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Orally Available Collagen Tripeptide: Enzymatic Stability, Intestinal Permeability, and Absorption of Gly-Pro-Hyp and Pro-Hyp

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ABSTRACT: Collagen-derived small peptides, such as Gly-Pro-Hyp (GPH) and Pro-Hyp (PH), play a role in various physiological functions. Although collagen degrades in the gastrointestinal tract randomly and easily, it is not readily cleaved into bioactive peptides. To increase the bioavailability of bioactive peptides, a collagen tripeptide (CTP) was prepared from fish scales by the digestion method using collagenase from nonpathogenic *Bacillus* bacteria. It was demonstrated that Hyp-containing peptides—GPH and PH—were better absorbed and reached higher plasma levels after the oral administration of CTPs in rats compared to high molecular weight collagen peptide (H-CP). GPH and PH were stable in gastrointestinal fluid and rat plasma for 2 h, and GPH was able to be transported across the intestinal cell monolayer. These results suggest that the ingestion of CTP is an efficient method for taking bioactive peptides orally due to the enzymatic stability and intestinal permeability of GPH and PH.

KEYWORDS: *collagen tripeptide, absorption, rat, Gly-Pro-Hyp, Pro-Hyp*

INTRODUCTION

Collagen and collagen peptides, including collagen tripeptide (CTP; tripeptide fractions from collagen), have been used as supplementary health foods with several biological effects. Peptides derived from collagen, including Pro-Hyp (PH) and Gly-Pro-Hyp (GPH), exert chemotactic effects on fibroblasts, peripheral blood neutrophils,[1,2] and monocytes[3] in Boyden chemotaxis chambers. These cells play a significant role in wound healing and inflammation.[4,5] GPH is also thought to be involved in platelet aggregation.[6] PH has been found to enhance mouse and human fibroblast cell proliferation and hyaluronic acid production.[7,8] Also, CTP has been reported to show osteotropic activity[9] and improved skin properties in the acetone-induced dry skin mouse model[10] and in human subjects.[11]

Collagen has a unique triple-helical structure composed of three α chains, including a frequently repeating sequence of Gly-X-Y, where X and Y are mostly Pro and Hyp.[12] The tripeptide, GPH, is considered to be a collagen-specific sequence. PH is a major active constituent of collagen-derived peptides, and it is absorbed from the gastrointestinal tract after collagen ingestion.[13,14] GPH is a minor component of the collagen-derived peptides remaining in human blood after the oral ingestion of collagen hydrolysates, although Gly-Pro-Hyp is an abundant sequence in collagen.[13,15] Marketed collagen peptides are usually manufactured from collagen by acid hydrolysis or by random site digestion using proteases. Recently, collagen tripeptide has been produced by several companies using collagenase to preserve the collagen-specific sequence, Gly-Pro-Hyp, during collagen hydrolysis and to increase the bioavailability of bioactive peptides. A high level of GPH in rat plasma was reported after the oral administration of low molecular weight collagen hydrolysates[16] or collagen tripeptide.[17] The smaller peptide is expected to be more stable against gastrointestinal and plasma enzymes and more convenient for transporting intestinal epithelial cell membranes. However, the exact reasons for why GPH is efficiently absorbed after the oral administration of CTP have not been clarified.

In this study, we demonstrate that Hyp-containing peptides GPH and PH are absorbed more effectively and reach a higher plasma level after the oral administration of CTP in rats compared to high molecular weight collagen peptide (H-CP). We confirmed that GPH and PH were stable in gastrointestinal fluid and rat plasma for 2 h. The ability of GPH and PH to permeate biological membranes was also studied using an in vitro model (Caco-2 cell monolayer) of human intestinal mucosa.

MATERIALS AND METHODS

CTP-L, CTP-H, and H-CP Preparation. Low-strength CTP (CTP-L) was prepared from fish scales by the digestion method using collagenase (Amicogen Inc., Jinju, South Korea) from nonpathogenic bacteria of the *Bacillus* species. The collagenase recognized the collagen-specific sequence Gly-Pro-X and produced larger amounts of Gly-X-Y tripeptides than other types of proteases, such as Alcalase. The mean molecular weight of CTP-L was approximately 500 Da. The contents of the total tripeptides, GPH, and PH in CTP-L were 25, 3.5, and 0.9%, respectively (Table 1). High-strength CTP (CTP-H) was prepared from CTP-L after further purification by activated carbon columns, ion-exchange columns, and microfiltration. The tripeptide fraction of CTP-H was 60% (Table 1). The specific characteristics of each collagen peptide are listed in Table 1. To compare the absorption efficiency of CTP with a general collagen hydrolysate, H-CP, which has an average molecular weight of 2 kDa, was prepared by random

