Food Chemistry 129 (2011) 1019–1024

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Identification of a novel food-derived collagen peptide, hydroxyprolyl-glycine, in human peripheral blood by pre-column derivatisation with phenyl isothiocyanate

Yasutaka Shigemura [a,b], Saeko Akaba [b], Eriko Kawashima [c], Eun Young Park [b], Yasushi Nakamura [b], Kenji Sato [b,*]

[a] Osaka Yuhigaoka Gakuen Junior College, 7-72 Ikutamadera-cho, Tennoji, Osaka 543-0073, Japan
[b] Division of Applied Life Sciences, Graduate School of Life and Environment Sciences, Kyoto Prefectural University, 1-5 Shimogamo, Kyoto 606-8522, Japan
[c] Yokohama Research Center, Chisso Corporation, 5-1, Okawa, Kanazawa-ku, Yokohama 236-8605, Japan

ARTICLE INFO

Article history:
Received 7 January 2011
Received in revised form 18 March 2011
Accepted 16 May 2011
Available online 23 May 2011

Keywords:
Collagen
Collagen hydrolysate
Peptide
Hyp-Gly
Pro-Hyp
Hyp
Skin
Plasma
Fibroblasts

ABSTRACT

Peptides in the blood of subjects before and after collagen hydrolysate ingestion were fractionated by ion exchange and size-exclusion chromatographies and then derivatised with phenyl isothiocyanate. The derivatives were characterised by reserved phase (RP)-HPLC. Prolyl-hydroxyproline (Pro-Hyp), which has been identified in the previous studies, was detected as a major food-derived collagen peptide in the blood of all subjects ($n = 5$). Another major peptide was identified as hydroxyprolyl-glycine (Hyp-Gly) in the blood of four subjects, which has not been detected in previous studies. The ratio of Hyp-Gly to Pro-Hyp depended on subjects and ranged from 0.00 to 5.04. Hyp-Gly was less susceptible to human serum peptidase than Pro-Hyp. Hyp-Gly enhanced the growth of mouse primary fibroblasts on collagen gels in a higher extent than Pro-Hyp. These findings suggest that Hyp-Gly plays a significant role in exerting the biological effects by ingestion of collagen hydrolysate.

1. Introduction

Collagen is a major constituent of the extracellular matrix. The denatured form of collagen is referred to as gelatin and is commonly used in foods, pharmaceuticals, photographic films, cosmetics, etc. In order to increase the solubility of gelatin, partially hydrolysed gelatin products have been prepared and are referred to as collagen hydrolysate. Recent human studies have suggested that daily ingestion of collagen hydrolysate increases the moisture content on the epidermis of women during winter, and also moderates joint pain in subjects with knee osteoarthritis (Deal & Moskowitz, 1999; Matsumoto, Ohara, Itoh, Nakamura, & Takahashi, 2006). In addition, several animal experiments support the beneficial effects of ingestion of collagen hydrolysate on skin and joint conditions (Moskowitz, 2000; Oesser, Adam, Babel, & Seifert, 1999; Tanaka, Koyama, & Nomura, 2009; Tsuruoka, Yamato, Sakai, Yoshitake, & Yonekura, 2007; Wu, Fujioka, Sugimoto, Mu, & Ishimi, 2004). After ingestion of collagen hydrolysate, Pro-Hyp, Pro-Hyp-Gly, Ala-Hyp, Ala-Hyp-Gly, Ser-Hyp, Ser-Hyp-Gly, Leu-Hyp, Ile-Hyp and Phe-Hyp have been identified as food-derived collagen peptides in human blood. Of these peptides, Pro-Hyp is the major component (Iwai et al., 2005; Ohara, Matsumoto, Ito, Iwai, & Sato, 2007). In a previous study, we demonstrated that Pro-Hyp stimulates the growth of mouse skin fibroblasts on collagen gels in a dose-dependent manner (Shigemura et al., 2009). Recently, Nakatani, Mano, Sampei, Shimizu, and Wada (2009) demonstrated that Pro-Hyp modulates rat osteoarthritis induced by a high phosphorus diet. These studies suggest that Pro-Hyp plays a significant role in the beneficial effects by ingestion of collagen hydrolysate. In these previous studies, the food-derived collagen peptides were isolated by a series of size exclusion chromatography (SEC) and reversed phase high performance liquid chromatography (RP-HPLC) (Iwai et al., 2005; Ohara et al., 2007). However, short-chain hydrophilic peptides are poorly resolved by RP-HPLC, as they are weakly retained in RP-HPLC columns. To solve these problems, a new approach based on pre-column derivatisation of peptides with phenyl isothiocyanate (PITC) has been developed (Aito-Inoue et al., 2006). This approach allows identification of short-chain hydrophilic peptides that were difficult to be isolated by RP-HPLC.

