JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Identification of Food-Derived Elastin Peptide, Prolyl-Glycine (Pro-Gly), in Human Blood after Ingestion of Elastin Hydrolysate

Yasutaka Shigemura,[†,‡] Misako Nakaba,[§] Eri Shiratsuchi,[§] Masashi Suyama,[§] Michio Yamada,[§] Tamami Kiyono,[‡] Kazuna Fukamizu,[‡] Eun Young Park,[‡] Yasusi Nakamura,[‡] and Kenji Sato*[,‡]

[†]Department of Nutrition, Faculty of Domestic Science, Tokyo Kasei University, 1-18-1 Kaga, Itabashi-ku, Tokyo, 173-8602, Japan

[§]Hayasikane Sangyo Co. Ltd., 2-4-8 Yamato-cho, Shimonoseki, Yamaguchi, 750-8608, Japan

[‡]Division of Applied Life Sciences, Graduate School of Life and Environment Sciences, Kyoto Prefectural University, 1-5 Shimogamo Kyoto 606-8522, Japan

ABSTRACT: Elastin hydrolysate has apparent beneficial effects, and the food-derived peptide prolyl-glycine (Pro-Gly) is present in human blood after oral ingestion. Following ingestion of elastin hydrolysate (10 g/60 kg body weight) by healthy human volunteers, peripheral blood was used to prepare plasma samples from which peptides were extracted by solid phase extraction and fractionated by size-exclusion chromatography (SEC). Peptides in the SEC fractions were derivatized with phenyl isothiocyanate (PITC) and resolved by reversed phase (RP)-HPLC. Pro-Gly was the major food-derived elastin peptide, reaching a maximum (18 μM) at 30 min after ingestion, and decreasing to approximately 20% at 4 h after ingestion. Finally, in cell culture, levels of Pro-Gly in the medium above 0.1 μg/mL significantly enhanced elastin synthesis of normal human dermal fibroblasts (NHDF) without affecting the rate of cell proliferation.

KEYWORDS: *elastin, peptide, elastin peptide, Pro-Gly, fibroblasts, skin, precolumn derivatization, food-derived peptide*

INTRODUCTION

Collagen and elastin are the major fibrous protein components of the extracellular matrix. Collagen, comprising a molecular family of more than 20 closely related gene products, can be classified into fibril-forming, which assembles into collagen fibrils, and non-fibril-forming collagens. Type I, II, III, V, and XI collagens are classified as fibril-forming collagen. Collagen fiber, which gives mechanical strength to connective tissues such as tendon and skin, is formed by the assembly of collagen fibrils. On the other hand, elastin forms elastic fiber and gives elasticity to organs such as lung, ligament, dermis, and blood vessels.

It has been demonstrated that ingestion of collagen hydrolysate at 5−10 g/60 kg body weight exerts biological activities in human, such as the increase of epidermal moisture content[1] and moderation of joint pain in knees.[2,3] The occurrence of food-derived collagen peptides such as Pro-Hyp, Pro-Hyp-Gly, Ala-Hyp, Ala-Hyp-Gly, Ser-Hyp, Ser-Hyp-Gly, Leu-Hyp, Ile-Hyp, Phe-Hyp, and Hyp-Gly has been demonstrated in human peripheral blood.[4−6] Pro-Hyp and Hyp-Gly, two major food-derived collagen peptides in human blood, enhance proliferation of primary mouse skin fibroblasts cultured on collagen gel[6,7] and stimulate synthesis of hyaluronic acid in those cells.[8] In addition, ingestion of Pro-Hyp moderates high phosphorus diet-induced osteoarthritis in rats.[9] These studies offer clues as to the mechanism underlying the beneficial effects of collagen hydrolysate ingestion.

Significant improvement in the morphological and physicochemical condition of human and mouse skin by ingestion of elastin hydrolysate in addition to collagen hydrolysate has been demonstrated previously.[10,11] These data suggest that food-derived elastin peptides might be absorbed into the blood circulation system and exert biological activity upon cells in the extracellular matrix.

The objective of the present study was to identify the food-derived elastin peptides present in human peripheral blood and to elucidate the mechanism for the beneficial effects on skin after the oral ingestion of elastin hydrolysate by examining peptide activity in cultured fibroblasts.