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Table 1. Characteristics of the High Molecular Weight Collagen Peptide (H-CP), Low-Strength Collagen Tripeptide (CTP-L), and High-Strength Collagen Tripeptide (CTP-H) Studied

	H-CP	CTP-L	CTP-H
mean molecular weight (Da)	2000	500	246
fraction of tripeptide (%, w/w)	<1	25	60
Gly-Pro-Hyp content (%, w/w)	nd[a]	3.5	15
Pro-Hyp content (%, w/w)	nd	0.9	2.5

[a]nd, not detected.

site digestion using Alcalase. H-CP was prepared from the same fish scales that had been used for preparing CTP-L and CTP-H. The contents of the tripeptides and the molecular weight distribution of the peptides in CTP-L, CTP-H, and H-CP were determined by gel filtration chromatography. The prepared collagen peptides all fulfilled the requirements for food grading.

Materials. The peptides, PH and GPH, were purchased from Bachem (Bubendorf, Switzerland) and Anygen (Kwangju, South Korea), respectively. The following materials were products of Sigma-Aldrich Co. (St. Louis, MO, USA): somatostatin, thyrotropin, oxytocin, pancreatin from porcine pancreas, formic acid, gabapentin, and Dulbecco's modified Eagle medium (DMEM). Other cell culture supplements and the 12-well Transwell plates were purchased from Corning Life Sciences (Foster City, CA, USA). HPLC grade acetonitrile and water were purchased from Fisher Scientific (Pittsburgh, PA, USA). All other chemicals were of analytical grade.

Animals. Eight-week-old male Sprague−Dawley rats (280−310 g) were purchased from Koatech Co. (Sungnam, South Korea). The animals were acclimatized for 1 week in a clean room (Laboratory Animal Research Center, Gyeongsang National University, Jinju, South Korea) at a temperature between 20 and 23 °C under a 12-h light−dark cycle and a relative humidity of 50 ± 5%. The Sprague−Dawley rats were fed irradiated pellet food and sterilized drinking water ad libitum. The protocol for this animal study (GNU-141210-R0060) was approved by the Animal Care and Use Committee of Gyeongsang National University.

Study of GPH and PH Stability in Rat Gastric Juice, Simulated Intestinal Fluid, and Rat Plasma. The stability of GPH and PH in gastric juice and simulated intestinal fluid was evaluated to assess whether GPH and PH are degraded in the gastrointestinal tract before absorption. Somatostatin and oxytocin were used as positive controls for gastric degradation and intestinal degradation, respectively.[18] The stability of GPH and PH in plasma was also tested to determine the stability of GPH and PH after sampling and during storage. Thyrotropin was used as a positive control for degradation by peptidase in plasma.[19] The gastric juice and plasma were collected from three rats and kept at −80 °C. The simulated intestinal fluid was prepared according to the United States Pharmacopeia and the National formulary (USP-NF) specifications (Test Solutions, United States, Pharmacopeia 35, NF 30, 2012). Briefly, 0.68 g of monobasic potassium phosphate was dissolved in 25 mL of water. Then, 7.7 mL of 0.2 N NaOH was added to adjust the pH to 6.8. Then 1 g of pancreatin was added to the solution, and the mixture was shaken gently to avoid precipitation until it was dissolved; after, the volume was adjusted to 100 mL with water.

A stock solution of GPH, PH, or a positive control compound was added to the rat gastric juice, simulated intestinal fluid, or rat plasma for a final concentration of 1 μM. The tubes were placed in a Thermomixer (Effendorf, Germany) and were maintained at 37 °C and 300 rpm. These samples were incubated for up to 2 h. At different time intervals, an aliquot (100 μL) of an ice-cold internal standard was added to a 50 μL aliquot of the sample. The same sample preparation method as that for plasma was used, and the amount of each peptide remaining after incubation was immediately determined by liquid chromatography−tandem mass spectrometry (LC-MS/MS).