The objectives of the present study were to identify the food-derived collagen peptides in human blood, which have not been isolated by the conventional method, by using the pre-column derivatisation method.

* Corresponding author. Tel.: +81 75 703 5405.
E-mail address: k_sato@kpu.ac.jp (K. Sato).

0308-8146/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2011.05.066

2. Materials and methods

2.1. Collagen hydrolysate

Collagen hydrolysate was prepared from fish scale gelatin by enzymatic hydrolysis and was a kind gift from Chisso Corporation (Tokyo, Japan), which can be obtained commercially (Marine collagen CF). This preparation mainly consisted of peptides with the average molecular weight of 1000 Da.

2.2. Chemicals

A standard mixture of amino acids (Type H), acetonitrile (HPLC-grade), trifluoroacetic acid (TFA) and phenyl isothiocyanate (PITC) were purchased from Wako Chemicals (Osaka, Japan). Pro-Hyp and Hyp-Gly were purchased from Bachem (Bubendort, Switzerland). Dulbecco's modified Eagle's medium (DMEM) and human serum were purchased from Sigma Chemicals (St. Louis, MO, USA), Dulbecco's phosphate-buffered saline (D-PBS) and gentamicin from Invitrogen (Carlsbad, CA, USA) and fetal bovine serum BWT S-1820 (FBS) from Biowest (Nuaillé, France). Calf acid-soluble type I collagen solution (0.5%) was purchased from Koken (Tokyo, Japan) and the Cell Counting Kit-8 from Dojin Glocal (Kumamoto, Japan). All other reagents were of analytical grade or better.

2.3. Human study design

The human study was carried out according to a protocol described previously (Iwai et al., 2005). These studies were performed according to the Helsinki Declaration under the supervision of medical doctors and were approved by the experimental ethical committees of the Chisso Corporation (Tokyo, Japan). Any negative effects have not been reported by collagen hydrolysate ingestion at 5–10 g/day for 12 weeks in human studies (Sumida et al., 2004; Ohara et al., 2007; Trč & Bohmová, 2011) and the safety of high dose of collagen hydrolysate ingestion (1.66 g/kg body weight) was also confirmed by animal experiments (Wu et al., 2004). The volunteers were informed that the objectives of the present study and the potential risks of ingestion of collagen hydrolysate such as diarrhoea, and abdominal pain. Before the experiment, five healthy volunteers (male forties, fifties, fifties; female twenties, fifties) fasted for 12 h, and then ingested the collagen hydrolysate (25 g/60 kg body weight) dissolved in 100 ml of water. Approximately 10 ml of venous blood samples were collected from the cubital vein before and 30, 60, 120, 240, 420 min and 24 h after the ingestion. The plasma prepared from the venous blood samples was then deproteinised by adding three volumes of ethanol and the ethanol-soluble fraction was stored at −80 °C until analysis.

2.4. Estimation of Hyp-containing peptides in human plasma

Amino acid analysis was performed by the method of Bidlingmeyer, Cohen, and Tarvin (1984) with slight modifications (Iwai et al., 2005; Sato et al., 1992). The Hyp-containing peptides in the ethanol-soluble fraction were estimated by subtracting the free Hyp from the total Hyp in the HCl hydrolysate, as described previously (Iwai et al., 2005).

2.5. Identification of food-derived collagen peptides in human blood

The peptides in 1 ml of the ethanol-soluble fraction were captured in the spin column packed with a strong cation exchanger (AG 50 W-8, Bio-Rad Laboratories, Hercules, CA, USA), and then fractionated by SEC using a Superdex Peptide 10/300 GL (GE Healthcare, Buckinghamshire, UK), as described previously (Aito-Inoue et al., 2006). The peptides in the SEC fractions were derivatised with PITC and the resultant phenyl thiocarbonyl (PTC)-peptides were resolved on an Inertsil ODS-3 column (GL Science, Tokyo, Japan). Binary gradient elution was performed with 0.01% TFA (solvent A) and 60% acetonitrile (solvent B) at a flow rate of 1 ml/min. The column was equilibrated with 15% B. The gradient profile was as follows: 0–30 min, 15–75% B; 30–35 min, 75–100% B; 35–40 min, 100% B; 40–40.1 min, 100–15% and 40.1–50 min, 15% B. The column was maintained at 45 °C and the absorbance at 254 nm was monitored. The sequence of the isolated PTC-peptides was determined using a peptide sequencer (PPSQ-21, Shimadzu, Kyoto, Japan) based on the Edman degradation method; the programme was changed to start from the cleavage step, as described previously (Higaki-Sato et al., 2003).