MATERIALS AND METHODS

Elastin Hydrolysate. Elastin hydrolysate was prepared from the bulbus arteriosus of skipjack tuna (Scombridae, *Katsuwonus pelamis*) caught in the Indian Ocean and the Pacific Ocean around Thailand by Hayashikane Sangyo Co., Ltd. (Shimonoseki, Japan), which can be commercially obtained as Katsuwo Elastin (Hayashikane Sangyo Co., Ltd., Shimonoseki, Japan).

Chemicals. A standard mixture of amino acids (type H), acetonitrile (HPLC-grade), trifluoroacetic acid (TFA), and phenyl isothiocyanate (PITC) was purchased from Wako Chemicals (Osaka, Japan). Pro-Gly was obtained from Kokusan Kagaku (Tokyo, Japan). Val-Gly and Gly-Val were purchased from Bachem (Bubendorf, Switzerland). AccQ·Tag was purchased from Waters (Milford, MA) and consists of the 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate reagent (AccQ), acetonitrile, and 0.2 mM sodium borate buffer (pH 8.8). Primary cultured normal human dermal fibroblasts (NHDF) were obtained from DS Pharma Biomedical (Osaka, Japan). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Invitrogen (Carlsbad, CA), and antibody against human tropoelastin was obtained from Cosmo Bio (Tokyo, Japan). The TMB Microwell Peroxidase Substrate System,

Received: February 4, 2012
Revised: April 16, 2012
Accepted: May 1, 2012
Published: May 1, 2012

dx.doi.org/10.1021/jf300497p | J. Agric. Food Chem. 2012, 60, 5128−5133

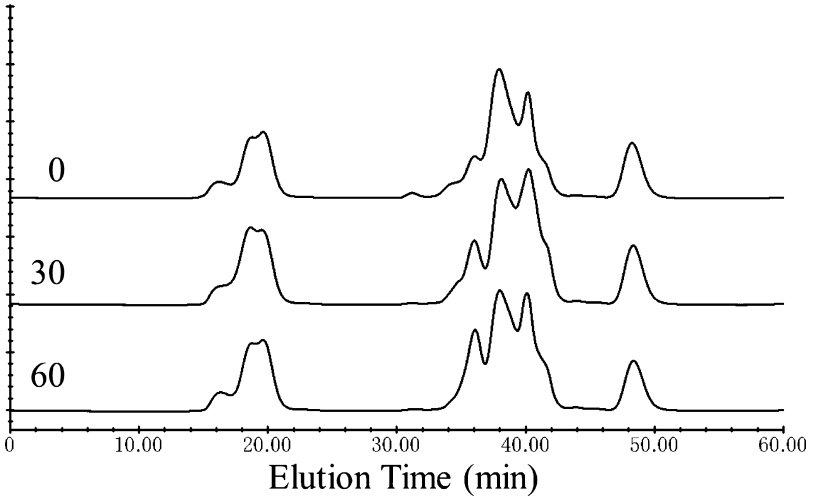

Figure 1. Fractionation of peptides from ethanol-soluble plasma fraction by size exclusion chromatography (SEC). Fractions were collected every minute. Plasma samples were prepared from blood collected at 0, 30, and 60 min after ingestion of elastin hydrolysate.