Caco-2 Cell Permeability Assay. The Caco-2 cell line was obtained from the Korean Cell Line Bank (Seoul, South Korea). Cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin−100 U/mL streptomycin, and 1% nonessential amino acid at 37 °C in a 95% air/5% CO_2 atmosphere. The cells were subcultured before reaching confluence, and the medium was changed every other day. For the permeability assay, cells were harvested and seeded at a density of 3×10^5 cells/cm^2 onto a polycarbonate Transwell membrane (pore size = 0.4 μm) coated with type I collagen and maintained for 21 days. The epithelial monolayer integrity was checked by measuring transepithelial electrical resistance (TEER). The Transwell monolayers with TEER values of >300 Ω·cm^2 were used for the assay. The permeability study was carried out as previously reported by van Breemen and Li.[20] In brief, the cell monolayer after 21 days of culture was used. Culture medium was removed and replaced by prewarmed transport medium, which was Hank's balanced salt solution (HBSS) containing 25 mM glucose, 350 mg/L sodium bicarbonate, and 10 mM HEPES. GPH or PH in transport medium (200 μM) was loaded to the apical (donor) chamber of the cell monolayer, and drug-free transport medium was added to the opposite, basolateral (receiver) chamber. An aliquot of 50 μL from the donor chamber was sampled to determine the initial concentration (C_0) at time 0. Aliquots of 50 μL were taken at 20, 40, 60, and 90 min from the receiver chamber and replaced with prewarmed drug-free transport medium to maintain sink conditions. The concentration of GPH and PH in the transport medium was determined by LC-MS/MS. Lucifer yellow (300 μM) was used as a paracellular transport marker compound to monitor the integrity of the monolayer during the experiment. The apparent permeability coefficient, measured using the cumulative amount of the peptide permeated across the membrane, was calculated with the equation

$$P_{app}\ (\mathrm{cm/s}) = \mathrm{d}Q/\mathrm{d}t \times 1/(A \times C_0)$$

where C_0 is the initial concentration in the donor solution (pmol/cm^3), A is the surface area of the inset filter membrane (cm^2), t is the incubation time (s), and $\mathrm{d}Q/\mathrm{d}t$ is the amount of peptides transported within a given time period (pmol/s).

In Vivo Absorption Study in Rats. The procedures for the pretreatment of rats, including the cannulation, have been reported previously.[21] One day before the administration of collagen peptides, the carotid artery of Sprague−Dawley rats was cannulated by insertion of polyethylene tubing under light ethyl ether anesthesia. The cannula tunneled under the skin was exteriorized to the dorsal side of the neck. A harness was used to allow free movement of the rats, and the cannula was embedded in the harness to protect it from damage. Rats were individually placed in each cage and allowed to recover from anesthesia before the start of the experiment. The rats were subjected to overnight fasting, with free access to water. Subsequently, they were assigned to receive either CTP-L, CTP-H, or H-CP at a dose of 500 mg/kg (5 mL/kg, dissolved in distilled water) orally with a feeding tube. There are some reports[9,10] that orally administered 500 mg/kg showed biological activity in vivo. An oral administration of 500 mg/kg CTP to rats with a femur fracture accelerated the fracture healing.[9] The content of tripeptides in the CTP (20%) used for rats with femur fracture was similar to the content of tripeptides in CTP-L used in our study (25%). Okawa et al. reported that oral administration of 500 mg/kg CTP improved dryness and pruritus in acetone-induced dry skin model mice.[10] Therefore, the 500 mg/kg dose was selected. The doses of GPH were estimated as 17.5 and 75 mg/kg for CTP-L and CTP-H on the basis of GPH content in CTP-L and CTP-H, respectively. The GPH dose of the H-CP group could not be estimated because GPH was not detected in H-CP (Table 1). An approximately 120 μL aliquot of blood sample was collected at 0 (before administration), 15, 30, 45, 60, 90, 120, 180, 240, and 360 min after oral administration, repeatedly. Heparinized 0.9% NaCl-injectable solution (20 U/mL) was used to flush immediately after each blood sampling to prevent blood clotting. Plasma was separated by centrifugation at 10000 rpm for 10 min and kept frozen at −20 °C before analysis. The concentration of GPH and PH in rat plasma was determined by LC-MS/MS.

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LC-MS/MS Analysis. Hyp-containing peptides in rat plasma were determined by LC-MS/MS, as described by Ichikawa et al.[15] with slight modifications. Standard solutions of GPH and PH for obtaining the calibration curve were prepared in phosphate-buffered saline, yielding final concentrations from 20 to 2000 ng/mL. Quality control samples (20, 50, 500, and 2000 ng/mL) were prepared like the standard solutions to evaluate accuracy and precision. Accuracy and precision (intrabatch and interbatch) were assessed by analysis of five replicate quality control samples on different batches. An aliquot (100 μL) of acetonitrile containing the internal standard (gabapentin 0.1 μg/mL) was added to 50 μL of plasma sample, vortex-mixed, and then kept for 30 min at 4 °C for protein precipitation. After centrifugation at 4 °C and 10000*g* for 10 min, the supernatant was transferred to a clean tube and then evaporated under a stream of nitrogen. The residue was dissolved in 200 μL of 0.1% formic acid in water and filtered using a 0.2 μm pore syringe filter; then 2 μL was injected into the LC-MS/MS system for analysis.

