uniquely contained substantial amounts of X-Hyp-Gly-type tripeptides (approximately 2.5%; w/w). We demonstrated that oral administration of the ginger-degraded collagen hydrolysate significantly increased the levels of X-Hyp-Gly in blood. Thus, it is expected that an oral ingestion of the X-Hyp-Gly-rich collagen hydrolysate has distinct health benefits. However, during the heat treatment in the manufacturing process, we observed that X-Hyp-Gly-type tripeptides in the collagen hydrolysate unexpectedly diminished, especially during the sterilization step (data not reported previously). In the present study, we aimed to identify the products from the heat treatment of X-Hyp-Gly-type tripeptides. In addition, oral bioavailability of the thermal conversion products was investigated to assess their potential as novel active ingredients in collagen hydrolysates.

MATERIALS AND METHODS

Chemicals. Dithiothreitol (DTT), L-alanine, and glycine 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 *trans*-4-hydroxy-L-proline was purchased from Sigma-Aldrich (St. Louis, MO). H-Pro-Hyp-OH, H-Hyp-Gly-OH, H-Gly-Ala-Hyp-OH, H-Gly-Pro-Ala-OH, H-Gly-Pro-Arg-OH, H-Gly-Pro-Hyp-OH, H-Ala-Pro-Gly-OH, and cyclo(Gly-Pro) were purchased from Bachem (Bubendorf, Switzerland). Other peptides were custom synthesized by AnyGen (Kwangju, Korea). Ginger rhizomes were purchased from a local supermarket. Gelatin was prepared from pepsin-solubilized bovine skin collagen as described previously.[13]

Ethics Statement. All animal studies were approved by the Experimental Ethical Committee of Nippi Research Institute of Biomatrix.

Preparation of Ginger-Degraded Collagen Hydrolysates. Ginger-degraded collagen hydrolysates for heating experiments or oral administration experiments were prepared as described previously.[13] Briefly, for heating experiments, 50 mg/mL gelatin solution in 2 mM DTT/100 mM sodium acetate buffer (pH 4.8) was hydrolyzed at 50°C for 16 h with shaking using 1/10 (w/w) of ginger powder, which was prepared by homogenizing ginger rhizomes in chilled acetone. After filtration using a 0.8 μm filter, the reactant was stored at −30°C until analysis. For oral administration experiments, 50mg/mL gelatin solution (pH 4.0 adjusted by HCl) containing 2 mM DTT was similarly hydrolyzed with ginger powder. The reactant was filtered to remove the ginger powder, acidified with HCl, filtered through a 0.8 μm filter, and subjected to ultrafiltration using Vivaspin 20-10K devices to remove ginger proteases. The flow-through fraction was lyophilized using a Virtis Genesis 25EL freeze dryer (SP Industries, Gardiner, NY). After washing with acetone, the collagen hydrolysate was redried using a centrifugal evaporator CVE-3100 (EYELA, Tokyo, Japan).

Preparation of Cyclo(Ala-Hyp) and Cyclo(Leu-Hyp). Ala-Hyp-Gly or Leu-Hyp-Gly was heated at 85°C for 8 h in 100 mM sodium acetate buffer (pH 4.8). The heat-treated peptide was subjected to reversed-phase chromatography using a Zorbax Eclipse XDB-C18 column (5 μm particle size, L × I.D. 150 mm × 4.6 mm; Agilent Technologies, Palo Alto, CA) with monitoring at 220 nm on an Alliance 2690 separation module equipped with a 2487 dual absorbance detector (Waters, Milford, MA). Separation was performed at a flow rate of 1 mL/min with a binary gradient as follows: 98% solvent A (0.1% formic acid) for 5 min, linear gradient of 0–99% solvent B (100% acetonitrile) for 10 min, and 98% solvent A for 5 min. Fractions of

cyclo(Ala-Hyp) at 3.0–3.4 min and cyclo(Leu-Hyp) at 9.7–9.9 min were collected from heat-treated Ala-Hyp-Gly and Leu-Hyp-Gly, respectively. After drying using the centrifugal evaporator, the purified cyclic dipeptide was dissolved in distilled water and stored at −30°C.

Heating Experiments of Collagenous Oligopeptides. The ginger-degraded collagen hydrolysate solution (pH 4.8) was heated at 85°C for 1 or 3 h. Synthetic di- and tripeptides were incubated at 4–100°C for 1 or 3 h in 100 mM citrate-NaOH buffer (pH 2.4), 100 mM sodium acetate buffer (pH 3.6 and 4.8), 100 mM sodium phosphate buffer (pH 6.0 and 7.2), or 100 mM sodium carbonate buffer (pH 8.4 and 9.6). The samples were diluted in 1% formic acid for liquid chromatography (LC)–mass spectrometry (MS) analysis.