2.6. Mass spectrometric analysis

The LCQ mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) was used for the analysis. Electrospray ionisation (ESI) in the positive ion mode was used. Detection of ion was achieved by scanning the range from 50 to 400 atomic mass units at an auto tune mode. Synthetic Pro-Hyp and Hyp-Gly were dissolved in 50% acetonitrile containing 0.05% formic acid to give a final concentration of 200 μM and then directly injected into the ESI-MS system using a micro syringe at a flow rate of 3 μl/min.

2.7. Digestion of Pro-Hyp and Hyp-Gly by serum peptidase

Aliquots (20 μl) of 20 mM Pro-Hyp or Hyp-Gly were mixed with 180 μl of human serum and incubated at 37 °C. Aliquots (20 μl) were collected from the reaction mixture before and 30, 90 and 180 min after the incubation and then mixed with 180 μl of ethanol to terminate the peptidase reaction. The content of free Hyp in the supernatant (50 μl) liberated from Pro-Hyp or Hyp-Gly was estimated by amino acid analysis. Degradation rate was calculated using the following equation.

$$\text{Degradation rate}(\%) = \text{Hyp concentration in the supernatant}(\text{pmol}) / \text{Peptide concentration}(10\,\text{nmol}) \times 100$$

2.8. Cell culture

Balb/c mice were purchased from SLC Japan (Shizuoka, Japan). Fibroblasts were obtained from the skin of these mice by the method of Rittié and Fisher (2005) with slight modifications (Shigemura et al., 2009). The abdominal skin was cut into square pieces (approximately 6–7 mm in width). The eight pieces were placed at the bottom of a culture dish (75 mm in diameter) so that they were not in contact with each other. Cultivation was carried out in 8 ml DMEM containing 584 mg/l L-glutamine, 0.01 mg/ml gentamicin, and 10% FBS in a humidified incubator at 37 °C under 5% CO_2. During cultivation, the medium was changed every 2 days. After cultivation for 2 weeks, the skin discs were removed and fibroblasts were recovered using a 0.25% trypsin–EDTA solution. The primary cultured fibroblasts were suspended to give a concentration of 5×10^4 cells/ml in DMEM containing 584 mg/l L-glutamine, 0.01 mg/ml gentamicin, 10% FBS, and the test component. The fibroblasts were then incubated on a collagen gel-coated plate as described previously (Shigemura et al., 2009). The collagen solution (0.5%) was mixed with the same volume of the double-concentrated DMEM medium containing L-glutamine and gentamicin and the test component in the absence of FBS. The mixture (100 μl) was then poured into each well of the 96-well plastic plate

and placed in the humidified incubator for 24 h at 37 °C under 5% CO_2 to allow gelation. Growth of the cells on the gel after suitable intervals was estimated using the Cell Counting Kit-8.

2.9. Statistical analysis

The 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, USA).

3. Results

3.1. Concentration of Hyp-containing peptide in plasma

As shown in Fig. 1, only negligible amounts of Hyp-containing peptide were observed in human plasma before the collagen hydrolysate ingestion. The concentration of Hyp-containing peptide increased to maximum level (approximately 120 nmol/ml) 1–2 h after the ingestion. The concentration decreased to half of the maximum level 4 h after the ingestion and negligible levels 7 h after the ingestion.

3.2. Identification of food-derived collagen peptides in human blood

Elution profiles of SEC for the ethanol soluble fraction of plasma are shown in Fig. 2. The amino acid analysis revealed that Hyp was contained in the HCl-hydrolysates of SEC Fr. 35–40 (data not shown). The PTC-derivatives of SEC Fr. 31–40 were resolved by RP-HPLC. As shown in Fig. 3, the peak (a) was observed in the SEC Fr. 36–37 of the subjects 1 and 2 only after the ingestion. This peak was identified as PTC-Pro-Hyp by sequence analysis, which was confirmed by comparison with the retention time of synthetic PTC-Pro-Hyp. On the other hand, another peak (b) was observed in the SEC Fr. 37 only in the subject 2 after the ingestion (Fig. 3). This peak was identified as PTC-Hyp-Gly by similar manner. There are no significant differences of elution profiles of PTC-derivatives in the SEC Fr. 31–35 before and after the ingestion (data not shown). In SEC Fr. 39–40, only PTC-Hyp was detected.