The LC-MS/MS system consisted of an Agilent 1260 HPLC system (Agilent, Germany) with an Agilent 6460 triple-quadrupole mass spectrometer (Agilent, Singapore) equipped with an electrospray ionization (ESI) source. A Zorbax SB-Aq (particle size 1.8 μm, 2.0 mm, i.d. × 100 mm, *l*; Agilent, Palo Alto, CA, USA) reversed phase column was used for HPLC separation. Column temperature was set as 30 °C. The HPLC mobile phase consisted of 0.1% aqueous formic acid (A) and acetonitrile (B), and a gradient program was used at a flow rate of 0.25 mL/min. The initial composition of 100% A was maintained for 2 min; it was then increased linearly from 0 to 90% B for 2 min and held for 1 min. The gradient was then changed back to the initial condition in 1 min and kept at the initial condition for 2 min. The total run time was 8 min. ESI was performed in the positive mode with nitrogen as the nebulizing, turbo spray, and curtain gas. The multiple reaction monitoring (MRM) detection method was used for the detection of peptides. Transitions monitored for peptides were m/z 229 → 70 for PH, m/z 286 → 155 for GPH, and m/z 172 → 154 for internal standard. The concentrations of peptides in the samples were determined from a calibration curve of the peak area ratio of the analyte to internal standard. The retention times of PH, GPH, and internal standard were approximately 2.3, 2.8, and 6.7 min, respectively. The calibration curves of peptides were linear over the ranges studied, with r^2 > 0.999. The limit of quantitation of PH and GPH was 20 ng/mL. The accuracy for PH and GPH ranged from 85.9 to 104.5%, whereas the coefficients of variation of the assay (intrabatch and interbatch precisions) were <8%.

Pharmacokinetic and Statistical Analysis. The total area under the plasma concentration−time curve from time zero to the last measured time, 6 h ($AUC_{0-6\ h}$) was calculated by the linear trapezoidal method.[22] The peak plasma concentration (C_{max}) and time to reach C_{max} (T_{max}) were retrieved directly from the experimental data.

A p value of <0.05 was considered to be statistically significant when using a t test between two means for the unpaired data or when using a one-way analysis of variance (ANOVA), followed by Dunnett's t test for multiple comparisons among three means for the unpaired data. All results, except the median (range) for T_{max}, are expressed as the mean ± standard deviation (SD).

RESULTS

Stability of GPH and PH. GPH and PH were stable in the rat gastric juice, simulated intestinal fluid, and rat plasma for up to 2 h (Figure 1). More than 90% of the initial amount remained in all solutions tested for the 2-h incubation period; however, the positive controls were not stable. The half-life (43 min) of somatostatin determined (Figure 1A) was similar to the values presented in the literature (58 min in human gastric fluid and 49 min in simulated gastric fluid).[18] Oxytocin was degraded almost within 15 min in the simulated intestinal fluid. Only 0.96% of oxytocin remained after the 2-h incubation period (Figure 1B). Thyrotropin also rapidly degraded in the rat plasma, and the concentration was reduced to 1.09% after the 2-h incubation period (Figure 1C). Accordingly, the rat gastric juice, simulated intestinal fluid, and rat plasma used showed enough peptidase activity and were thus suitable for the evaluation of peptide stability. This result suggests that the chemical and enzymatic degradation of GPH and PH in the rat gastric juice, gastrointestinal enzymes, and rat plasma is almost negligible.