Preparation of An Internal Standard Mixture of Collagen-Derived Oligopeptides and Cyclic Dipeptides. Stable isotope-labeled collagen (SI-collagen) was first prepared in human lung fibroblast cultures using stable isotopically heavy Pro, Lys, and Arg as described previously.[12, 13, 43, 44] After denaturation at 60°C for 30 min, SI-collagen was digested using trypsin and chymotrypsin (1:50 enzyme/substrate ratio, respectively) in 100 mM ammonium bicarbonate/1 mM $CaCl_2$ at 37°C for 16 h, and further digested with freshly prepared dialyzed mouse plasma at 37°C for 24 h as described previously.[12, 13] After ethanol deproteinization and drying of the ethanol-soluble fraction using the centrifugal evaporator, the protease digest of SI-collagen (SI-digest) was heated in 50 mM sodium acetate buffer (pH 4.8) at 85°C for 1 h. The heat-treated SI-digest was then stored at −30°C.

Oral Administration of Collagen Hydrolysates. The ginger-degraded collagen hydrolysate was dissolved in 50 mM sodium acetate buffer (pH 4.8) and divided into two aliquots. One aliquot was heated at 85°C for 3 h to convert X-Hyp-Gly to cyclo(X-Hyp), while the other was excluded from the heat treatment to retain X-Hyp-Gly. These two hydrolysates were orally

130 administered to 3-month-old male ICR mice (Japan SLC, Shizuoka, Japan), as reported
131 previously.[13] Briefly, blood from the tail vein was collected 1 h after oral administration of 10
132 mg of the collagen hydrolysates. The mice had been fed a collagen free-diet, AIN-93M (Oriental
133 Yeast, Tokyo, Japan), from a day before the experiment. Plasma was prepared by centrifugation
134 and was mixed with heat-treated SI-digest used as an internal standard. After ethanol
135 deproteinization and drying of the ethanol-soluble fraction, the sample was reconstituted in 0.1%
136 formic acid for LC–MS analysis. Heat-treated SI-digest was mixed into the calibration standards
137 as the internal standard.

138 **Oral Administration of Synthetic Peptides.** Synthetic peptides were orally administered to
139 9-month-old male ICR mice (Japan SLC) as per the method above for the collagen hydrolysates.
140 Mice were divided into two groups and orally administered peptide mixtures containing 100 μg
141 each of the following peptides: group 1 [Ala-Hyp, Pro-Hyp, Hyp-Gly, cyclo(Ala-Hyp),
142 cyclo(Leu-Hyp), and cyclo(Gly-Pro)]; group 2 [Gly-Ala-Hyp, Gly-Pro-Ala, Gly-Pro-Hyp, Ala-
143 Hyp-Gly, Pro-Hyp-Gly, and cyclo(Ala-Hyp)]. Plasma was prepared before administration (0 h)
144 and at 0.5, 1, 2, 4, and 6 h after administration. LC–MS samples were prepared after the addition
145 of heat-treated SI-digest as described above. The area under the plasma concentration−time curve
146 ($AUC_{0–6\ h}$) was calculated using the trapezoidal rule.

147 **LC–MS Analysis.** Samples prepared in the peptide heating experiments and oral
148 administration experiments were analyzed by LC–MS in multiple reaction monitoring (MRM)
149 mode using a hybrid triple quadrupole/linear ion trap 3200 QTRAP mass spectrometer (AB
150 Sciex, Foster City, CA) coupled to an Agilent 1200 Series HPLC system (Agilent Technologies).
151 The samples were loaded onto an Ascentis Express F5 HPLC column (5 μm particle size, L ×
152 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 (for Ala-Hyp, Pro-Hyp, Hyp-Gly, and Ala-Hyp-Gly in serum samples); or 100% solvent A (0.1% formic acid) for 7.5 min, linear gradient of 0–90% solvent B (100% acetonitrile) for 12.5 min, 90% solvent B for 5 min, and 100% solvent A for 5 min (for other analytes). Analyst 1.6.2 (AB Sciex) was used to perform the data acquisition and analysis. The MRM transitions of amino acids, oligopeptides, and cyclic dipeptides are shown in Table S1 in the **Supporting Information**. 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.

Direct Infusion MS Analysis. Before or after heating synthetic Ala-Hyp-Gly at 85°C for 3 h in distilled water with a clean glass vial, MS spectra were obtained by direct infusion analysis using an ultra-high resolution quadrupole time-of-flight (QTOF) mass spectrometer (maXis II, Bruker Daltonics, Bremen, Germany). The theoretically simulated MS spectrum of cyclo(Ala-Hyp) was generated by Compass DataAnalysis version 4.3 (Bruker Daltonics).

