Absorption and metabolism of orally administered collagen hydrolysates evaluated by the vascularly perfused rat intestine and liver \textit{in situ}

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ABSTRACT

A number of studies have shown that oral administration of collagen hydrolysate (CH) results in the absorption of di- and tri-peptides. In order to understand the dynamics of CH absorption and metabolism, molecular profiles of hydroxyproline (Hyp) and Hyp-containing peptides (HCPs) were analyzed by \textit{in situ} perfusion of rat intestine and liver. The total amount of absorbed HCPs during 1 h of perfusion was 16.6 μmol, which was significantly higher than that of free Hyp (6.6 μmol). In addition, HCPs were also reliably detected in hepatic perfusate at the level higher than free Hyp. Thus, the results demonstrated that CH is absorbed predominantly as peptides, which subsequently enter systemic circulation. Size exclusion chromatography showed that perfusates include significant amount of HCPs larger than tripeptides, leading us to analyze these peptides in detail. Mass spectrometric analysis of intestinal perfusate finally identified three CH-derived peptides, which are surprisingly large as food-derived circulating peptides. Peptide quantitation by liquid chromatography-tandem mass spectrometry (LC-MS/MS) revealed that di- and tri-peptides, which are previously identified as major peptides in circulating blood, comprise only a part of HCPs in intestinal and liver perfusate. Finally, analysis of portal vein blood revealed that the larger peptides, such as pentadecapeptide identified in this study, could be absorbed \textit{in vivo}. Taken all together, this study showed that peptides which are larger than tripeptide could reach to the circulation system after administration of CH, revealing previously unknown dynamics of absorption of CH.
regulation by CH. However, several studies have identified at least 29 di- and tri-peptides other than PO in plasma after oral administration of CH (9, 19, 22, 25, 26, 31, 34). Recently, some of these peptides have been shown to play a regulatory role in skin barrier function (13, 20, 22, 24). Thus, in order to understand molecular mechanisms of beneficial effect of CH administration, it is important to clarify the detailed molecular profiles of CH-derived metabolites circulating in the blood. Recent studies have shown the delivery of CH-derived peptides to target tissues. Watanabe-Kamiyama et al. have analyzed the intestinal absorption and tissue distribution of radio-labeled collagen-derived tripeptide (glycyl-prolyl-hydroxyproline: GPO) (32). They reported that radioactivity was predominantly detected in specific tissues, such as the liver, kidney, cartilage and skin. Kusubata et al. have shown the delivery of food-derived PO is delivered abundantly to the area of damaged skin (13). These results indicate that circulating HCPs including PO have some functions in the regulation oftarget tissues.

Although these studies provide important information about dynamics of absorption and metabolism of orally administrated CH, the profile of molecules just after absorption remains largely elusive. In many studies, evaluation of these circulating peptides has been carried out after 30 min–several hours post oral administration of CH (9, 19, 22, 25, 26, 31, 34). Therefore, the analytes were the mixture of peptides circulating just after absorption and their metabolites stably circulating for a while. In addition, endogenous peptides, residual materials and peptidases in blood sample could interfere the identification of CH-derived peptides with short half-lives or lower content, which could have some functions to regulate target tissues.

In this study, we used in situ vascular perfusion technique to analyze the dynamics of CH-derived peptides. We prepared perfusates from portal vein and inferior vena cava, in order to understand intestinal absorption and hepatic metabolism of CH-derived peptides, respectively. Interestingly, size exclusion chromatography revealed that intestinal perfusates include significant amount of HCPs which are larger than tripeptides, suggesting existence of unidentified HCPs in perfusate. Mass-spectrometric analysis identified novel HCPs, including pentadeca-peptides which are surprisingly large as food-derived circulating peptides. Importantly, these peptides were reliably detected in hepatic perfusate, suggesting that larger peptides could pass through the liver and reach the systemic circulation system at least once. Together, these results have revealed previously unknown aspect of absorption and metabolism of HCPs after oral administration of CH.