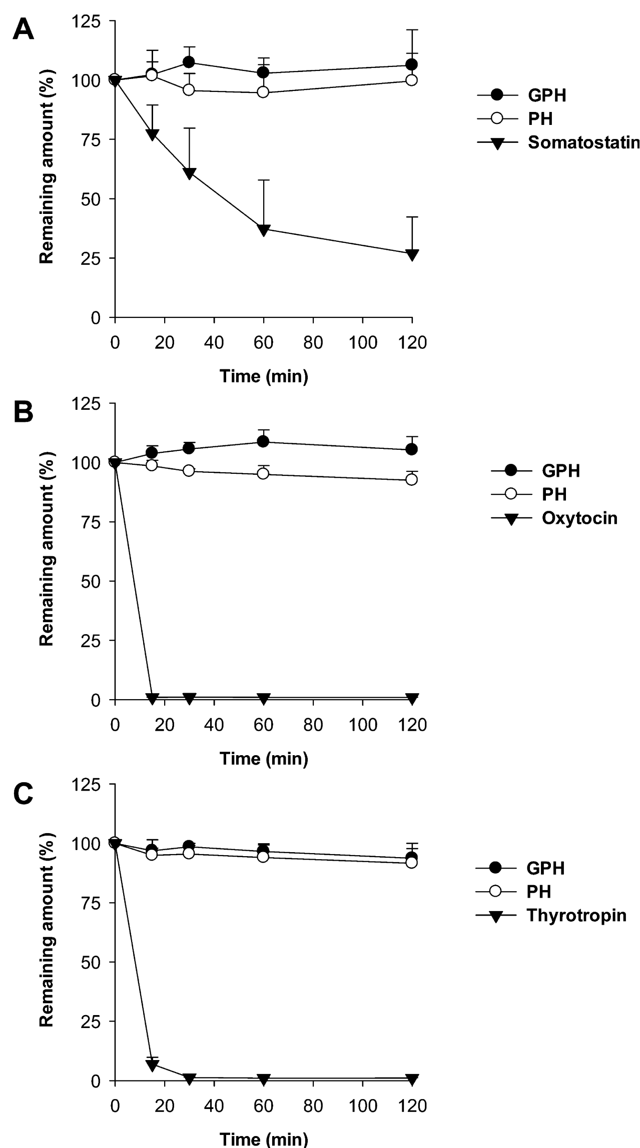

Figure 1. Stability of Gly-Pro-Hyp (GPH) and Pro-Hyp (PH) in rat gastric juice (A), simulated intestinal fluid with pancreatin (B), and rat plasma (C) (n = 3 for each). Bars represent standard deviation.

Caco-2 Cell Permeability Assay. To confirm the suitability of the Caco-2 cell monolayer permeability method, propranolol, atenolol, and lucifer yellow were tested as high- and low-permeability model drugs and paracellular transport marker compound, respectively. The mean apparent permeability coefficient (P_{app}) of propranolol was 5.82 × 10^{-5} cm/s, which is similar to the range ((1.12 ± 0.05)−(4.30 ± 3.6) × 10^{-5} cm/s) published in the literature.[23] The lucifer yellow flux values for the monolayer for 90 min were <2% both before and after the experiment, thus ensuring the integrity of the cell monolayer. Accordingly, our Caco-2 cell monolayer system was determined as suitable for the evaluation of peptides

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permeability. The time courses of GPH and PH transport profiles are shown in Figure 2. When the GPH was loaded onto

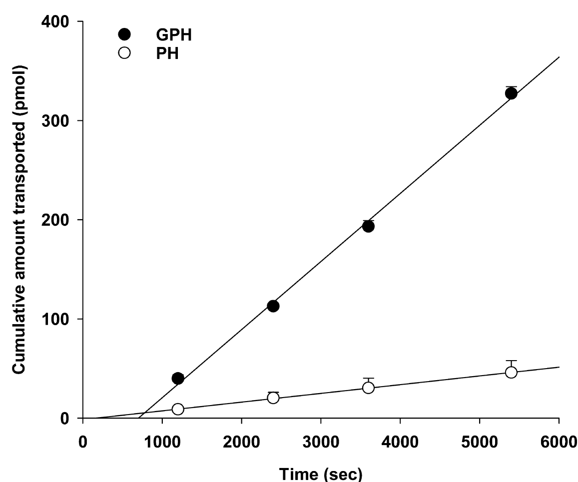

Figure 2. Mean cumulative amount of Gly-Pro-Hyp (GPH; ●, n = 3) and Pro-Hyp (PH; ○, n = 3) transported across the Caco-2 cell monolayer after loading of 200 μM of each peptide onto apical chamber, respectively. Bars represent standard deviation.

the donor chamber, GPH and PH transported across the epithelial cell monolayer were both detected in the receiver chamber from the first sampling time (20 min). The P_{app} value of GPH was $(1.09 \pm 0.03) \times 10^{-6}$ cm/s, which is slightly higher than that of the low-permeability marker drug, atenolol ($(0.50 \pm 0.08) \times 10^{-6}$ cm/s). The values were statistically different from those of atenolol. The P_{app} value of atenolol was also within the reported range ($(0.13-0.57) \times 10^{-6}$ cm/s).[23] The cumulative amount of PH transported across the Caco-2 cell monolayer 90 min after the GPH was loaded onto the donor chamber was 11.8 ± 0.21 pmol. It was only 3.59% of GPH amount transported across the monolayer. Meanwhile, the P_{app} value ($(0.13 \pm 0.03) \times 10^{-6}$ cm/s) of PH was significantly lower than that of GPH as the PH was loaded onto the donor chamber.