RESULTS AND DISCUSSION

Heat-Induced Decrease in X-Hyp-Gly. To evaluate thermal stability, we subjected a ginger-degraded collagen hydrolysate solution, which contains high levels of Gly-Pro-Y and X-Hyp-Gly-type tripeptides,[13] to heat treatment at 85°C and pH 4.8 for 1 or 3 h. LC–MS analysis showed that while Gly-Pro-Y-type tripeptides (initially 78.9 mg/g gelatin in total) only slightly decreased after 3 h heating (71.7 mg/g) (Fig. 1A), X-Hyp-Gly-type tripeptides (initially 16.2 mg/g) dramatically decreased by the heat treatment (6.2 mg/g after 1 h and 1.2 mg/g after 3 h)

(Fig. 1B). Similarly, synthetic Ala-Hyp-Gly and Leu-Hyp-Gly showed 65% and 67% decreases, respectively, by 1 h of heating at 85°C and pH 4.8 (Fig. 1C). In contrast, other types of collagenous synthetic peptides, including Ala-Hyp, Pro-Hyp, Hyp-Gly, and Gly-Ala-Hyp, were thermally stable except for Gly-Pro-Ala and Gly-Pro-Hyp, which showed 15% and 7% decreases, respectively. The slight decreases in Gly-Pro-Y-type tripeptides are likely due to thermal conversion into cyclo(Gly-Pro), as previously reported.[39]

The heat-induced significant decrease in X-Hyp-Gly suggests that this type of collagen-derived tripeptide is thermally degraded into amino acids and/or dipeptides. Thus, we next monitored the degradation products of Ala-Hyp-Gly by LC–MS after heating at 85°C and pH 4.8 for 1 or 3 h (Fig. 1D). Gly was found to be generated concomitantly with the decrease in Ala-Hyp-Gly. However, the complement degradation product, Ala-Hyp, was completely undetectable, as were other potential degradation products (Hyp-Gly, Ala, and Hyp). These observations indicate that Ala-Hyp-Gly was converted into an unexpected form by heating.

Identification of Cyclo(X-Hyp) Converted from X-Hyp-Gly by Heating. We performed mass spectrometric analysis of heat-treated Ala-Hyp-Gly using high resolution QTOF–MS to identify the thermal conversion product. After 3 h heating of Ala-Hyp-Gly (*m/z* 260.13, *z* = 1) in distilled water, we observed the generation of a large unknown peak "X" (*m/z* 185.09, *z* = 1) (Fig. 2A). From the observed mass and the appearance of Gly shown in Fig. 1D, we predicted that cyclo(Ala-Hyp) was formed from Ala-Hyp-Gly by cyclodehydration that occurred simultaneously or after cleavage of the peptide bond between Hyp and Gly. Thus, we compared the measured spectrum of peak X with the simulated spectrum of cyclo(Ala-Hyp). As shown in Fig. 2B, the monoisotopic peak of X (*m/z* 185.0948) was highly matched to that of cyclo(Ala-Hyp) (*m/z* 185.0921, $C_8H_{12}N_2O_3$+H), and the isotopic distribution of peak X was also identical to

that of the simulated spectrum. These results demonstrate that peak X was cyclo(Ala-Hyp) thermally converted from Ala-Hyp-Gly. The cyclic dipeptide is assumed to be configured as *cis*-cyclo(L-Ala-L-Hyp) based on the fact that natural constituents of the original tripeptide are L-amino acids.[26] However, epimerization is possibly occurred by the thermal treatment. NMR analysis is needed to definitively determine the steric configuration.

The heat-induced cyclization of Ala-Hyp-Gly was quantitatively estimated by LC–MS with authentic cyclo(Ala-Hyp), which was purified from heat-treated Ala-Hyp-Gly by reversed-phase chromatography. A substantial amount of cyclo(Ala-Hyp) was generated by heating at 85°C and pH 4.8, which was inversely proportional to the decrease in Ala-Hyp-Gly (Fig. 3A and Fig. S1 in the **Supporting Information**). After 3 h of heating, the molar concentration of cyclo(Ala-Hyp) reached ~100% of that of the original peptide. In contrast, only a trace amount of cyclo(Ala-Hyp) was generated from Ala-Hyp after heating at 85°C for 3 h (Fig. 3B).

To define the conditions for the formation of Hyp-containing cyclic dipeptides from X-Hyp-Gly-type tripeptides, we analyzed three parameters related to the cyclization reaction: peptide sequence, reaction pH, and reaction temperature. First, we heat-treated various types of X-Hyp-Gly at 85°C and pH 4.8 for 1 h and observed decreases of more than 50% for all of the peptides except Pro-Hyp-Gly (Fig. 4A). Ala-Pro-Gly and Leu-Pro-Gly were also decreased by the heat treatment, probably due to cyclo(X-Pro) formation. Second, we measured the generation of cyclo(Ala-Hyp) from Ala-Hyp-Gly heated at 85°C for 1 h while varying the pH. Cyclization efficiently occurred at pH 4.8–7.2 (63.0–72.7%), while the reaction was markedly suppressed at pH 2.4 (8.7%) (Fig. 4B). Finally, we confirmed that the peptide cyclization was highly dependent on the reaction temperature (0.3–3.3% at 4–55°C, 57.6% at 85°C, and 82.7% at 100°C, under 1 h heating at pH 4.8) (Fig. 4C). These data indicate that we can both promote and

suppress cyclization of X-Hyp-Gly (other than Pro-Hyp-Gly) by controlling the process conditions; high temperature with weakly acidic/neutral pH to produce cyclo(X-Hyp) and low temperature with acidic pH to maintain X-Hyp-Gly-type tripeptides.