3.3. The ratio of food-derived Pro-Hyp and Hyp-Gly in human plasma

Pro-Hyp was detected in all subjects. On the other hand, Hyp-Gly was observed in the subjects1, 3–5. The ratio of Hyp-Gly to Pro-Hyp in human plasma 1 h after the ingestion was semi-quantitatively estimated on the basis of PTC-peptide peak area. As shown in Table 1, it was distributed ranging from 0.00 to 5.04 and extensively varied between subjects.

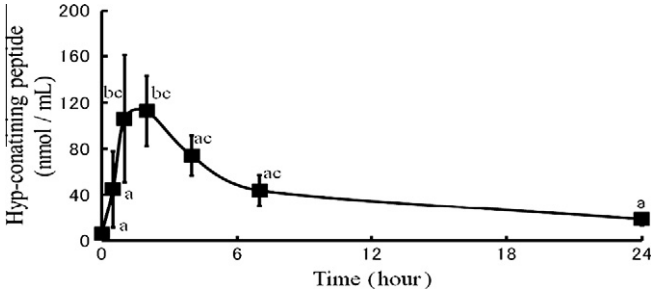

Fig. 1. Plasma level of hydroxyproline in peptide form after oral ingestion of the collagen hydrolysate. Average concentration of 5 subjects are shown. The data are shown as the mean ± SD; $n = 5$. The different letters on the values indicate significant difference ($p < 0.05$).

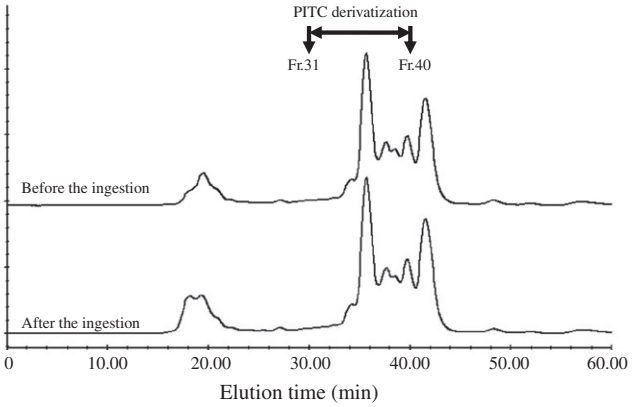

Fig. 2. Fractionation of peptides in ethanol-soluble fractions by size exclusion chromatography. Sample was collected every 1 min.

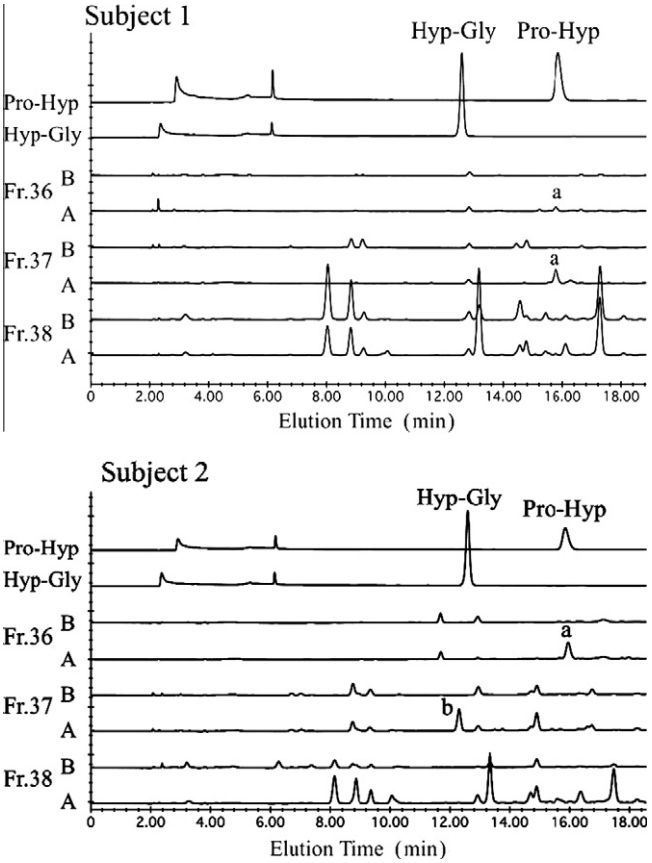

Fig. 3. SEC fractionation of the PTC-derivatives. Samples were prepared from Subject 1 and 2 before (B) and after (A) the ingestion of collagen hydrolysate. Synthetic PTC-Pro-Hyp and PTC-Hyp-Gly were also resolved.

Table 1
Ratios of Hyp-Gly to Pro-Hyp in human blood 1 h after the ingestion of collagen peptide preparation.