In Vivo Absorption Study. The plasma concentrations of GPH and PH were determined by the LC-MS/MS method. The validated analysis method was enough to quantify GPH and PH in plasma after oral administration of CTP-L, CTP-H, and H-CP at a dose of 500 mg/kg to rats. The calibration curves of GPH and PH covered the concentration range of samples. After the oral administration of peptides, the mean concentration versus time profiles of GPH and PH are shown in Figures 3 and 4, respectively, and relevant absorption parameters are listed in Tables 2 and 3. GPH and PH were absorbed rapidly and reached peak concentrations (C_{max}), which were at least 10 times higher than their initial concentrations, within 15−60 min after oral administration of CTP-L and CTP-H. The concentration of PH was slightly increased and reached C_{max} in the plasma 0.5−6 h after oral administration of H-CP. Meanwhile, the plasma level of GPH was not significantly different from initial concentration in the H-CP ingestion group for 6 h. The GPH C_{max} values were significantly higher in the CTP-L and CTP-H groups than in the H-CP group (15 and 79 times the corresponding H-CP group values). The GPH $AUC_{0-6\ h}$ values in the CTP-L and CTP-H groups were also significantly higher than those in the H-CP group (2.6 and 12 times the corresponding H-CP group

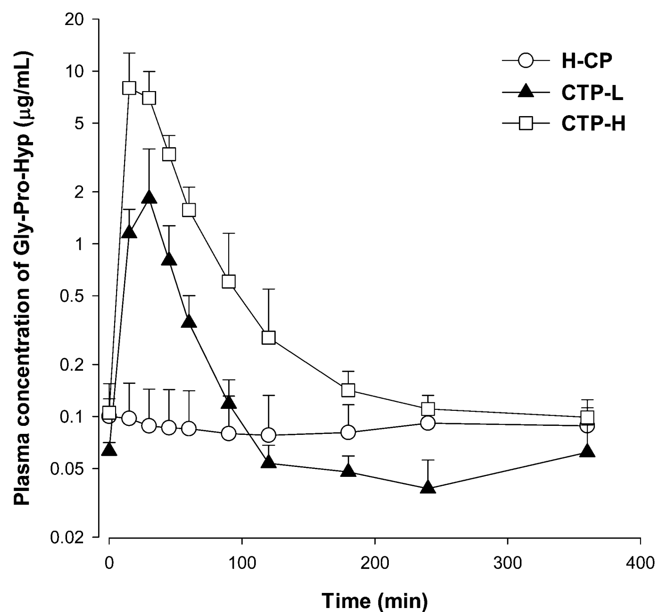

Figure 3. Mean plasma concentration−time profile of the tripeptide, Gly-Pro-Hyp (GPH), after oral administration (500 mg/kg) of high molecular weight collagen peptide, H-CP (○; n = 6), low-strength collagen tripeptide, CTP-L (▲; n = 6), and high-strength collagen tripeptide, CTP-H (□; n = 5) to male Sprague−Dawley rats. Bars represent standard deviation.

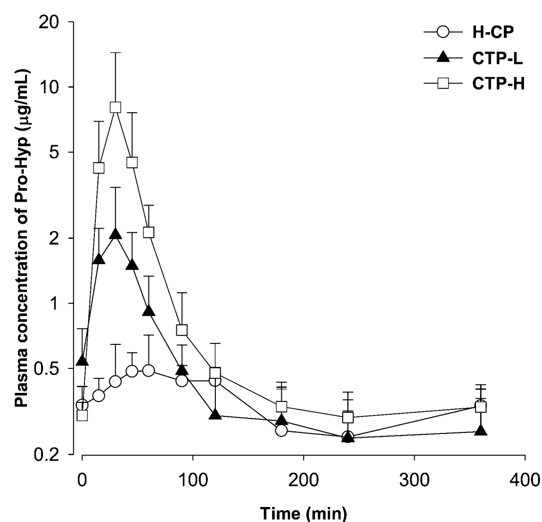

Figure 4. Mean plasma concentration−time profile of the dipeptide, Pro-Hyp (PH), after oral administration (500 mg/kg) of high molecular weight collagen peptide, H-CP (○; n = 6), low-strength collagen tripeptide, CTP-L (▲; n = 6), and high-strength collagen tripeptide, CTP-H (□; n = 5) to male Sprague−Dawley rats. Bars represent standard deviation.

values). However, the GPH T_{max} values were comparable among the three rat groups. The PH C_{max} values in the CTP-L and CTP-H groups were significantly higher than those in the H-CP group (3.7 and 14 times the corresponding H-CP group values). The $AUC_{0-6\ h}$ values of PH were also significantly higher in the CTP-L and CTP-H groups than in the H-CP group (1.5 and 3.4 times the corresponding H-CP group values). However, the PH T_{max} values were comparable among the three rat groups.