MATERIALS AND METHODS

Chemicals. The synthetic peptides which have amino acid sequences in type I collagen, such as Pro-Hyp (PO), Pro-Hyp-Gly (POG), Hyp-Gly-Pro (OGP), Gly-Glu-Thr-Gly-Pro-Ala-Gly-Arg-Hyp-Gly (GETGPAGROG), Asp-Gly-Glu-Ala-Gly-Lys-Pro-Gly-Arg-Hyp-Gly-Glu (DGEAGKPGROGE), and Gly-Glu-Ala-Gly-Pro-Ala-Gly-Pro-Ala-Gly-Pro-Arg (GEAGPAGPAGPAGPR), were purchased from PH Japan (Hiroshima, Japan). Gly-Gly (GG) was purchased from Kanto Chemical (Tokyo, Japan). Gly-Pro (GP), Leu-Gly-Gly (LOG), Pro-Hyp-Gly-Pro (POGP), Hyp-Gly-Pro-Hyp (OGPO), (Pro-Pro-Gly)₅, (Pro-Hyp-Gly)₅, and α-Cyano-4-hydroxycinnamic acid (CHCA) were purchased from Peptide Institutes (Osaka, Japan). Hyp-Gly (OG), Ala-Hyp (AO), Ser-Hyp (SO), and Gly-Pro-Ala (GPA) were synthesized in our laboratory, and used after crystallization. Enzymatic hydrolysate of porcine skin gelatin (SCP-5000; average molecular weight is 5000 Da) was provided by Nitta Gelatin (Osaka, Japan) (10). L-amino acids, including L-hydroxyproline (Hyp), and the other analytical grade chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan). The HPLC solvents were obtained from Sigma Chemical (St. Louis, MO, USA).

Animals and diets. Six-week-old male Wistar rats (approximately 200 g) were purchased from Charles River Japan (Kanagawa, Japan) and were individually housed in stainless-steel wire cages in a room maintained at 22 ± 2°C with a 12-h light-dark cycle. The rats were fed with a solid feed (MF; Oriental Yeast, Tokyo, Japan) for more than three days before the experiments. The rats were fasted overnight prior to the experiments. The animal care protocol was approved by the Utsunomiya University Animal Research Committee under the Guidelines for Animal Experiments of Utsunomiya University (authorization number: AP16-0017). CH was solubilized by water at the concentration of 0.5 g/mL, then 2 mL of CH solution was intragastrically administered to rats by using teflon sonda.
Vascular perfusion of the rat small intestine and liver: Intestinal perfusion was performed by the reported method of Liu et al. (15). Briefly, rats were anesthetized with sodium thiopental (12.5 mg/200 g BW, i.p.) at 40 min after injection of CH, and then fixed to a surgical platform in perfusion apparatus maintained at 37°C using a thermoregulatory-controlled heating unit. The thoracic cavity was disclosed by cutting upwards and bilaterally along the rib wall, leading to the expose of the heart. An anticoagulant was injected into the heart, and then a small incision was made through the apex of the left ventricle. A catheter was inserted into the aorta, which was clamped in order to fix the position of the catheter. Then, the portal vein was exposed, and another catheter was inserted into the vessel. A single-pass perfusion was established by securing the in-flow catheter in the aorta, and the perfusate was collected through an out-flow catheter of the portal vein. The exposed surface of the small intestine was covered with wet gauze and plastic film to avoid surface drying. Portal venous perfusion was performed with a Krebs Ringer bicarbonate solution (pH 7.4) containing 2% bovine serum albumin and 15 mM glucose. The perfusate solution was supplemented with dexamethasone and noradrenaline to counteract the effects of denervation and continuously bubbled with 95% O₂–5% CO₂. This perfusion was started at 1 h after injection of CH. For first 5 min, the flow rate was adjusted to approximately 5 mL/min to prevent vascular occlusion. Then, the flow rate was re-adjusted to approximately 1 mL/min, finally achieving a blood pressure of 110–115 mm of Hg.

Hepatic perfusion was performed basically by the same method. For collecting the liver perfusate, an out-flow catheter was inserted into the inferior vena cava which connect to the right atrium of the heart. In order to prevent the leakage of perfusate, the inferior vena cava which connect to the lower trunk and abdomen, as well as the hepatic artery, were occluded tightly by a klemme.

Preparation of plasma samples. The sampling was carried out by decapitating the rats after 2 h post administration of CH. Plasma samples were collected by the centrifugation at 1000 × g for 20 min, followed by the sedimentation of proteins by adding 3 volumes of ethanol. The supernatants were used for the analyses. For collecting blood samples from portal vein, rats were anesthetized by intraperitoneal injection of thiopental sodium after 2 h post administration of CH. After surgical operation, blood was collected directly from the portal vein by using 21-gauge needles. In order to suppress the serum peptidase activity toward CH-derived peptides, blood samples were transferred immediately into ice-cold ethanol solution. This step was carried out within 2 min after collection of blood samples. Plasma samples were prepared after centrifugation of ethanol-treated blood at 3000 × g for 10 min and used for the analyses.