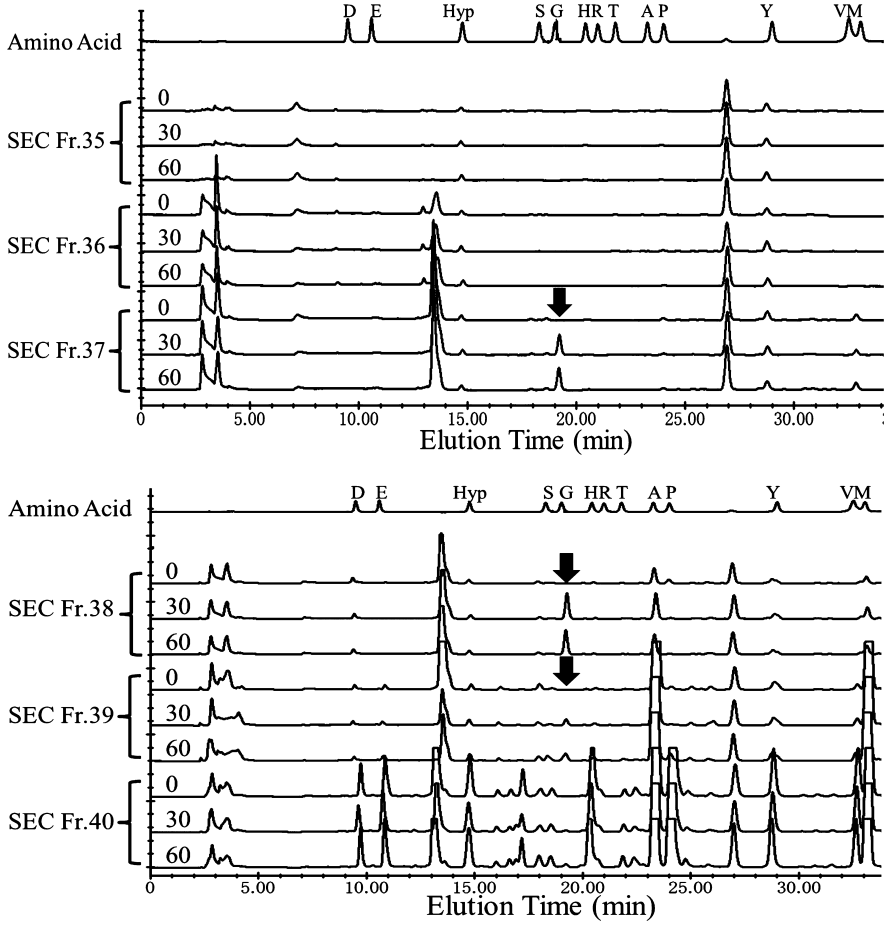

Figure 2. Resolution of PTC-amino acids and PTC-peptides by RP-HPLC. Amino acids and peptides in SEC fractions 35−40 were derivatized with PITC. The resultant derivatives were resolved by RP-HPLC. For comparison, PTC-amino acid mixtures were also resolved. SEC fractions were prepared from blood collected at 0, 30, and 60 min after ingestion. Peaks marked with arrows were collected and subjected to amino acid sequence analysis.

goat anti-rabbit IgG polyclonal antibody−horseradish peroxidase conjugate was purchased from KPL (Gaithersburg, MD). Solubilized elastin prepared from bovine neck ligament was purchased from Sigma (St. Louis, MO). The Cell Counting Kit-8 was purchased from Dojin Glocal (Kumamoto, Japan). All other reagents were of analytical grade or higher.

Blood Collection and Preparation of Plasma Samples. The study was carried out as described previously.[4,5] The study was performed according to the Helsinki Declaration under the supervision of medical doctors, and approved by the experimental ethical committee of Hayasikane Sangyo. Volunteers were informed of the objectives of the present study and the potential risks of ingestion of

