Note

Fecal Excretion of Orally Administered Collagen-Like Peptides in Rats: Contribution of the Triple-Helical Conformation to Their Stability

Takaki Koide,^{*,a} Naoyuki Yamamoto,^a Kazuma B. Taira,^a and Hiroyuki Yasui^b

^aDepartment of Chemistry and Biochemistry, School of Advanced Science and Engineering, Waseda University; Tokyo 169–8555, Japan; and ^bDepartment of Analytical and Bioinorganic Chemistry, Division of Analytical and Physical Chemistry, Kyoto Pharmaceutical University; Kyoto 607–8414, Japan.

Received July 14, 2015; accepted October 8, 2015

Orally ingested peptides are generally digested in the gastrointestinal (GI) tract and absorbed in the form of oligopeptides. We previously reported that intravenously administered collagen-like triple-helical peptides circulated in the bloodstream and were excreted in their intact forms in urine nearly quantitatively. In the present study, we investigated the fates of orally administered collagen-like peptides in rats. (Pro-Hyp-Gly)₁₀ (Hyp: 4-hydroxyproline), which formed a stable triple-helical structure, was stable in the GI tract, and 72.3±13.0% of the peptide was excreted in the feces. Its recovery ratio was similar to that of all-D-(Pro-Pro-Gly)₁₀ (75.1±15.7%), the indigestible control. In contrast, (Pro-Hyp-Gly)₅ and (Pro-Pro-Gly)₁₀, the random coil conformations of which were dominant at body temperature, were not detected in fecal samples, indicating that they were digested by proteases. The high stability of the triple-helical conformation in mammalian bodies suggests the potential use of collagen-like peptides as novel scaffolds of peptide drugs.

Key words collagen; peptide; triple helix; digestion; feces

The collagen triple helix is a unique tertiary structure found in collagen family proteins. The triple helix is a right-handed supercoil of three left-handed polyproline II-like helices. All three peptide strands in the triple helix require the primary structure of (Xaa-Yaa-Gly)_n, the Xaa and Yaa positions of which are frequently occupied by Pro and 4-hydroxyproline (Hyp), respectively. The rigid triple-helical structure contributes to the high stability of tissue collagen by providing resistance to proteases. Chemically synthesized peptides with the similar sequence of (Xaa-Yaa-Gly)_n to those of native collagen have an inherent propensity to self-trimerize, thereby forming the triple helix. In addition, the thermal stabilities of the triple helices may be tuned according to the compositions of the Xaa and Yaa-amino acids and lengths of the constituent peptides.

Prompted by the unique structure and stability of the collagen triple helix, we have been investigating the biomedical applications of water-soluble collagen-like triple-helical peptides. We previously designed and synthesized collagen-like cell-penetrating peptides by introducing Arg residues to triple-helical peptides. These peptides showed efficient protein-delivery activity into cultured cells and high stability against serum proteases.¹⁾ We also recently developed anti-microbial peptides with a collagen-like triple-helical scaffold.²⁾ Furthermore, we investigated the fates of intravenously administered collagen-like peptides in rodents. The findings obtained revealed that the triple-helical peptides circulated in the bloodstream in their intact forms, and this was followed by quantitative excretion in the urine.³⁾ In the present study, we further analyzed the fates of orally ingested collagen-like peptides in rats in order to determine whether the triple-helical peptides were also resistant to digestive enzymes in the gastrointestinal (GI)-tract.

MATERIALS AND METHODS

Peptide Synthesis and Characterization (Pro-Hyp-Gly)₁₀, (Pro-Hyp-Gly)₅, and (Pro-Pro-Gly)₁₀ were purchased from the Peptide Institute (Osaka, Japan). Other peptides were constructed according to the standard Fmoc-based solid-phase method, purified by reversed-phase HPLC, and characterized by matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) MS, as described previously.³⁾ The conformation of the peptides (1 mg/mL) in phosphate-buffered saline (PBS, pH 7.4) or in diluted HCl (pH 1.8) was analyzed by circular dichroism (CD) spectrometry after refolding at 4°C. CD spectra were recorded with a J-820 CD spectropolarimeter (Jasco Co., Hachioji, Japan) equipped with a Peltier thermo controller using a 0.5-mm quartz cuvette. Thermal denaturation of the triple helix was monitored by $[\theta]_{225}$ values with increases in temperature from 4°C at increments of 18°C per hour. $[\theta]_{225}$ values were normalized to the fraction folded and melting temperature (T_m) values were taken when the fraction folded was 0.5.⁴⁾

Animal Studies Male Wistar rats (age, 7–8 weeks; weight, 215–265 g) were purchased from Shimizu Experimental Material Co. (Kyoto, Japan), and were maintained on a 12-h light/dark cycle in our central animal facility. They were allowed free access to standard solid food (MF; Oriental Yeast Co., Tokyo, Japan) and tap water. Prior to the experiment, they were housed in individual metabolism cages and fasted for 16 h. Peptide samples were orally administered using feeding gavages at a dose of 4.0 mg/head. Feces and urine were differently collected over periods of 0–24 and 24–48 h after administration of the peptide samples. All animal experiments were approved by the Experimental Animal Research Committee of Kyoto Pharmaceutical University (KPU) and were performed according to the KPU Guidelines for Animal Experimentation.