Quantitation of hydroxyproline and HCPs by biochemical method. The amount of free Hyp and HCPs in perfusate was determined before and after hydrolysis by hydrochloric acid. Therefore, the amount of HCPs was evaluated by the amount of Hyp generated by the hydrolysis of HCP. The deproteinized samples were analyzed for the quantitation of free Hyp and HCPs. An aliquot of the deproteinized supernatant was hydrolyzed in 6N HCl at 110°C for 24 h and subsequently dried under vacuum. Finally, the amount of peptide form of Hyp was estimated by subtracting free Hyp from total Hyp.

Size exclusion chromatography analysis. Size exclusion chromatography was used for the analysis of molecular size distribution of absorbed peptides. The ethanol treated sample was dried under vacuum and dissolved in 30% acetonitrile solution in the presence of 0.1% trifluoroacetic acid (TFA). A spin column (0.22 μm, Ultrafree-MC GV; Merck Millipore, Billerica, MA, USA) was used for the clarification. The filtrated sample was applied to Superdex Peptide HR 10/30 column (GE Healthcare, Chicago, IL, USA). Elution was carried out with 30% acetonitrile solution in the presence of 0.1% TFA for 60 min at flow rate of 0.5 mL/min. Fractions were collected every 1 min and used for the quantitation of Hyp.

Analysis of perfusate and plasma by LC-MS/MS. The quantitation of the peptides was carried out for three times and the results were expressed as the mean values ± standard error. For the analysis of di- or tri-peptides, one sample was prepared from each rat. For the analysis of large peptides identified in this study, one sample was prepared by pooling samples from five rats.

The samples were analyzed by LC-MS/MS. Inert-sustain peptide C18 column (20 × 150 cm, 3 μm; GL Sciences, Tokyo, Japan) was used for the separation of peptides. For the quantitation of CH-derived peptides, elution was performed with 0.1% TFA for 5 min followed by a linear gradient to 15% acetonitrile.
trile in the presence of 0.1% TFA over 15 min. The flow rate was 0.4 mL/min. For the analysis of free Hyp, isocratic elution was performed with 0.1% TFA for 3 min.

Mass spectrometry was performed with a Triple TOF 5600 mass spectrometer (AB Sciex, Framingham, MA, USA) equipped with an electro-spray ionization source. The pressure values of nebulizer gas, the heater gas, and Curtain Gas were adjusted to 50, 50, and 25 psi, respectively. The source temperature was adjusted to 500°C. The ion spray voltage was adjusted to 5.5 kV in the positive ionization mode. MS/MS experiments were carried out using nitrogen as the collision gas. The collision energy was optimized for each peptide. Data acquisition and analyses were performed by using Analyst software (ver. 1.6.; AB Sciex, Framingham, MA, USA). Quantification of the corresponding CH-derived peptides in the sample by an LC-MS/MS analysis was carried out by using MRM (Multiple Reaction Monitoring), in which the transition of m/z 133.061 > 76.039 (CE: 15) for GG, m/z 173.092 > 116.071 (CE: 20) for GP, m/z 189.087 > 86.061 (CE: 25) for OG, m/z 203.103 > 132.066 (CE: 20) for AO, m/z 219.098 > (CE: 20) for SO, m/z 229.118 > 132.066 (CE: 20) for PO, m/z 244.129 > 155.082 (CE: 15) for GPA, m/z 260.124 > 189.087 (CE: 20) for AOG, m/z 276.119 > 189.087 (CE: 20) for SOG, m/z 286.140 > 189.087 (CE: 25) for POG, m/z 286.140 > 155.082 (CE: 25) for GPO, m/z 286.140 > 173.092 (CE: 25) for PO, m/z 302.171 > 189.087 (CE: 25) for LOG, m/z 318.130 > 225.087 (CE: 20) for EOG, m/z 399.187 > 186.140 (CE: 30) for POG, m/z 399.187 > 229.118 (CE: 25) for OGPO, m/z 457.720 (divalent cation) > 570.299 (CE: 25) for GETGPAGROG, m/z 395.855 (trivalent cation) > 628.305 (CE: 25) for DGEAGKPGROGE, m/z 631.318 (divalent cation) > 779.416 (CE: 35) for GEAGPAGPAGPR, m/z 637.830 (divalent cation) > 1005.515 (CE: 30) for (PPG)$_2$, m/z 677.817 (divalent cation) > 1069.495 (CE: 35) for (POG)$_2$, and m/z 132.066 > 86.061 (CE: 25) for Hyp, was selected.