Rizzi previously described that tripeptides (Gly-Leu-Ala, Ala-Leu-Gly, Leu-Gly-Phe, and Pro-Gly-Gly) were converted into cyclic dipeptides consisting of N-terminal dipeptides by heating at 120°C for 0.5 h, showing 7–23% yields.[36] The study explained that the cyclodehydration reaction occurs by attack of the N-terminal amino group on the carbonyl group of the peptide bond between the internal and C-terminal amino acid residues followed by the loss of the C-terminal amino acid. However, the reported cyclization rate of the tripeptides used was markedly lower than that of the dipeptide (Val-Ala, 94%). In contrast, X-Hyp-Gly was found to be more easily converted into cyclo(X-Hyp) under the relatively moderate heating conditions. We speculate that the efficient cyclization of X-Hyp-Gly is attributed to the presence of the imino acid at the internal position. In addition, the C-terminal Gly is also assumed to be important for the efficient cyclization based on the comparison with the thermal conversion of Gly-Pro-Y-type tripeptides into cyclo(Gly-Pro) that were previously reported (24 h heating at 95°C for ~80% cyclization).[39] However, present data are insufficient to completely support these speculations. Further experiments are needed to determine the key sequence features for the highly efficient cyclization of X-Hyp-Gly.

Preparation of Stable Isotope-Labeled Internal Standards of Cyclo(X-Hyp). Using LC–MS, we next investigated whether the Hyp-containing cyclic dipeptides are absorbed into the blood. A recent study reported simultaneous quantification of 31 kinds of cyclic dipeptides using LC–MS with good repeatability and linearity for standards of the analytes.[45] However, the matrix effect was suggested to decrease the recovery rate of some of the cyclic dipeptides when spiked

into tea extracts. Ion suppression (or enhancement) effects in LC–MS caused by coeluting matrix components sometimes critically impair the quantitative accuracy. This is especially true in complex biological samples, as reported in our previous study analyzing Hyp-containing peptides in blood.[12] To accurately quantitate the cyclic dipeptides in blood, we utilized a recently developed internal standard mixture named SI-digest, in which various kinds of collagen-derived oligopeptides, including X-Hyp-Gly, are stable isotopically labeled.[12, 43] We further treated the SI-digest with heat (85°C and pH 4.8 for 1 h) to generate internal standards of cyclic dipeptides. Generation of stable isotope-labeled cyclo(Ala-Hyp), cyclo(Leu-Hyp), and also cyclo(Gly-Pro) was confirmed as shown in Fig. S2 in the **Supporting Information**. In the following experiments, the heat-treated SI-digest was mixed into plasma samples as an internal standard for LC–MS analysis.

Absorption of Cyclo(X-Hyp) into Blood after Oral Administration of Ginger-Degraded Collagen Hydrolysates. The collagen hydrolysate prepared using ginger protease was heated at 85°C and pH 4.8 for 3 h to generate cyclic dipeptides. We confirmed that the contents of cyclo(Ala-Hyp) and cyclo(Leu-Hyp) were increased from 0.7 to 5.2 mg/g and from 1.0 to 9.1 mg/g, respectively, following the heat treatment (Fig. 5A). A heat-induced increase was also detected for cyclo(Gly-Pro) (0.6 to 5.7 mg/g), which was likely derived from Gly-Pro-Y-type tripeptides, as reported previously.[39] The heat-treated and non-treated (control) collagen hydrolysates were orally administered to mice, and plasma samples for LC–MS analysis were obtained 1 h later. After oral administration of the control collagen hydrolysate, only slight amounts of the cyclic dipeptides were detected in the blood (Fig. 5B). In contrast, all three cyclic dipeptides displayed markedly higher plasma levels after the administration of the heat-treated collagen hydrolysate. The plasma concentrations of cyclic dipeptides paralleled their contents in

the administered collagen hydrolysates. These results demonstrate that orally administered collagen-derived cyclic dipeptides were absorbed into the blood.

Although we focused on cyclo(Ala-Hyp) and cyclo(Leu-Hyp) as representatives of the Hyp-containing cyclic dipeptides in this study, other cyclo(X-Hyp) were also detected in the ginger-degraded collagen hydrolysate after heat treatment (data not shown). As shown in Fig. 5A, the amount of cyclo(Ala-Hyp) and cyclo(Leu-Hyp) was comparable or higher than cyclo(Gly-Pro). While cyclo(Gly-Pro) is probably derived from various kinds of Gly-Pro-Y-type tripeptides, the Hyp-containing cyclic dipeptides are from their respective precursors (Ala-Hyp-Gly and Leu-Hyp-Gly), indicating that the total amounts of cyclo(X-Hyp) in the heat-treated collagen hydrolysate would be considerably high.