Subject 1	0
Subject 2	5.04
Subject 3	1.20
Subject 4	0.21
Subject 5	0.30

3.4. Intensity of Pro-Hyp and Hyp-Gly Ions by ESI-MS

ESI-MS analysis revealed that intensity of Hyp-Gly ion (188.93 m/z) was approximately 20% of Pro-Hyp ion (228.93 m/z) in same content (Fig. 4). When PTC-derivatives were used, no significant ion intensity was observed for PTC-Pro-Hyp and PTC-Hyp-Gly in the present condition (data not shown).

3.5. Degradation of Pro-Hyp and Hyp-Gly by human serum peptidase

Synthetic Pro-Hyp and Hyp-Gly were treated with human serum. As shown in Fig. 5, the degradation rates of Pro-Hyp and Hyp-Gly were 5.35% and 0.51% 180 min after the incubation, respectively. Only negligible amounts of Hyp were liberated from Hyp-Gly by human serum peptidase, which indicated that Hyp-Gly is more resistant to serum peptidase than Pro-Hyp.

3.6. Effect on growth of fibroblasts on collagen gel

As shown in Fig. 6, Pro-Hyp and Hyp-Gly significantly enhanced growth of fibroblasts on collagen gel in comparison to control. Hyp-Gly showed significantly higher stimulatory activity to fibroblasts growth than Pro-Hyp.

4. Discussion

In the previous studies, food-derived collagen peptides in human blood have been identified using RP-HPLC-UV detection (Iwai et al., 2005; Ohara et al., 2007) and RP-HPLC–ESI-MS/MS detection (Ichikawa et al., 2010). Pro-Hyp has been identified as a major food-derived collagen peptide in human blood in these studies. In the present study, Hyp-Gly is identified as a major food-derived collagen peptide in four of the five subjects in addition to Pro-Hyp

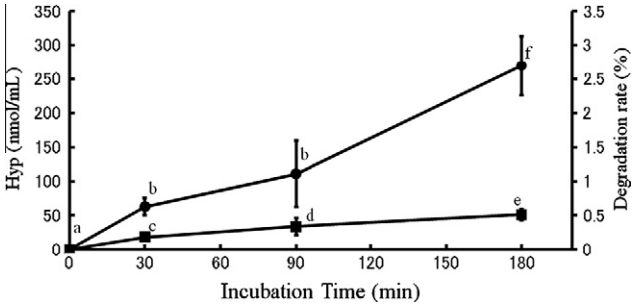

Fig. 5. Degradation of Pro-Hyp and Hyp-Gly by human serum peptidase. 100 mM of Pro-Hyp and Hyp-Gly were mixed with human serum and incubated at 37 °C. The concentration of free Hyp liberated from Pro-Hyp(●) and Hyp-Gly(■) and degradation rate was shown. The data are shown as the mean ± SD; $n = 4$. Degradation rate was calculated by following equation. Degradation rate (%) = Hyp concentration in the supernatant (pmol)/Peptide concentration (10 nmol) × 100.

by using pre-column derivatisation technique. It is difficult to resolve Hyp-Gly by the RP-HPLC without PITC derivatisation due to its hydrophilic nature and small molecular mass. In addition, relatively low ion intensity of Hyp-Gly was observed by ESI-MS when compared to Pro-Hyp. Therefore, Hyp-Gly had not been detected in the previous studies (Ichikawa et al., 2010; Iwai et al., 2005; Ohara et al., 2007). The pre-column derivatisation technique used in the present study is useful for detecting and identifying short-chain hydrophilic food-derived peptides in complex biological samples.

Although the same collagen hydrolysate was ingested by the five volunteers, the ratio of Pro-Hyp to Hyp-Gly in the blood samples extensively depended on subjects (Table 1). This finding suggests that some polymorphisms might exist in the peptide transporter, endopeptidase and/or exopeptidase. It has been