For the quantitation of trace amount of peptides, the height of the MS/MS peak was carefully analyzed with respect to the recommended criteria reported by the Japan Society for Analytical Chemistry (29). Briefly, a specific MS/MS peak was judged as reliable when the peak intensity exceeds the detection limit value calculated by using background peak value detected in blank sample. According to the recommended method, we configured the detection limit as background peak value plus 10 times of the background standard errors.

**Stability of CH-derived peptides against the digestion by plasma peptidase.** The stability of the peptides was analyzed by using rat plasma. The plasma was prepared and incubated for 10 min at 37°C. Five μL of each peptide dissolved in PBS was added to 45 μL of pre-warmed rat plasma at the concentration of 1 μg/mL. After 30 min, the samples were deproteinized by adding 3 volumes of ethanol. The supernatants were used for the LC-MS/MS analyses.

**Statistical analysis.** One way ANOVA with Tukey’s post hoc test was used for the statistical analysis. Values of $P < 0.05$ were considered significant. All data was expressed as the mean ± SEM.

**RESULTS**

**Amount of free Hyp and HCPs in intestinal and hepatic perfusate**

Previously, we used in situ vascular perfusion technique to analyze the transport of synthetic HCPs across the rat small intestine (15). In this study, we applied this method for the evaluation of intestinal absorption and hepatic metabolism of CH which is orally administrated. After intragastric injection of CH, perfusates were collected for 60 min from the catheter which is inserted into portal vein or inferior vena cava. First, we assessed the amount of CH-derived free Hyp and HCPs in perfusates. As shown in Fig. 1, free Hyp was observed constantly in the intestinal perfusate throughout the perfusion period. The average level of free Hyp was 109 nmol/mL, which was significantly higher than the basal levels of free Hyp in perfusate (around 20 nmol/mL, Liu et al.) (15), suggesting the transport of CH-derived Hyp into portal vein. Interestingly, the concentration of HCPs significantly increased approximately 20 min after the start of perfusion, finally reaching a maximum level of 400 nmol/mL. Thus, molecular mechanism for the absorption of HCPs diverges from that for Hyp. Absorbed amount of HCPs during 1 h after oral administration of CH was 16.6 μmol, which was significantly higher than that of free Hyp (6.6 μmol). Together, these results have indicated that CH is absorbed predominantly as peptides in the small intestine. Similar results were observed in hepatic perfusates (Fig. 1, Table 1). Although the concentration of HCPs decreased to one-half after passing through the liver, significant amount of HCPs appeared to enter the systemic circulation.
Absorption of collagen peptide

The ratio of the HCPs to the free Hyp was approximately 3 : 1 and 1.5 : 1 just after absorption and after passing through the liver, respectively. These results are at variance with the previous studies reporting that free Hyp is dominant in the serum after administration of CH.

HCPs larger than tripeptide in perfusate

Our analysis has suggested that the ratio of HCPs to free Hyp in absorbed fraction would be much higher than previously considered, thus we analyzed the molecular profile of the perfusates. As shown in Fig. 2A, size exclusion chromatography showed that CH used in this study consists of peptides with molecular mass ranged from 1,000 to 20,000 Da. Only trace amount of peptide with molecular mass less than 300 Da was detected. Thus, CH does not include di- and tri-peptide as major ingredients. Elution profiles of perfusates were shown in Fig. 2B and 2C. Interestingly, significant amount of compounds with molecular mass ranging from 100 to 1,500 Da was detected in intestinal perfusate (Fig. 2B), showing clear contrast to the elution profile of CH. The result indicated the existence of peptides larger than tripeptides in intestinal perfusate. Also, the data indicated that intestinal perfusate includes free amino acids, as well as di- and tri-peptides, as metabolites of administered CH. In hepatic perfusate, a main peak was detected at the elution time of free amino acid, whereas considerable amount of compounds larger than tripeptides was still detected. In contrast, elution profile of carotid artery serum showed that a main peak was detected at the position of free Hyp, suggesting the advanced turnover of the peptides during circulation. In order to confirm that elution profiles accurately represent the distribution of molecular masses of CH-derived peptides and amino acids, fractions were collected and the amount of Hyp was analyzed after hydration by hydrochloric acid. Similar elution profiles were observed in perfusates and blood sample, confirming that the data represent the dynamics of CH-derived peptides. Taken together, HCPs including peptides larger than tripeptide would pass through the liver and enter the systemic circulation at least once.