Efficient Absorption of Cyclo(X-Hyp) Compared to Collagenous Oligopeptides. Further evaluation of the absorption of the cyclic dipeptides was performed using synthetic peptides. Mice were orally administered peptide mixtures (100 μg each), which included cyclic dipeptides and various kinds of collagenous oligopeptides reported to appear in the blood after oral ingestion of collagen hydrolysate.[12, 46] Since some peptides cannot be discriminated after partial peptide hydrolysis in the gastrointestinal tract and blood (e.g., from Ala-Hyp-Gly to Ala-Hyp and Hyp-Gly), we divided the peptides into two experimental groups. After the administration, most of the peptides were observed to be increased in the plasma, except for Ala-Hyp, Gly-Ala-Hyp, and Gly-Pro-Ala (Fig. 6). Plasma concentrations of the collagenous dipeptides and tripeptides peaked at 0.5 or 1 h and returned to the basal level at 2 h. Among the oligopeptides, Pro-Hyp showed the highest maximum plasma concentration (C_{max}, 0.118 μg/mL). The $AUC_{0–6h}$ of the dipeptides and tripeptides ranged from 0.065 to 0.431 μg/mL·h (Table 1). On the other hand, Hyp-containing cyclic dipeptides showed higher values of C_{max} [0.748 and 0.626 μg/mL for

cyclo(Ala-Hyp) and 1.495 μg/mL for cyclo(Leu-Hyp)]. These higher concentrations were maintained for a relatively longer period of time. At 2 h after the administration, the plasma level relative to the C_{max} was still 43–100%. Due to the characteristic profiles of cyclo(X-Hyp), the $AUC_{0–6h}$ of the cyclic dipeptides reached >2 μg/mL·h, which was dramatically higher than that of the collagenous oligopeptides (Table 1). Similarly, a significantly higher plasma level was observed for another type of cyclic dipeptide, cyclo(Gly-Pro). Previous studies by Mizuma et al. demonstrated that cyclic dipeptides are more efficiently absorbed from the small intestine than the linear forms due to their high stability to proteolysis and high transportability via peptide transporters.[47, 48] Hydrophobicity is one of the determinants for the recognition of peptides by peptide transporters.[49, 50] Thus, the high hydrophobicity of cyclic dipeptides potentially enhances their intestinal absorption. These properties of cyclic dipeptides are assumed to contribute to the high oral bioavailability of cyclo(X-Hyp). *In vitro* transport studies using intestinal epithelial cells (Caco-2) are now in progress.

In conclusion, we demonstrate that X-Hyp-Gly-type tripeptides are almost completely converted into Hyp-containing cyclic dipeptides by heating at 85°C and pH 4.8 for 3 h, and that the cyclic dipeptides are more efficiently absorbed into the blood than conventional collagenous oligopeptides, including Pro-Hyp. Previous studies have reported that cyclo(Ser-Hyp) found in a hydrolysate of human placenta has various beneficial effects, including protection against liver injury,[33, 34] improvement of acute renal failure,[41] and promotion of corneal wound healing.[42] Such beneficial biological activities of cyclo(Ser-Hyp) could provide an indication that other Hyp-containing cyclic dipeptides might demonstrate beneficial effects as well.

ABBREVIATIONS USED

Hyp, hydroxyproline; DTT, dithiothreitol; LC, liquid chromatography; MS, mass spectrometry; SI-collagen, stable isotope-labeled collagen; AUC, area under the plasma concentration–time curve; MRM, multiple reaction monitoring; QTOF, quadrupole time-of-flight; C_{max}, maximum plasma concentration

Supporting Information. Figure S1: MRM chromatograms of Ala-Hyp-Gly and cyclo(Ala-Hyp). **Figure S2**: MRM chromatograms of stable isotope-labeled cyclo(Ala-Hyp), cyclo(Leu-Hyp), and cyclo(Gly-Pro) in SI-digest. **Table S1**: MRM transitions of amino acids, oligopeptides, and cyclic dipeptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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472 **Figure captions**

473 **Figure 1.** Heating experiments of collagenous oligopeptides. Contents of (A) Gly-Pro-Y-type
474 tripeptides and (B) X-Hyp-Gly-type tripeptides after heating of ginger-degraded collagen
475 hydrolysate at 85°C and pH 4.8 for 1 or 3 h. (C) Residual ratio of various types of synthetic
476 collagenous peptides after heating at 85°C and pH 4.8 for 1 h. (D) Concentrations of Ala-Hyp-
477 Gly and its potential degradation products after heating of Ala-Hyp-Gly (10 μg/mL) at 85°C and
478 pH 4.8 for 1 or 3 h. The data represent the mean ± SD of three separate experiments.

479

480 **Figure 2.** Identification of cyclo(Ala-Hyp) by mass spectrometric analysis. (A) QTOF–MS
481 spectra of Ala-Hyp-Gly (control) and heat-treated Ala-Hyp-Gly (85°C, 3 h). (B) Measured and
482 simulated QTOF–MS spectra of cyclo(Ala-Hyp).

483

484 **Figure 3.** Comparison of heat-induced cyclo(Ala-Hyp) formation beginning with either Ala-
485 Hyp-Gly or Ala-Hyp. (A) Ala-Hyp-Gly (10 nmol/mL) and (B) Ala-Hyp (10 nmol/mL) were
486 heated at 85°C and pH 4.8 for 1 or 3 h. The data represent the mean ± SD of three separate
487 experiments.