dx.doi.org/10.1021/jf300497p | J. Agric. Food Chem. 2012, 60, 5128−5133

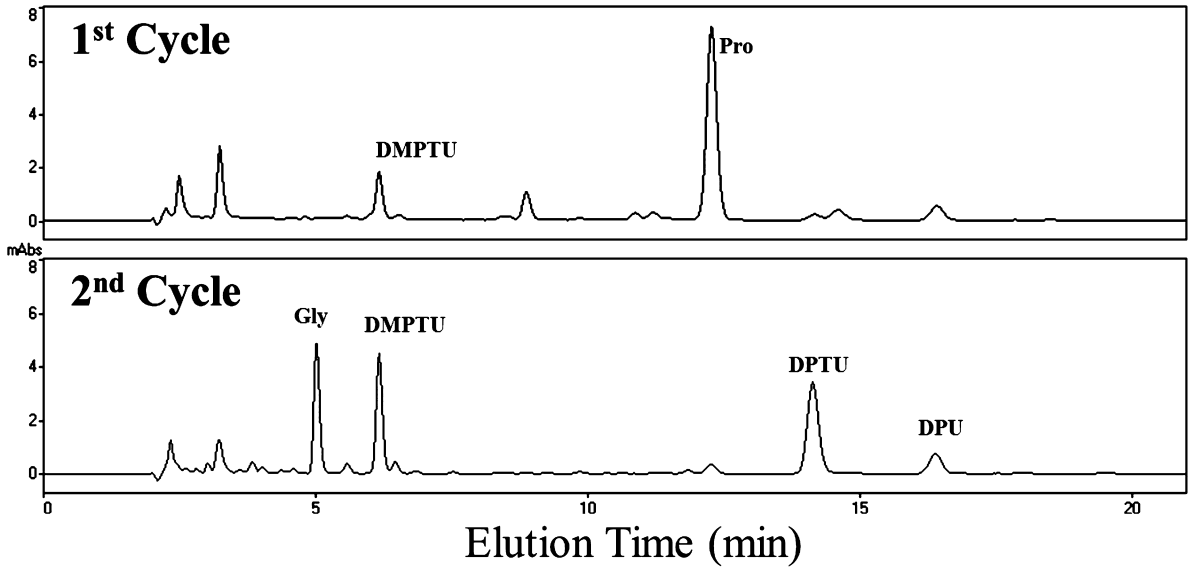

Figure 3. Peptide sequence analysis of peaks marked in Figure 2. Phenylthiohydantoin (PTH) amino acid elution profiles at the 1st and 2nd steps are shown. DMPTU: *N*-dimethyl-*N*′-phenylthiourea. DPTU: *N*,*N*′-diphenylthiourea. DPU: *sym*-diphenylurea.

elastin hydrolysate such as stomach discomfort, diarrhea, and abdominal pain. Healthy volunteers fasted for 12 h before ingesting elastin hydrolysate (10 g/60 kg body weight). Elastin hydrolysate was dissolved in water to 20% (w/v). This preparation consisted mainly of elastin peptides with an average molecular weight of 500 Da. Approximately 10 mL of venous blood was collected from the cubital vein of volunteers (n = 4 or n = 6). The plasma obtained from the venous blood samples was then deproteinized by addition of three volumes of ethanol, and the ethanol-soluble fraction was collected by centrifugation at 1000g for 10 min and stored at −80 °C until analysis.

Identification of Food-Derived Elastin Peptides in Human Blood. Blood was collected from four healthy volunteers (mean age, 50.5 ± 7.9) 0, 30, and 60 min after ingestion, and ethanol-soluble blood sample fractions were prepared as described above. Peptides were extracted from the mixture of ethanol-soluble blood sample fractions using a spin column packed with a strong cation exchanger (AG 50W × 8, Bio-Rad Laboratories, Hercules, CA), and further fractionated by SEC using Superdex Peptide 10/300 GL (GE Healthcare, Buckinghamshire, U.K.), as described previously.[6] The peptides in the SEC fractions were dried under vacuum and derivatized with PITC using the method of Bidlingmeyer et al.[12] with slight modifications.[13−15] The derivatives, phenyl thiocarbamyl (PTC)-peptides, were resolved on an Inertsil ODS-3 column (4.6 mm i.d. × 250 mm; GL Science, Tokyo, Japan) by binary gradient elution, as described previously.[6] The sequence of isolated PTC-peptides was determined using a peptide sequencer (PPSQ-21, Shimadzu, Kyoto, Japan) based on the Edman degradation method; the program was modified to start from the cleavage step, as described previously.[13]

Estimation of Pro-Gly Content in Human Plasma. The concentration of elastin-derived peptides Pro-Gly, Val-Gly, and Gly-Val in plasma from 6 healthy volunteers (mean age 37.4 ± 14.8) at 0, 30, 60, 120, 240, and 420 min after ingestion was estimated by LC−MS/MS using Q-TRAP 3200 (AB SCIEX, Foster City, CA). Peptides in the 75% ethanol-soluble fraction were derivatized with AccQ as described previously.[16,17] The derivatives were resolved on an Inertsil ODS-3 column (2 mm i.d. × 250 mm). Binary gradient elution was performed with 0.1% formic acid (solvent A) and 80% acetonitrile containing 0.1% formic acid (solvent B) at a flow rate of 0.2 mL/min. The column was equilibrated with 100% solvent A, and the gradient profile was as follows: 0−12 min, 0−50% B; 12−20 min, 50−100% B; 20−24 min, 100% B; 24−24.1 min, 100−0% B; and 24.1−30 min, 0% B. The column was maintained at 40 °C throughout. MS/MS condition was optimized in positive mode by using Analyst Version 4.2 (AB SCIEX) in auto select mode.

Cell Culture. Normal human dermal fibroblast (NHDF) cell suspension in DMEM containing 1% FBS was aliquoted to 96-well plastic plates (2 × 10^4 cell/well) and placed in a humidified incubator at 37 °C under 5% CO_2. After 24 h, 0, 0.1, 1.0, and 10 μg/mL of Pro-Gly peptide was added to the medium. Three days later, elastin synthesis was determined as a constituent of tropoelastin, a precursor protein of mature elastin in elastic fiber. Tropoelastin concentration in the medium was determined by ELISA according to the method of Prosser et al.[18] The calibration curve was generated using the bovine neck ligament elastin. Proliferation of NHDF was estimated by Cell Counting Kit-8 (CCK-8).