It is generally thought that food derived peptides larger than tripeptide are not transported from intestinal lumen to portal vein. Thus, molecular weight distribution of HCPs in perfusate showed very unique character of CH absorption, leading us to identify larger HCPs. Peptides in intestinal perfusate were separated by reverse phase chromatography and fractions were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.
Interestingly, the amino acid sequences of these peptides were GETGPAGROG, DGEAGKPGROGE and GEAGPAAGPAGPAGPR, which are quite large as food-derived and circulating peptides.

**Quantitation of HCPs in perfusate and serum**

Next, we made quantitative evaluation of each peptide, including the peptides identified in this study. We used liquid chromatography-tandem mass spectrometry (LC-MS/MS) for the analysis. As shown in Table 2, all peptides were reliably detected in perfusates prepared after administration of CH. In contrast, the amount of all peptides in control perfusates was near or below the detection limit. Therefore, we judged that the data for CH-administered group represent approximately the actual amount of food-derived peptides, although statistical comparison to control group was impossible. As reported in previous studies (10), peptides such as prolyl-hydroxyproline (PO) and prolyl-hydroxyprolyl-glycine (POG) were main constituent of perfusates. The total amount of analyzed di- and tri-peptides was 75 nmol/mL in intestinal perfusate, corresponding to only a fraction of all HCPs absorbed in small intestine (calculated as 385 nmol/mL from Table 1). The average concentration of di- and tri-peptides was 5.7 nmol/mL, whereas that of larger peptides was 280 pmol/mL. Thus, the data indicated that CH is absorbed mainly as di- and tri-peptides but many kinds of larger peptides are also absorbed at lower content. In order to confirm that the larger peptides are absorbed in vivo, we analyzed portal vein serum by LC-MS/MS. As shown in Table 3, the amount of DGEAGKPGROGE and GEAGPAAGPAGPAGPR was apparently higher in CH-administered group compared to control group. Similarly, the amount of GETGPAGROG showed a tendency to be higher in CH-administered group. Together, these results indicated in vivo absorption of peptides larger than tripeptide in rat small intestine. These peptides were not detected in carotid artery serum prepared at 2 h after administration of CH, suggesting their turnover in the systemic circulation. The average amount of these peptides in portal vein serum was 76 fmol/mL, which was equivalent to one hundred thousandth of that of di- and tri-peptides (7.8 nmol/mL). Therefore, in vivo absorption of HCPs larger than tripeptide occurred at lower level compared to di- or tri-peptides. Another possibility was the rapid degradation of these peptides just after intestinal absorption.

**Stability of HCPs toward serum peptidases**

The data also suggested that the liver performs im-
Absorption of collagen peptide

Table 2  The amount of CH-derived peptides in perfusate

A

| Peptide | Concentration (nmol/mL) | Intestinal perfusate | Hepatic perfusate |
|---------|-------------------------|----------------------|-------------------|
|         |                         | CH      | Water | CH    | Water |
| GP      | 13.59 ± 3.89*           | 0.15 ± 0.04 | 9.64 ± 1.14* | 0.11 ± 0.02 |
| OG      | 2.24 ± 0.21             | N.D.   |       | 1.29 ± 0.05 | N.D.   |
| AO      | 3.15 ± 0.43             | (0.02, N.D., N.D.) | 3.59 ± 0.38 | (0.02, N.D., N.D.) |
| SO      | 2.96 ± 0.48             | N.D.   |       | 3.33 ± 0.3 | N.D.   |
| PO      | 24.62 ± 1.73            | (0.11, 0.36, N.D.) | 17.44 ± 1.38* | 0.21 ± 0.03 |
| GPA     | 1.74 ± 0.10             | (0.012, 0.023, N.D.) | 0.28 ± 0.04 | N.D.   |
| AOG     | 3.36 ± 0.41             | (0.014, N.D., N.D.) | 1.31 ± 0.09 | N.D.   |
| SOG     | 2.48 ± 0.28             | N.D.   |       | 1.24 ± 0.12 | N.D.   |
| POG     | 14.03 ± 1.41            | (0.019, N.D., N.D.) | 2.54 ± 0.15 | N.D.   |
| GPO     | 1.07 ± 0.20*            | 0.04 ± 0.02 | 0.62 ± 0.06* | 0.050 ± 0.007 |
| OGP     | 0.12 ± 0.03*            | 0.0019 ± 0.0005 | 0.059 ± 0.009 | (0.001, 0.002, N.D.) |
| LOG     | 1.15 ± 0.23             | N.D.   |       | 0.21 ± 0.02 | (0.001, N.D., N.D.) |
| EOG     | 3.23 ± 0.45             | (0.013, 0.038, N.D.) | 1.99 ± 0.23* | 0.026 ± 0.003 |