488

489 **Figure 4.** Effects of amino acid composition, reaction pH, and reaction temperature on
490 cyclization of X-Hyp-Gly. (A) Residual ratios of X-Hyp-Gly and X-Pro-Gly-type tripeptides
491 heated at 85°C and pH 4.8 for 1 h. (B and C) Conversion ratios for Ala-Hyp-Gly heated at (B)
492 85°C for 1 h with varying pH (2.4–9.6), and (C) pH 4.8 for 1 h with varying temperature (4–
493 100°C). The conversion ratio was calculated according to the concentration of generated

494 cyclo(Ala-Hyp) relative to the original concentration of Ala-Hyp-Gly. The data represent the
495 mean ± SD of three separate experiments.
496
497 **Figure 5.** Absorption of cyclic dipeptides after oral administration of ginger-degraded collagen
498 hydrolysates. (A) Contents of cyclo(Ala-Hyp), cyclo(Leu-Hyp), and cyclo(Gly-Pro) in ginger-
499 degraded collagen hydrolysates with (heat) or without (control) heating at 85°C and pH 4.8 for 3
500 h. The data represent the mean ± SD of three separate measurements. (B) Plasma concentrations
501 of cyclo(Ala-Hyp), cyclo(Leu-Hyp), and cyclo(Gly-Pro) after oral administration of 10 mg of the
502 collagen hydrolysates to ICR mice. The data represent the mean ± SD (n = 3).
503
504 **Figure 6.** Plasma concentrations of collagenous oligopeptides and cyclic dipeptides after oral
505 administration of the peptides. Synthetic peptide mixtures (100 μg each) were administered to
506 ICR mice, and plasma concentrations of the peptides were measured. Group 1: Ala-Hyp, Pro-
507 Hyp, Hyp-Gly, cyclo(Ala-Hyp), cyclo(Leu-Hyp), and cyclo(Gly-Pro). Group 2: Gly-Ala-Hyp,
508 Gly-Pro-Ala, Gly-Pro-Hyp, Ala-Hyp-Gly, Pro-Hyp-Gly, and cyclo(Ala-Hyp). The data represent
509 the mean ± SD (n = 5). ND, not detected.

Tables

Table 1. $AUC_{0–6h}$ of orally administered collagenous oligopeptides and cyclic dipeptides

	Peptide	μg/mL·h
Group 1	Ala-Hyp	0.070 ± 0.008
	Pro-Hyp	0.431 ± 0.089
	Hyp-Gly	0.069 ± 0.021
	Cyclo(Ala-Hyp)	2.350 ± 0.524
	Cyclo(Leu-Hyp)	3.206 ± 1.128
	Cyclo(Gly-Pro)	2.672 ± 0.870
Group 2	Gly-Pro-Hyp	0.196 ± 0.036
	Ala-Hyp-Gly	0.065 ± 0.015
	Pro-Hyp-Gly	0.184 ± 0.032
	Cyclo(Ala-Hyp)	2.036 ± 0.157

The data represent the mean ± SD (n = 5).