Mouse skin primary fibroblasts were cultured on collagen gel as described previously,[9] and cell proliferation was estimated by CCK-8.

Statistical Analysis. Differences between the means were evaluated by analysis of variance followed by Fisher's PLSD method (p < 0.05) using StatView Version 5.0 (Abacus Concepts, Berkeley, CA).

RESULTS

Identification of Food-Derived Elastin Peptide in Human Blood. The chromatograms of SEC fractions of plasma peptides are shown in Figure 1. Amino acid analysis revealed that peptides and/or amino acids were eluted between 35 and 40 min (i.e., fractions 36−40), and resolution of these derivatized fractions by RP-HPLC (Figure 2) shows that the peak concentration was observed 30 and 60 min after ingestion (Figure 2, arrow indicating fractions 37, 38, and 39). As the retention time of this peak differed from that of PTC-derivatized standard amino acids, the peak was collected and subjected to sequence analysis. As shown in Figure 3, the first and second residues of the peptide sequence were identified as proline (Pro) and glycine (Gly), respectively. No significant signals were observed in the following cycles, indicating that this peak represents PTC-Pro-Gly. In other SEC fractions, no significant difference was observed between RP-HPLC chromatograms before and after ingestion of elastin hydrolysate, indicating that the peaks in these SEC fractions are derived from amino acids and endogenous peptides. Apart from Pro-Gly, increases in other peptide levels after ingestion of elastin hydrolysate at 20 g/60 kg body weight were not detected (data not shown).

Quantification of Food-Derived Pro-Gly in Human Plasma. Pro-Gly levels reached a maximum level (18 μM) at 30 min after ingestion (10 g/60 kg body weight), decreasing to 20% of the maximum level at 240 min and to negligible levels at 420 min after ingestion (Figure 4). With ingestion of elastin hydrolysate at 20 g/60 kg body weight, the maximum level was approximately 45 μM at 60 min after ingestion (data not shown). Negligible amounts of other dipeptides (Val-Gly and Gly-Val), which could potentially have been derived from the repeated motif of elastin, were detected in human blood after ingestion (data not shown).

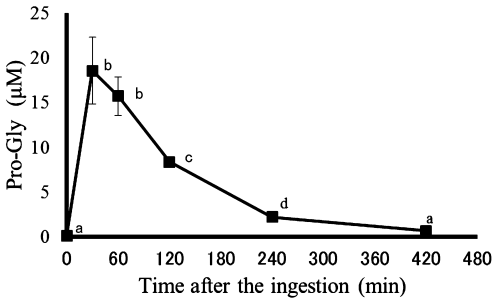

Figure 4. Concentration of food-derived Pro-Gly in human plasma after oral ingestion of elastin hydrolysate. The means of 6 subjects are shown. Data are shown as the mean ± SD; $n = 6$. The letters indicate significant difference ($p < 0.05$).

Effects of Pro-Gly on Fibroblasts. As shown in Figure 5, compared with the control, elastin synthesis by NHDF was significantly enhanced by addition of 0.1 to 10 μg/mL of Pro-Gly after 72 h of incubation. On the other hand, MTT assay using CCK-8 revealed that Pro-Gly did not affect the proliferation of NHDF cultured in DMEM containing 1% FBS on plastic plates or mouse primary fibroblasts on collagen gel (data not shown).

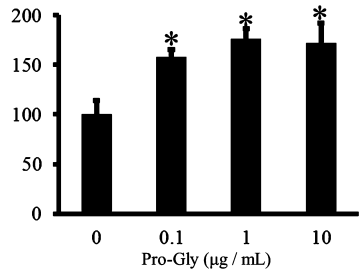

Figure 5. Effect of Pro-Gly on elastin synthesis in NHDF. At 3 days after addition of Pro-Gly at 0, 0.1, 1, and 10 μg/mL, tropoelastin level in the medium was determined by ELISA and normalized against a control (0). Data are shown as the mean ± SD; $n = 4$. Asterisks indicate significant difference from the control ($p < 0.05$).