Peptide Extraction from Feces Feces were minced with

* To whom correspondence should be addressed. e-mail: koi@waseda.jp

Fig. 1. CD Spectroscopic Analysis of Peptides in PBS

(A) CD spectra recorded at 37°C. (B) Thermal melting curves for triple helices represented by fraction folded calculated according to the literature.⁴⁾ An asterisk indicates a solution in diluted HCl (pH 1.8).

scissors and homogenized in 10% acetonitrile containing 0.05% trifluoroacetic acid (34 mL/g feces) using a Polytron PT2500E homogenizer (Kinematica, Luzern, Switzerland) and then heated at 80°C for 3 min. The homogenates were further sonicated using an Astrason S-300 sonicator (Misonix, Farmingdale, NY, U.S.A.). After the debris had been spun down at 2200×*g* for 15 min, the supernatants were collected and diluted to 35 mL/g. The debris was extracted again in the same manner, and the first and second extracts were finally combined.

Purification and Quantification of Peptides in Fecal Extracts and Urine Samples The concentrations of peptides in fecal and urine samples were determined by MALDI-TOF MS followed by data fitting using the Isotopica software, as described previously.^{3,5,6)} The corresponding ¹⁵N-enriched peptide, as an internal standard, was added to 1 mL of each fecal extract or urine sample cleared by centrifugation. Each sample was then applied to an InertSep RPI column (30 mg, GL Science, Tokyo, Japan) and eluted with 20–35% (v/v) acetonitrile containing 0.05% trifluoroacetic acid (TFA). The eluent was further separated by a Cosmosil 5C18-ARII column (4.6 mm i.d.×250 mm, Nacalai Tesque) on a Shimadzu HPLC 20A system (Shimadzu, Kyoto, Japan). MALDI-TOF MS spectra of peptide-containing fractions were obtained on an Autoflex III (Bruker Daltonics, U.S.A.), using α-cyano-4-hydroxycinnamic acid as the matrix. The relative abundances of the feces- and urine-derived peptides and ¹⁵N-enriched internal standards were determined by deconvoluting the isotopic peaks into each molecular species using the Isotopica software. Standard curves were also created by a similar analysis of the samples of known mixing ratios of non-enriched and ¹⁵N-enriched peptides, and used for the quantification of peptides.

RESULTS

Characterization of Peptides The thermal stabilities of the triple-helical conformation of test peptides in phosphate buffered saline (PBS) were analyzed by CD spectrometry. As

Table 1. *T_m* Values and Triple-Helical Contents of Peptides

Peptide	<i>T_m</i> (°C)	Triple helical content at 37°C (%)
(Pro-Hyp-Gly) ₁₀	63	100
(Pro-Hyp-Gly) ₁₀ *	64	100
D-(Pro-Pro-Gly) ₁₀	35	24
(Pro-Pro-Gly) ₁₀	35	24
(Pro-Hyp-Gly) ₅	<4	0

* At pH 1.8.

shown in Fig. 1 and Table 1, most of the population of (Pro-Hyp-Gly)₁₀ molecules had the triple-helical conformation at 37°C, and the conformational stability was not significantly changed at pH 1.8. On the other hand, the triple-helical conformation of (Pro-Pro-Gly)₁₀ was not stable at the same temperature and the random-coil conformation was dominant. (Pro-Hyp-Gly)₅ was mostly a random coil, even at 4°C.

Excretion of Peptides into Feces and Urine The PBS solutions of the test peptides (4.0 mg/head) were orally administered to rats, and feces and urine were collected 0–24 h and 24–48 h after their administration. The amounts of the peptides in the fecal extracts and urine samples were determined by MALDI-TOF MS using corresponding ¹⁵N-enriched peptides as internal standards for quantification. The results are summarized in Table 2. Within 48 h of its administration, 72.3±13.0% of intact (Pro-Hyp-Gly)₁₀ was excreted into the feces (0–24 h, 54.0±17.6% and 24–48 h, 18.3±11.2%). This value was similar to that of all-D-(Pro-Pro-Gly)₁₀, which was used as an indigestible control (total 75.1±15.7%, 0–24 h, 51.2±18.6% and 24–48 h, 23.9±15.6%). In contrast, intact peptides were not detected in the feces or urine of (Pro-Hyp-Gly)₅-administered or (Pro-Pro-Gly)₁₀-administered rats, indicating that these peptides were digested in the GI-tract. Negligible amounts (less than 1% of the administered amounts) of (Pro-Hyp-Gly)₁₀ and all-D-(Pro-Pro-Gly)₁₀ were also detected in urine samples.