Figure Graphics

Figure 1

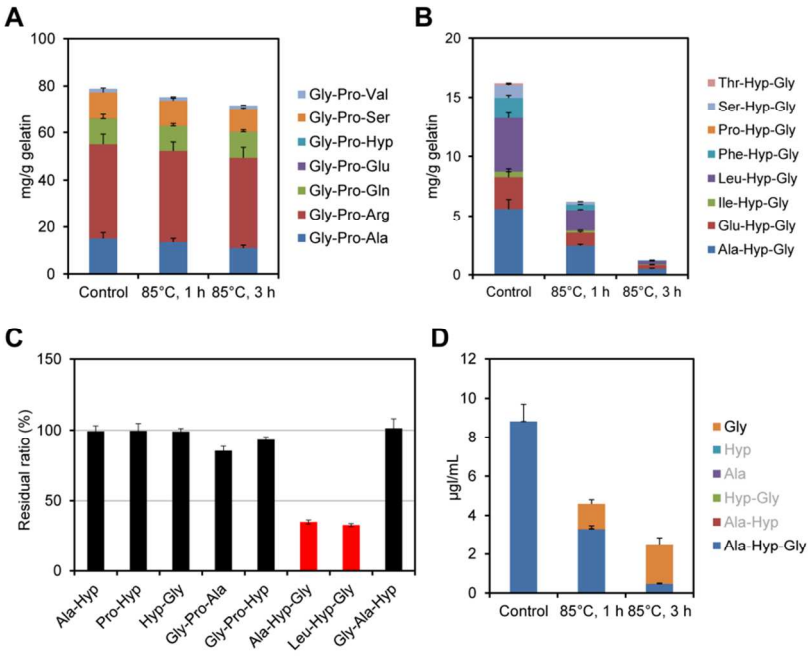


Figure 2

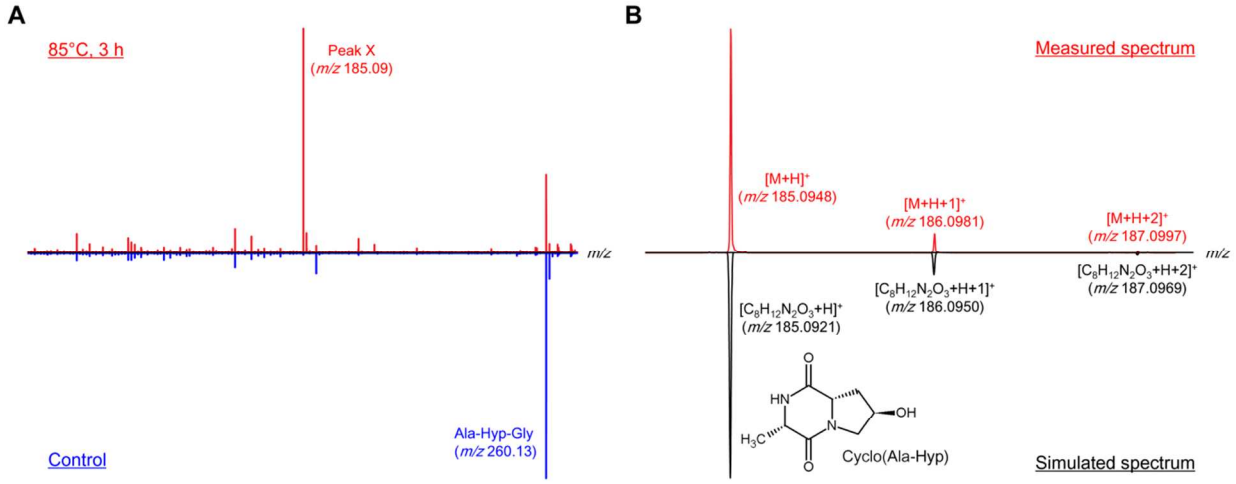

A
85°C, 3 h
Peak X
(m/z 185.09)
m/z
Ala-Hyp-Gly
(m/z 260.13)
Control
B
Measured spectrum
[M+H]+
(m/z 185.0948)
[M+H+1]+
(m/z 186.0981)
[M+H+2]+
(m/z 187.0997)
m/z
[C8H12N2O3+H+2]+
(m/z 187.0969)
[C8H12N2O3+H+1]+
(m/z 186.0950)
[C8H12N2O3+H]+
(m/z 185.0921)
HN
OH
N
H3C
O
O
Cyclo(Ala-Hyp)
Simulated spectrum

Figure 3

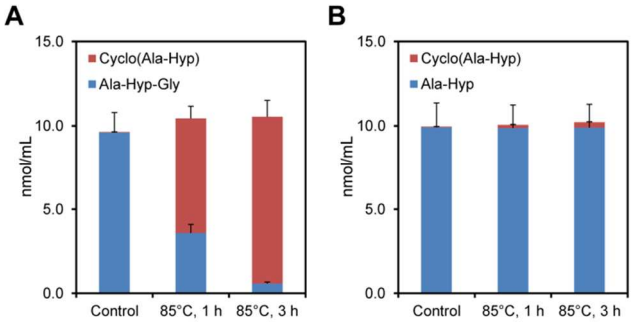

A
15.0
10.0
5.0
0.0
nmol/mL
Cyclo(Ala-Hyp)
Ala-Hyp-Gly
Control
85°C, 1 h
85°C, 3 h
B
15.0
10.0
5.0
0.0
nmol/mL
Cyclo(Ala-Hyp)
Ala-Hyp
Control
85°C, 1 h
85°C, 3 h

Figure 4

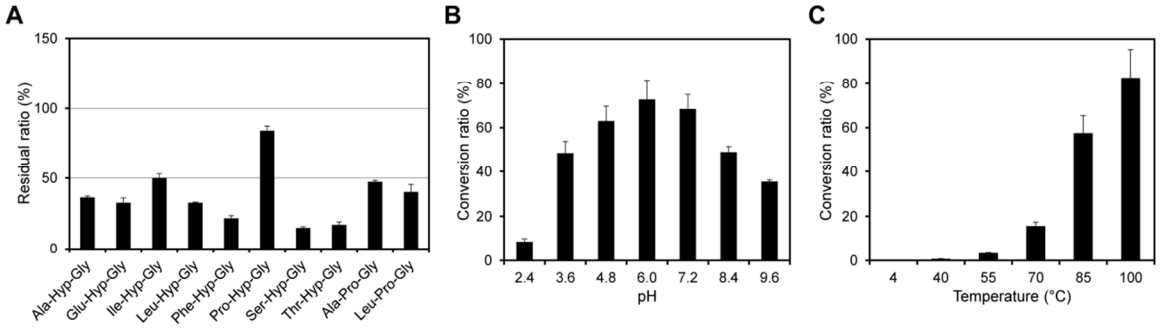

A
Residual ratio (%)
150
100
50
0
Ala-Hyp-Gly
Glu-Hyp-Gly
Ile-Hyp-Gly
Leu-Hyp-Gly
Phe-Hyp-Gly
Pro-Hyp-Gly
Ser-Hyp-Gly
Thr-Hyp-Gly
Ala-Pro-Gly
Leu-Pro-Gly
B
Conversion ratio (%)
100
80
60
40
20
0
2.4
3.6
4.8
6.0
7.2
8.4
9.6
pH
C
Conversion ratio (%)
100
80
60
40
20
0
4
40
55
70
85
100
Temperature (°C)