DISCUSSION

In the present study, Pro-Gly was identified in human peripheral blood after ingestion of the elastin hydrolysate, while only negligible amounts of Pro-Gly were detected before the ingestion. The Pro-Gly could be produced by degradation of endogenous protein, such as host elastin. However, such high levels (μM) of degradation products of extracellular matrix components have been observed only under severe pathological conditions such as bone metastasis of cancer.[19,20] If most of the Pro-Gly in human blood after the ingestion were degradation product of the endogenous proteins, serious tissue damage might occur. However, such adverse effect by ingestion of elastin hydrolysate has not been observed in the present and previous studies.[10,11] Therefore, it is quite unlikely that increase of Pro-Gly to μM level is caused by degradation of endogenous protein of healthy volunteers. Alternatively, Pro-Gly could be synthesized from amino acids by ingestion of elastin hydrolysate, if food-derived elastin peptides were completely degraded into amino acids. In this case, Pro-Gly might be induced by ingestion of other food protein and peptides. However, Pro-Gly has not been detected in human plasma after the ingestion of other protein hydrolysates, such as collagen, sardine muscle, and cow milk.[4−6,21,22] These facts indicate that increase of Pro-Gly to μM order in human blood after ingestion of the elastin hydrolysate could not be explained by the increase of endogenous peptides. On the basis of these facts, we concluded that Pro-Gly is a food-derived elastin peptide.

Relatively low levels of food-derived peptides, e.g. Val-Tyr (1.934 nM)[21] and Ile-Pro-Pro (0.897 nM),[22] have been detected in human plasma after ingestion of sardine protein hydrolysate and fermented milk, respectively. On the other hand, food-derived peptides containing the post-translationally modified amino acid hydroxyproline (Hyp) have been detected at micromolar levels in human plasma after ingestion of collagen hydroysate. These reports suggest that the presence of post-translationally modified amino acids in peptides might be essential for their occurrence at elevated concentrations in human plasma. However, the present study reveals the occurrence of Pro-Gly, which consists of unmodified amino acids, in human plasma at micromolar levels.

Pro-Hyp and Hyp-Gly, major food-derived collagen peptides in human blood, can be derived from the repeating motif in collagen (Pro-Hyp-Gly). Pro-Gly can also be derived from the repeating motif in fish elastin (Val-Pro-Gly-Val-Gly, Val-Pro-Gly).[23,24] Thereby, other dipeptides such as Val-Gly and Gly-Val could be potentially derived from a repeating motif in elastin. However, LC−MS/MS analysis coupled with the AccQ precolumn derivatization technique detected only negligible amounts of these peptides in human plasma after ingestion of elastin hydrolysate, which indicates that other peptides from the repeating motif might be degraded during preparation of elastin hydrolysate solution, or during the digestion and absorption process in the human gastrointestinal tract. Alternatively, those other peptides might be rapidly incorporated into cells. Nevertheless, in both cases, Pro-Gly is a major food-derived elastin peptide, which can be delivered to peripheral tissues.

Tajima et al. demonstrated, in a cell culture system, that the repeating motif in elastin, Val-Gly-Val-Ala-Pro-Gly, enhances elastin synthesis and proliferation of fibroblasts.[25] The present study indicates that Pro-Gly enhances elastin synthesis at a lower level, which suggests that Pro-Gly could be the crucial motif for enhancement of elastin synthesis in fibroblasts. On the other hand, Pro-Gly did not affect proliferation of NHDF cultured on plastic, or primary mouse skin fibroblasts cultured on collagen gel. Hence, it appears that ingestion of elastin hydrolysate might increase synthesis of elastin without enhancing fibroblast proliferation. It has been demonstrated that Pro-Hyp and Hyp-Gly, both food-derived collagen peptides found in human blood, enhance proliferation of mouse skin primary fibroblasts cultured on collagen gel. These facts indicate that food-derived elastin and collagen peptides in human blood induce different biological responses in

fibroblasts, though Pro-Gly and Hyp-Gly differ only in the occurrence of a hydroxyl group.

Placebo-controlled double-blind studies have demonstrated that moisture content of epidermis and subjective skin condition of women are improved by ingestion of collagen hydrolysate (5−6 g/day).[1] Recently, Nakaba et al. reported that supplementation of a relatively low dose of elastin hydrolysate (100 mg/day) with collagen hydrolysate (1 g) significantly increased skin elasticity and improved subjective perception of skin condition compared to collagen hydrolysate alone.[10] The present study demonstrates the occurrence of food-derived elastin peptides with different biological activity from collagen peptides, which could partially explain the beneficial health effects of supplementation with elastin hydrolysate.