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Table 2. Mean (±SD[a]) Absorption Parameters of Gly-Pro-Hyp of High Molecular Weight Collagen Peptide (H-CP), Low-Strength Collagen Tripeptide (CTP-L), and High-Strength Collagen Tripeptide (CTP-H) to Male Sprague−Dawley Rats

parameter[b]	H-CP (n = 6)	CTP-L (n = 6)	CTP-H (n = 5)
$AUC_{0-6\ h}$ (μg·min/mL)	31.02 ± 15.96	79.34 ± 41.18*[c]	365.8 ± 101.8***
C_{max} (μg/mL)	0.1099 ± 0.0546	1.658 ± 1.786*	8.658 ± 3.908***
T_{max} (min)	37.5 (0−240)[d]	30 (15−30)	15 (15−45)

[a]SD, standard deviation. [b]$AUC_{0-6\ h}$, total area under the plasma concentration−time curve from time zero to last measured time, 6 h; C_{max}, peak plasma concentration; T_{max}, time to reach C_{max}. [c]Significantly different from H-CP group (*, $p < 0.05$; ***, $p < 0.001$). [d]Median (range).

Table 3. Mean (±SD[a]) Absorption Parameters of Pro-Hyp of High Molecular Weight Collagen Peptide (H-CP), Low-Strength Collagen Tripeptide (CTP-L), and High-Strength Collagen Tripeptide (CTP-H) to Male Sprague−Dawley Rats

parameter[b]	H-CP (n = 6)	CTP-L (n = 6)	CTP-H (n = 5)
$AUC_{0-6\ h}$ (μg·min/mL)	122.9 ± 17.36	181.9 ± 55.62*[c]	411.9 ± 169.9**
C_{max} (μg/mL)	0.5851 ± 0.0817	2.151 ± 1.299*	8.236 ± 6.158*
T_{max} (min)	60 (30−360)[d]	30 (15−60)	30 (30−60)

[a]SD, standard deviation. [b]$AUC_{0-6\ h}$, total area under the plasma concentration−time curve from time zero to last measured time, 6 h; C_{max}, peak plasma concentration; T_{max}, time to reach C_{max}. [c]Significantly different from H-CP group (*, $p < 0.05$; **, $p < 0.01$). [d]Median (range).

DISCUSSION

In the present study, we quantified GPH and PH, which are both Hyp-containing peptides, in rat plasma after the oral administration of CTP and H-CP. Collagenase from non-pathogenic *Bacillus* bacteria was used for CTP production. Some collagenases have high activity levels, but they are usually isolated from the pathogenic *Clostridium* species.[24] The CTP prepared in our study can be safely used as a functional food.

In our preliminary study, we measured the rat plasma levels of GPH, Ser-Hyp-Gly, Ala-Hyp-Gly, PH, and Ala-Hyp after the oral administration of CTP-L. In agreement with previous studies,[17] GPH and PH were the major peptides in the plasma, and the levels of other peptides were not significantly changed after the ingestion of CTP-L. PH is reportedly a major Hyp-containing peptide in human blood after the oral ingestion of gelatin hydrolysates, and it can be absorbed as an intact dipeptide by the gastrointestinal tract.[13,15] GPH has been considered to be hydrolyzed to PH in the gastrointestinal tract before absorption[25] because GPH is a minor component detected in human blood after the oral ingestion of collagen or collagen hydrolysates.[12,14] However, recent papers state that GPH can be absorbed as an intact form after the oral administration of low molecular weight collagen hydrolysates[16] and collagen tripeptide.[17] To clarify why CTP is efficiently absorbed, we evaluated the stability and permeability of major Hyp-containing peptides GPH and PH.

GPH and PH were found to be stable, with a >90% recovery rate in gastrointestinal fluid and plasma after a 2-h incubation period. They were not decomposed by gastric acid, gastric enzymes, pancreatin, or plasma peptidases of rats (Figure 1). These results were consistent with the PH stability reported in human plasma by Meilman et al.[26] PH is not hydrolyzed by plasma peptidase activity and is excreted into urine without degrading in humans.[26]