Figure 5

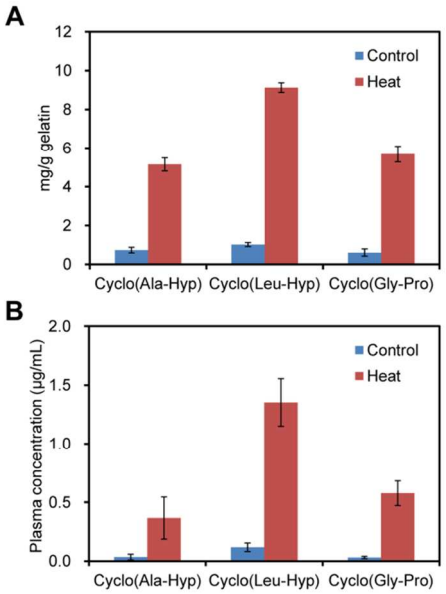
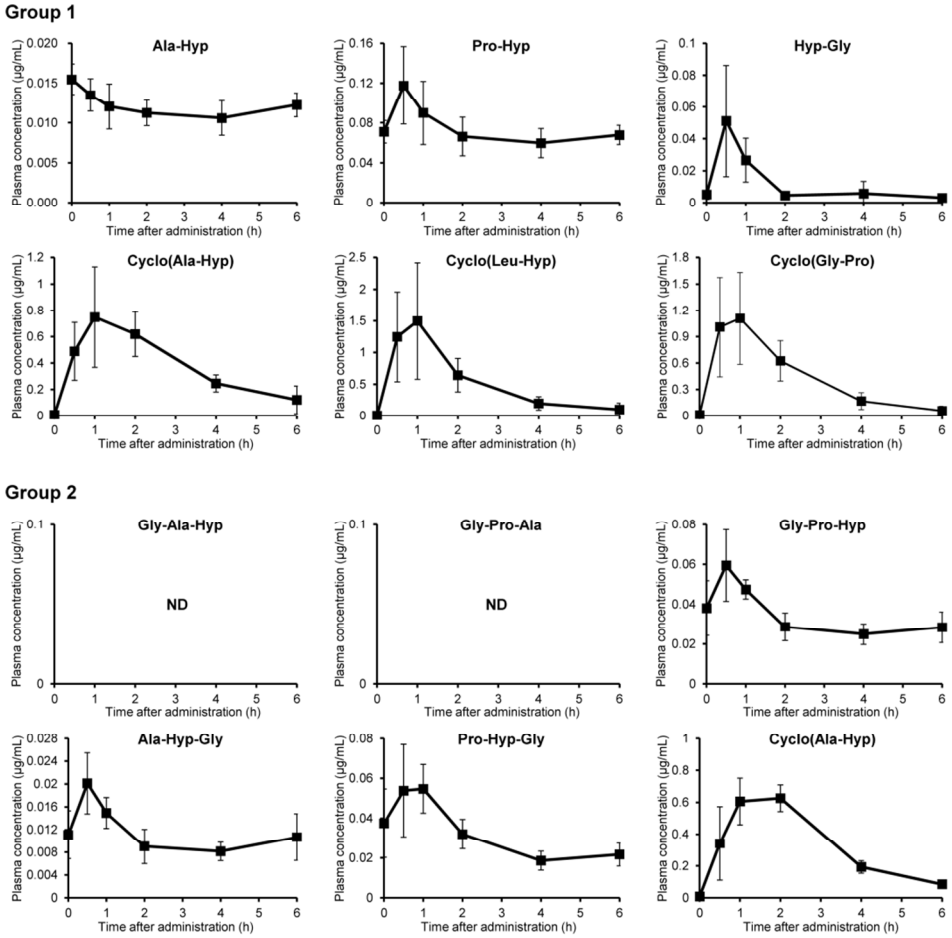

A
12
10
8
6
4
2
0
mg/g gelatin
Control
Heat
Cyclo(Ala-Hyp)
Cyclo(Leu-Hyp)
Cyclo(Gly-Pro)
B
2.0
1.5
1.0
0.5
0.0
Plasma concentration (μg/mL)
Control
Heat
Cyclo(Ala-Hyp)
Cyclo(Leu-Hyp)
Cyclo(Gly-Pro)

Figure 6


Group 1
Ala-Hyp
Pro-Hyp
Hyp-Gly
Cyclo(Ala-Hyp)
Cyclo(Leu-Hyp)
Cyclo(Gly-Pro)
Group 2
Gly-Ala-Hyp
ND
Gly-Pro-Ala
ND
Gly-Pro-Hyp
Ala-Hyp-Gly
Pro-Hyp-Gly
Cyclo(Ala-Hyp)
Plasma concentration (μg/mL)
Time after administration (h)

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