Further studies on the effects of Pro-Gly on synthesis of the other extracellular matrix proteins and growth and differentiation of other cell types, as well as evaluation of the distribution of food-derived elastin peptides in organs, are currently in progress in our laboratory.

AUTHOR INFORMATION

Corresponding Author

*Phone: +81-75-703-5405. E-mail: k_sato@kpu.ac.jp.

Funding

This work was partially supported by a Grant-in-Aid for Young Scientists (B) No. 23700944 from the Japan Society for the Promotion of Science.

Notes

The authors declare no competing financial interest.

Safety. The human study was performed according to the Helsinki Declaration under the supervision of medical doctors, and approved by the experimental ethical committee of Hayasikane Sangyo.

ABBREVIATIONS USED

AccQ, 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate reagent; CCK-8, cell counting kit-8; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosolbent assay; FBS, fetal bovine serum; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; NHDF, normal human dermal fibroblasts; ODS, octadecylsilyl; PITC, phenyl isothiocyanate; PLSD, protected least significant difference; PTC, phenyl thiocarbamyl; PTH, phenylthiohydantoin; RP-HPLC, reversed phase high performance liquid chromatography; SEC, size-exclusion chromatography; TFA, trifluoroacetic acid; DMPTU, *N*-dimethyl-*N*′-phenylthiourea; DPTU, *N*,*N*′-diphenylthiourea; DPU, *sym*-diphenylurea

REFERENCES

(1) Matsumoto, H.; Ohara, H.; Itoh, K.; Nakamura, Y.; Takahashi, S. Clinical effect of fish type I collagen hydrolysate on skin properties. *ITE Lett. Batteries, New Technol. Med.* **2006**, *7*, 386−390.

(2) Trč, T.; Bohmová, J. Efficacy and tolerance of enzymatic hydrolysed collagen (EHC) vs. glucosamine sulphate (GS) in the treatment of knee osteoarthritis (KOA). *Int. Orthop.* **2010**, *35* (3), 341−348.

(3) Deal, C. L.; Moskowitz, R. W. Nutraceuticals as therapeutic agents in osteoarthritis. The role of glucosamine, chondroitin sulfate, and collagen hydrolysate. *Rheum. Dis. Clin. North Am.* **1999**, *25*, 379−395.

(4) Iwai, K.; Hasegawa, T.; Taguchi, Y.; Morimatsu, F.; Sato, K.; Nakamura, Y.; Higashi, A.; Kido, Y.; Nakabo, Y.; Ohtsuki, K. Identification of food-derived peptides in human blood after oral ingetion of gelatin hydrolysates. *J. Agric. Food Chem.* **2005**, *53*, 6531−6536.

(5) Ohara, H.; Matsumoto, H.; Itoh, 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*, 1532−1535.

(6) 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* (3), 1019−1024.

(7) 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*, 444−449.

(8) 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*, 330−338.

(9) Nakatani, S.; Mano, H.; Sampei, C.; Shimizu, J.; Wada, M. Chondroprotective effect of the bioactive peptide prolyl-hydroxyproline in mouse cartilage in vitro and in vivo. *Osteoarthritis Cartilage.* **2009**, *12*, 1620−1627.

(10) Nakaba, M.; Koikeda, T.; Saitou, Y. Effect of the elastin peptide derived from fish species for human skin. *J. New Rem. Clin.* **2007**, *56*, 109−115.

(11) Hayakawa, T.; Sato, M.; Saiga-Egusa, A.; Takahata, Y.; Morimatsu, M.; Nomura, Y. Effect of porcine arterial elastin peptide to the moisture content of mice skin. *Nihon Chikusan Gakkaiho* **2009**, *80* (2), 215−222.

(12) Bidlingmeyer, B. A.; Cohen, S. A.; Tarvin, T. L. Rapid analysis of amino acids using pre-column derivatization. *J. Chromatogr.* **1984**, *336*, 93−104.

(13) Aito-Inoue, M.; Ohtsuki, K.; Nakamura, Y.; Park, E. Y.; Iwai, K.; Morimatsu, F.; Sato, K. Improvement in isolation and identification of food-derived peptides in human plasma based on precolumn derivatization of peptides with phenyl isothiocyanate. *J. Agric. Food Chem.* **2006**, *54*, 5261−5266.