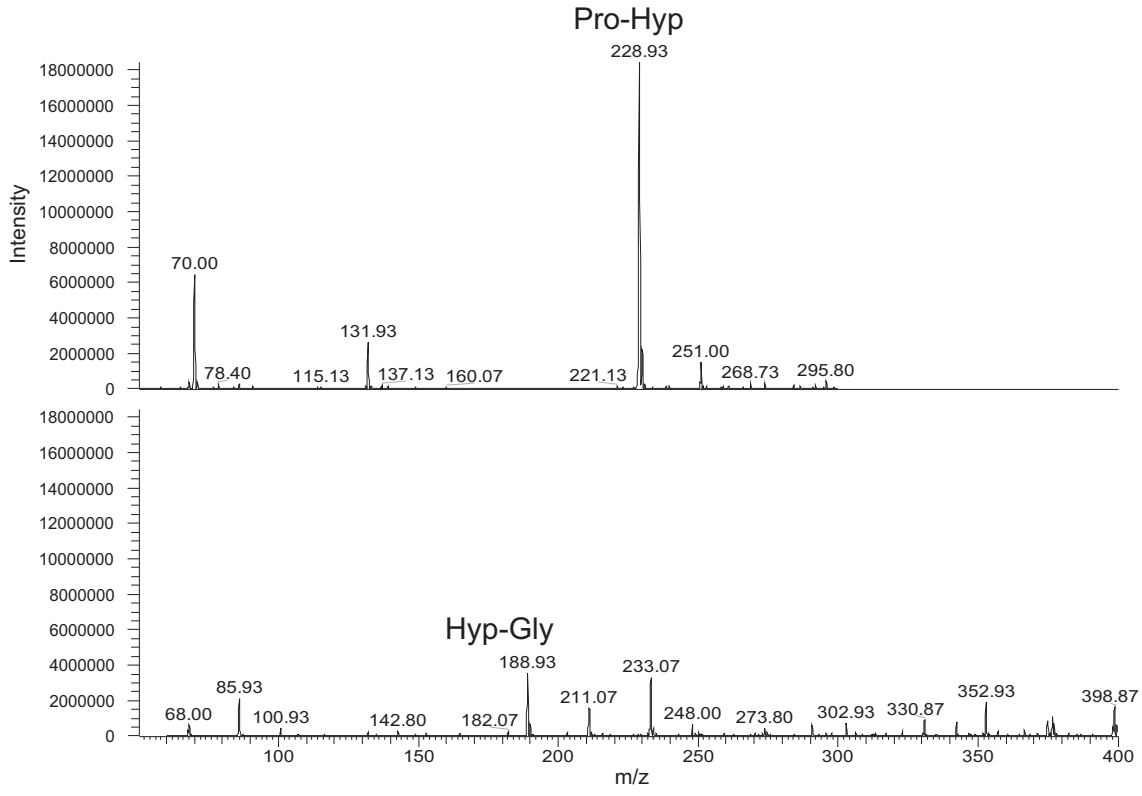

Fig. 4. Electrospray ionisation mass spectrum (ESI-MS) of Pro-Hyp and Hyp-Gly in same concentration (200 nmol/ml).

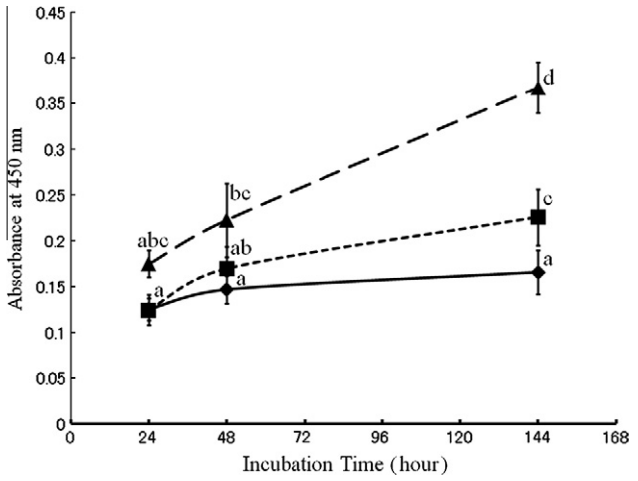

Fig. 6. Effect of Hyp-Gly and Pro-Hyp on the growth of fibroblast on collagen gel. Mouse skin primary fibroblasts (5×10^3 cells) were cultured on a 96-well plate paved with collagen gel for 144 h. 200 nmol/ml of Hyp-Gly (▲), and Pro-Hyp (■) were added into the control medium containing 10% FBS (♦). Growth of fibroblasts was estimated by the absorbance of the medium treated with Cell Counting Kit-8 after 24, 48 and 144 h from incubation. The data are shown as the mean ± SD; $n = 4$. The different letters on the values indicate significant difference ($p < 0.05$).

demonstrated that the peptide transporter 1 (PEPT1) plays a significant role in di- and tri-peptides absorption in enterocytes (Adibi, 1997; Aito-Inoue, Lackeyram, Fan, Sato, & Mine, 2007). However, it has been demonstrated that PEPT1 transports dipeptides in a non-specific manner. In addition, both Pro-Hyp and Hyp-Gly are resistant to exopeptidase in human blood (Fig. 5) compared to non-collagenous dipeptides (Druml et al., 1991; Hubl, Druml, Langer, & Lochs, 1989). Therefore, it is likely that some polymorphisms might be present in endopeptidase that results in cleavage of large collagen peptides into small peptides, rather than peptide transporter and exopeptidase. Alternatively, exogenous factor such as dietary habit may affect peptide transportation and/or digestion of collagen peptides.