Table 2. Excretion of Peptides in Feces and Urine

Peptide	Excretion (%)					
	Feces			Urine		
	0–24 h	24–48 h	Total	0–24 h	24–48 h	Total
(Pro-Hyp-Gly) ₁₀	54.0±17.6	18.3±11.3	72.3±13.0	0.42±0.48	0.12±0.09	0.54±0.52
D-(Pro-Pro-Gly) ₁₀	51.2±18.6	23.9±15.6	75.1±15.7	0.49±0.58	0.11±0.11	0.60±0.61
(Pro-Hyp-Gly) ₁₀ *	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
(Pro-Hyp-Gly) ₅	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Values are the mean±S.D. ($n=5$, $*n=4$); n.d., not detected.

DISCUSSION

Although Pro- and Hyp-containing collagenous peptides were reported to be relatively resistant to enzymatic hydrolysis,⁷⁾ the results of the present study revealed that the digestion of collagen-like peptides in the GI-tract significantly varied according to their conformations at body temperature. The triple-helical conformation was shown to be highly stable, even in the GI-tract, in which many digestive enzymes function. The indigestible profile of triple-helical (Pro-Hyp-Gly)₁₀ was similar to that of the control all-D-peptide. The non-recovered 27.7% of (Pro-Hyp-Gly)₁₀ was speculated to remain in the gut because a positive correlation was observed between the recovery of the peptides in the 24–48-h feces extracts and the amounts of feces (data not shown). However, we were unable to eliminate the possibility of enzymatic degradation in the GI-tract. Furthermore, the recovery of trace amounts of intact (Pro-Hyp-Gly)₁₀ and all-D-(Pro-Pro-Gly)₁₀ from urine samples may be attributed to contamination due to small particles of feces in urine samples.

Although our results clearly demonstrated the indigestible properties of the triple-helical peptide, they may not be extrapolated to the digestibility of native triple-helical collagen in mammals. Native animal-derived collagen ingested by rats was reported to be completely digested in the GI-tract.⁸⁾ This may be due to the lower thermal stability of the triple helix of mammalian collagen than that of (Pro-Hyp-Gly)₁₀, an ideal regular triplet repeat for the triple-helical model. Previous studies reported that the triple helix of native mammalian collagen was not completely stable at a body temperature of 37°C, resulting in micro-unfolding.^{9,10)} However, it is tempting to speculate that fish preying on birds (*i.e.* European catfish hunting pigeons)¹¹⁾ may not efficiently digest the collagen of the victim because the triple helix of avian collagen has been suggested to have a higher T_m value than the body temperature of the poikilothermic predator.

Peptides are recognized as privileged structures for drugs that may elicit potent and specific pharmacological effects. However, the pharmacokinetic profiles of peptides are generally below that required, mainly because of their susceptibility to proteases. In order to overcome this drawback, peptide backbones are often cyclized or tied up with multiple disulfide

bridges. Since triple-helical peptides were shown to be endowed with certain biological activities (*i.e.*, cell-penetrating and antimicrobial activities),^{1,2)} the high stability of the conformation offers new opportunities for the use of collagen-like peptides as scaffolds for drugs that may be effective, even in the GI-tract.

Acknowledgment This work was supported by a research grant from Urakami Foundation.

Conflict of Interest The authors declare no conflict of interest.

REFERENCES

- 1) Yamazaki CM, Nakase I, Endo H, Kishimoto S, Mashiyama Y, Masuda R, Futaki S, Koide T. Collagen-like cell-penetrating peptides. *Angew. Chem. Int. Ed. Engl.*, **52**, 5497–5500 (2013).
- 2) Masuda R, Kudo M, Dazai Y, Mima T, Koide T. Collagen-like Antimicrobial Peptides, submitted.
- 3) Yasui H, Yamazaki CM, Nose H, Awada C, Takao T, Koide T. Potential of collagen-like triple helical peptides as drug carriers: Their *in vivo* distribution, metabolism and excretion profiles in rodents. *Biopolymers*, **100**, 705–713 (2013).
- 4) Mizuno K, Boudko SP, Engel J, Bächinger HP. Kinetic hysteresis in collagen folding. *Biophys. J.*, **98**, 3004–3014 (2010).
- 5) <http://coco.protein.osaka-u.ac.jp/Isotopica>
- 6) Fukuda M, Takao T. Quantitative analysis of deamidation and isomerization in β 2-microglobulin by ¹⁸O labeling. *Anal. Chem.*, **84**, 10388–10394 (2012).
- 7) 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.*, **73**, 1741–1747 (2009).
- 8) Harkness ML, Harkness RD, Venn MF. Digestion of native collagen in the gut. *Gut*, **19**, 240–243 (1978).
- 9) Leikina E, Merts MV, Kuznetsova N, Leikin S. Type I collagen is thermally unstable at body temperature. *Proc. Natl. Acad. Sci. U.S.A.*, **99**, 1314–1318 (2002).
- 10) Kadler KE, Hojima Y, Prockop DJ. Assembly of type I collagen fibrils *de novo*. Between 37 and 41 degrees C the process is limited by micro-unfolding of monomers. *J. Biol. Chem.*, **263**, 10517–10523 (1988).
- 11) Cucherousset J, Boulêtreau S, Azémar F, Compin A, Guillaume M, Santoul F. “Freshwater killer whales”: beaching behavior of an alien fish to hunt land birds. *PLoS ONE*, **7**, e50840 (2012).