(14) Sato, K.; Tsukamasa, Y.; Imai, C.; Ohtsuki, K.; Shimizu, Y.; Kawabata, M. Improved method for identification and determination of ε-(γ-glutamyl)-lysine cross-link in protein using proteolytic digestion and derivatization with phenyl isothiocyanate followed by high-performance liquid chromatography separation. *J. Agric. Food Chem.* **1992**, *40*, 806−810.

(15) Higaki-Sato, N.; Sato, K.; Esumi, Y.; Okumura, T.; Yoshikawa, H.; Tanaka-Kuwajima, C.; Kurata, A.; Kotaru, M.; Kawabata, M.; Nakamura, Y.; Ohtsuki, K. Isolation and identification of indigestible pyroglutamyl peptides in an enzymatic hydrolysate of wheat gluten prepared on an industrial scale. *J. Agric. Food Chem.* **2003**, *51*, 8−13.

(16) Urado, D.; Park, E. Y.; Nakamura, Y.; Sato, K. Identification of food-derived peptides in human blood by peptide derivatization and mass spectrometric analysis. *J. Jpn. Soc. Food Eng.* **2009**, *29* (4), 175−184.

(17) de Mejia, E. G.; Martinez-Villaluenga, C.; Fernandez, D.; Urado, D.; Sato, K. Bioavailability and Safety of Food Peptides. In *Food proteins and peptides: Chemistry, Functionality, Interactions, and Commercialization*, Hettiarachchy, N. S., Sato, K., Marshall, M. R., Kannan , A., Eds.; CRC Press: Boca Raton, FL, 2012; pp 284−312.

(18) Prosser, I. W.; Whitehouse, L. A.; Parks, W. C.; Stahle-Backdahl, M.; Hinek, A.; Park, P. W.; Mecham, R. P. Polyclonal antibodies to tropoelastin and the specific detection and measurement of tropoelastin in vitro. *Connect. Tissue Res.* **1991**, *25* (3−4), 265−279.

(19) Inoue, H; Iguch, H; Kouno, A; Tsuruta, Y. Fluorometric determination of N-terminal prolyl dipeptides, proline and hydroxyproline in human serum by pre-column high-performance liquid chromatography using 4-(5,6-dimethoxy-2-phthalimidinyl)-2- methoxyphenylsufonyl chloride. *J. Chromatogr., B* **2001**, *757* (2), 369−373.

(20) Mazzi, G; Fioravanzo, F; Burti, E. New marker of bone resorption: hydroxyproline-figurescontaining peptide. High-performance liquid chromatographic assay without hydrolysis as an alternative to hydroxyproline determination: a preliminary report. *J. Chromatogr., B* **1996**, *678* (2), 165−172.
(21) Matsui, T.; Tamaya, K.; Seki, E.; Osajima, K.; Matsumoto, K.; Kawasaki, T. Val-Tyr as a natural antihypertensive dipeptide can be absorbed into the human circulatory blood system. *Clin. Exp. Pharmacol. Physiol.* **2002**, *29*, 204−208.
(22) Foltz, M.; Meynen, E. E.; Bianco, V.; van Platerink, C.; Koning, T. M.; Kloek, J. Angiotensin converting enzyme inhibitory peptides from a lactotripeptide-enriched milk beverage are absorbed intact into the circulation. *J. Nutr.* **2007**, *137*, 953−958.
(23) Miao, M.; Bruce, A. E.; Bhanji, T.; Davis, E. C.; Keeley, F. W. Differential expression of two tropoelastin genes in zebrafish. *Matrix Biol.* **2007**, *26* (2), 115−124.
(24) He, D.; Chung, M.; Chan, E.; Alleyne, T.; Ha, K. C.; Miao, M.; Stahl, R. J.; Keeley, F. W.; Parkinson, J. Comparative genomics of elastin: Sequence analysis of a highly repetitive protein. *Matrix Biol.* **2007**, *26* (7), 524−540.
(25) Tajima, S.; Wachi, H.; Uemura, Y.; Okamoto, K. Modulation by elastin peptide VGVAPG of cell proliferation and elastin expression in human skin fibroblasts. *Arch. Dermatol. Res.* **1997**, *289*, 489−492.