It has been demonstrated by *in vitro* studies that Pro-Hyp, one of the quantitatively major constituents of food-derived collagen peptide, has a stimulatory activity on migration of mouse fibroblast from skin to medium (Shigemura et al., 2009) and hyaluronic acid synthesis (Ohara et al., 2010). Furthermore, Nakatani et al. (2009) demonstrated that Pro-Hyp suppresses osteoarthritis induced by a high phosphorus diet. The present authors also demonstrated that the proliferation of primary cultured fibroblast on collagen gel was enhanced by addition of Pro-Hyp in dose dependent manner from 50 to1000 nmol/ml (Shigemura et al., 2009). After single ingestion of collagen hydrolysate at 25 g/60 kg body weight, maximum level of Hyp-containing peptides is approximately from 50 to 200 nmol/ml. The concentration largely depends on subjects. Thus, these studies partially explain the beneficial effects of collagen hydrolysate ingestion in human studies. In addition, the present study demonstrates that Hyp-Gly has significantly higher stimulatory activity on fibroblast growth than Pro-Hyp, which suggests that Hyp-Gly may have a significant role in exerting the biological effects associated with ingestion of collagen hydrolysate. Furthermore, subject variability in the composition of food-derived collagen peptides may cause differences in the efficacy of collagen hydrolysate ingestion between subjects.

It has been suggested that ingestion of smaller doses (5 g/day) of collagen hydrolysate for longer periods might improve skin conditions (Sumida et al., 2004; Ohara et al., 2007). Further studies on content and composition of food-derived collagen peptide in human blood by ingestion of smaller dose of collagen hydrolysate for longer periods and effects of these food-derived peptides on skin condition are now in progress.

Acknowledgements

This work was partially supported by Grant-in-Aid for Scientific Research (C) No. 20500712 and Grant-in-Aid for Young Scientists (B) No. 20700613 from the Japan Society for the Promotion of Science.

References

Adibi, S. A. (1997). The oligopeptide transporter (Pept-1) in human intestine: Biology and function. *Gastroenterology, 113*(1), 332–340.

Aito-Inoue, M., Lackeyram, D., Fan, M. Z., Sato, K., & Mine, Y. (2007). Transport of a tripeptide, Gly-Pro-Hyp, across the porcine intestinal brush-border membrane. *Journal of Peptide Science, 13*(7), 468–474.

Aito-Inoue, M., Ohtsuki, K., Nakamura, Y., Park, E. Y., Iwai, K., Morimatsu, F., et al. (2006). Improvement in isolation and identification of food-derived peptides in human plasma based on precolumn derivatization of peptides with phenyl isothiocyanate. *Journal of Agricultural and Food Chemistry, 54*(15), 5261–5266.

Bidlingmeyer, B. A., Cohen, S. A., & Tarvin, T. L. (1984). Rapid analysis of amino acids using pre-column derivatization. *Journal of Chromatography, 336*(1), 93–104.

Deal, C. L., & Moskowitz, R. W. (1999). Nutraceuticals as therapeutic agents in osteoarthritis. The role of glucosamine, chondroitin sulfate, and collagen hydrolysate. *Rheumatic Diseases Clinics of North America, 25*(2), 379–395.

Druml, W., Lochs, H., Roth, W., Hubl, W., Balcke, P., & Lenz, K. (1991). Utilization of tyrosine dipeptides and acetyltyrosine in normal and uremic humans. *American Journal of Physiology Endocrinology and Metabolism, 260*, 280–285.

Higaki-Sato, N., Sato, K., Esumi, Y., Okumura, T., Yoshikawa, H., Tanaka-Kuwajima, C., et al. (2003). Isolation and identification of indigestible pyroglutamyl peptides in an enzymatic hydrolysate of wheat gluten prepared on an industrial scale. *Journal of Agricultural and Food Chemistry, 51*(1), 8–13.

Hubl, W., Druml, W., Langer, K., & Lochs, H. (1989). Influence of molecular structure and plasma hydrolysis on the metabolism of glutamine-containing dipeptides in humans. *Metabolism, 38*(8 Suppl. 1), 59–62.