The Caco-2 cell monolayer system has been widely used as a model for the human intestinal absorption of drugs and nutrients.[20] Caco-2 cells derived from a human intestinal adenocarcinoma spontaneously differentiate to form tight junctions expressing brush-border proteases and transporters, including PEPT1.[27] Aito-Inoue et al. suggested that GPH could not cross the epithelial apical membrane in an intact form and be hydrolyzed to PH by aminopeptidase N. Then the resulting PH is transported into the small intestinal epithelial cells.[25] However, in our study, GPH was able to pass through the monolayer in an intact form, and the major form transported into the receiver chamber was GPH and not PH when GPH was loaded onto the donor chamber of the Caco-2 cell monolayer. Moreover, the mean P_{app} value of GPH was about 8 times higher than that of PH. The P_{app} values of PH were found to be similar to the reported P_{app} values of uncharged tripeptide (Gly-Asn-Ala) transported across the Caco-2 cell monolayer via the paracellular pathway.[28] In general, permeation across the cell monolayer is dependent on molecular size and charge if the compounds are transported mainly via the paracellular pathway. However, the permeation of GPH was much greater than that of PH, although GPH and PH are both uncharged peptides and the molecular size of GPH is larger than that of PH. This suggests that active transporters, such as oligopeptide transporters, might be involved in the permeation of GPH across the intestinal epithelial layer. The specific transporter for GPH transport, however, needs further investigation. The aforementioned data suggest that GPH could be stable in the gastrointestinal tract and absorbed in an intact form into systemic circulation after ingestion. The possibility remains that peptidases differ between rats and humans, although GPH was stable in rat plasma and gastric juice. Further studies using human plasma or human peptidases could be helpful to determine the efficiency of GPH in humans.

The absorption of GPH was confirmed by studying in vivo absorption in rats. When H-CP was administered to rats, it was difficult to detect changes in the plasma's GPH levels. This result is similar to that reported in a human study.[15] However, the GPH level in the plasma quickly increased and reached the C_{max}, which was 10 times higher than the basal level at around 1 h after CTP administration. Thereafter, the concentration of GPH in the plasma declined exponentially to its initial level within 3 h. This indicates that the increased GPH in the plasma originating from oral ingestion was not synthesized in rats. Watanabe-Kamiyama et al. also reported that GPH remained in rat plasma for several hours in its intact form after the oral ingestion of low molecular weight collagen hydrolysate using radiolabeled tracers.[16] Long peptides might be more prone to random degradation in the gastrointestinal tract. Meanwhile, dipeptides or tripeptides might be more resistant to proteases or peptidases in the blood and the gastrointestinal tract. We

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confirmed the stability of GPH and PH in gastrointestinal fluid and rat plasma.

It is well-known that the oral delivery of bioactive peptides is limited because they are unstable in the gastrointestinal tract, poorly penetrate through the intestinal mucosa, and metabolize rapidly in systemic circulation. For example, the AUC of the milk casein-derived pentapeptide HLPLP, which has an antihypertensive effect, was reported as 0.603 μg·min/mL after the oral administration of 40 mg/kg of HLPLP in rats.[29] The mean $AUC_{0-6\ h}$ value of GPH was 79.34 μg·min/mL after the oral administration of a 17.5 mg/kg dose of GPH in CTP-L (Table 2). Therefore, GPH is well absorbed and not metabolized or excreted rapidly in systemic circulation. The GPH level of plasma after CTP administration may increase due to the high GPH content in CTP; the higher the GPH content in CTP, the higher the GPH plasma concentration (Figure 3). CTP-H has a 4.3 times higher content of GPH than that of CTP-L (Table 1). The mean $AUC_{0-6\ h}$ value of GPH was proportionally increased to the GPH dose in CTP-H compared to that in CTP-L (79.34 and 365.8 μg·min/mL for CTP-L and CTP-H, respectively). The mean $AUC_{0-6\ h}$ value of PH was also proportionally increased to the PH dose in CTP-H compared to that in CTP-L (181.9 and 411.9 μg·min/mL for CTP-L and CTP-H, respectively). The content of PH in CTP-H was 2.7 times higher than that of CTP-L (Table 1). Similarly, Yamamoto et al. studied CTP with a higher GPH content than that in our CTP and obtained a higher GPH level in plasma than that obtained in our study.[17] The plasma levels of GPH and PH were dependent on the initial amount of GPH and PH administered. The Gly-X-Y units were likely not preserved, and long peptides were likely produced, as H-CP was prepared by random digestion of collagen using protease. Therefore, GPH and PH might be not efficiently generated from H-CP in rats compared to CTP even though the H-CP was prepared using the same fish scales. Taken together, the present results suggest that CTP, as an orally available form of collagen peptide, is highly efficient due to its gastrointestinal and plasma stability and due to the transcellular permeability of GPH and PH.

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ABBREVIATIONS USED

CTP-L, low-strength collagen tripeptide; CTP-H, high-strength collagen tripeptide; GPH, Gly-Pro-Hyp; PH, Pro-Hyp; H-CP, high molecular weight collagen peptide

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