Ichikawa, S., Morifuji, M., Ohara, H., Matsumoto, H., Takeuchi, Y., & Sato, K. (2010). Hydroxyproline-containing dipeptides and tripeptides quantified at high concentration in human blood after oral administration of gelatin hydrolysate. *International Journal of Food Sciences and Nutrition, 61*(1), 52–60.

Iwai, K., Hasegawa, T., Taguchi, Y., Morimatsu, F., Sato, K., Nakamura, Y., et al. (2005). Identification of food-derived collagen peptides in human blood after oral ingestion of gelatin hydrolysates. *Journal of Agricultural and Food Chemistry, 53*(16), 6531–6536.

Matsumoto, H., Ohara, H., Itoh, K., Nakamura, Y., & Takahashi, S. (2006). Clinical effect of fish type I collagen hydrolysate on skin properties. *ITE Letters, 7*, 386–390.

Moskowitz, R. W. (2000). Role of collagen hydrolysate in bone and joint disease. *Seminars in Arthritis and Rheumatism, 30*(2), 87–99.

Nakatani, S., Mano, H., Sampei, C., Shimizu, J., & Wada, M. (2009). Chondroprotective effect of the bioactive peptide prolyl-hydroxyproline in mouse articular cartilage in vitro and in vivo. *Osteoarthritis and Cartilage, 17*(12), 1620–1627.

Oesser, S., Adam, M., Babel, W., & Seifert, J. (1999). Oral administration of (14)C labeled gelatin hydrolysate leads to an accumulation of radioactivity in cartilage of mice (C57/BL). *Journal of Nutrition, 129*(10), 1891–1895.

Ohara, H., Ichikawa, S., Matsumoto, H., Akiyama, M., Fujimoto, N., Kobayashi, T., et al. (2010). Collagen-derived dipeptide, proline-hydroxyproline, stimulates cell proliferation and hyaluronic acid synthesis in cultured human dermal fibroblasts. *Journal of Dermatology, 37*(4), 330–338.

Ohara, H., Matsumoto, H., Ito, K., Iwai, K., & Sato, K. (2007). Comparison of quantity and structures of hydroxyproline-containing peptides in human blood after oral ingestion of gelatin hydrolysates from different sources. *Journal of Agricultural and Food Chemistry, 55*(4), 1532–1535.

Rittié, L., & Fisher, G. J. (2005). Isolation and culture of skin. *Methods in Molecular Medicine, 117*, 83–98.

Sato, K., Tsukamasa, Y., Imai, C., Ohtsuki, K., Shimizu, Y., & Kawabata, M. (1992). 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. *Journal of Agricultural and Food Chemistry, 40*, 806–810.

Shigemura, Y., Iwai, K., Morimatsu, F., Iwamoto, T., Mori, T., Oda, C., et al. (2009). Effect of Prolyl-hydroxyproline (Pro-Hyp), a food-derived collagen peptide in human blood, on growth of fibroblasts from mouse skin. *Journal of Agricultural and Food Chemistry, 57*(2), 444–449.

Sumida, E., Hirota, A., Kuwaba, K., Kusubata, M., Koyama, Y., Araya, T., et al. (2004). The effect of oral ingestion of collagen peptide on skin hydration and biochemical data of blood. *Journal of Nutritional Food, 7*(3), 45–52.

Sumida, E., Hirota, A., Kuwaba, K., Kusubata, M., Koyama, Y., Araya, T., et al. (2004). The effect of oral ingestion of collagen peptide on skin hydration and biochemical data of blood. *Journal of Nutritional Food, 7*(3), 45–52.

Tanaka, M., Koyama, Y., & Nomura, Y. (2009). Effects of collagen peptide ingestion on UV-B-induced skin damage. *Bioscience, Biotechnology, and Biochemistry, 73*(4), 930–932.

Trč, T., & Bohmová, J. (2011). Efficacy and tolerance of enzymatic hydrolysed collagen (EHC) vs glucosamine sulphate (GS) in the treatment of knee osteoarthritis (KOA). *International Orthopaedics, 35*(3), 341–348.

Tsuruoka, N., Yamato, R., Sakai, Y., Yoshitake, Y., & Yonekura, H. (2007). Promotion by collagen tripeptide of type I collagen gene expression in human osteoblastic cells and fracture healing of rat femur. *Bioscience, Biotechnology, and Biochemistry, 71*(11), 2680–2687.

Wu, J., Fujioka, M., Sugimoto, K., Mu, G., & Ishimi, Y. (2004). Assessment of effectiveness of oral administration of collagen peptide on bone metabolism in growing and mature rats. *Journal of Bone and Mineral Metabolism, 22*(6), 547–553.