Total  73.73 ± 4.08* | 0.39 ± 0.20 | 43.54 ± 3.24* | 0.40 ± 0.07 |

B

| Peptide | Concentration (pmol/mL) | Intestinal perfusate | Hepatic perfusate |
|---------|-------------------------|----------------------|-------------------|
|         |                         | CH      | Water | CH    | Water |
| POGP    | 631.9 ± 157.3           | N.D.   |       | 115.0 ± 8.3 | N.D.   |
| OGPO    | 15.1 ± 1.4              | (0.96, N.D., N.D.) | 9.0 ± 1.8 | N.D.   |
| GETGPAGROG | 170.2 ± 33.3* | 0.50 ± 0.17 | 78.2 ± 3.5* | 0.46 ± 0.10 |
| DGEAGKPGRGEO | 107.0 ± 20.2 | N.D.   | 42.3 ± 2.5 | N.D.   |
| GEAGPAGPAGPR | 485.1 ± 121.7 | N.D.   | 191.0 ± 50.2 | N.D.   |

Total  1409.3 ± 322.3* | 0.50 ± 0.17 | 487.0 ± 67.6* | 0.46 ± 0.10 |

The amount of each peptide was determined by calculating the peak area of the specific fragment ion on the LC-MS/MS chromatogram with or without synthetic peptide as internal standard. Table A represents the amount of di- and tripeptides. Table B represents the amount of the peptides larger than tripeptide. Each value is expressed as the mean ± SEM (n = 3). In some samples, peaks of fragment ion fell below the fixed threshold (detection limit), which were expressed by N. D. In samples including N. D., all quantitation data was shown.

important functions related to the metabolism of HCPs, as clearly indicated by the decrease of peptide concentration in hepatic perfusate (Fig. 2, Table 2). However, all peptides were reliably detected in hepatic perfusate, indicating that significant amount of HCPs could pass through the liver, finally entering the systemic circulation. In contrast, most of Hyp-containing materials was free Hyp in carotid artery serum prepared 2 h after oral administration of CH (Fig. 2D). Thus, serum proteases could have functions in degrading HCPs during circulation. In order to analyze the effect of serum peptidases on HCPs, synthetic peptides were treated with serum for 30 min, then the amount of remaining peptides was determined by LC-MS/MS analysis. As shown in Table 4, many HCPs were highly resistant to serum peptidases. The remaining ratios of thirteen HCPs have exceeded 50%, in marked contrast to the peptides that do not contain Hyp, whose remaining ratio was less than 40%. The exact position of Hyp in the peptide sequences appeared to be irrelevant to their resistance toward serum peptidases. Exceptionally, prolyl-hydroxyprolyl-glycyl-proline (POGP) was extremely susceptible to the digestion, indicating specific turnover of POGP. DGEAGKPGRGEO and GETGPAGROG were strikingly highly resistant to serum peptidases, as indicated by remaining ratio of 71% and 36%, respectively. These results suggested that HCPs larger than tripeptide would reach certain organs other than the liver, via systemic circulatory system.

DISCUSSION

Our study provides novel insights into how CH is digested, absorbed and transported to systemic cir-
In situ intestinal perfusion analysis revealed that CH was absorbed predominantly as peptides. The ratio of HCPs to free Hyp was much higher than previously reported (10). The liver played an important role in the metabolism of HCPs, but it became clear that significant amount of HCPs could pass through the liver and reach the systemic circulation. The present biochemical analysis revealed that intestinal perfusates contained a large number of HCPs with sizes larger than tripeptide. The analysis of portal vein serum revealed that some of these larger peptides are actually transported to the vascular network in vivo after oral administration of CH. In addition, many HCPs were shown to be highly resistant to the digestion by serum peptidases. Taken together, our results suggest that far more kinds of CH-derived peptides are absorbed in intestinal tract than conventionally assumed and could reach several tissues by the systemic circulation.

Our in situ perfusion strategy involved profiling of the peptides transported from intestinal tract to portal vein, and then carrying out secondary profiling of the peptides finally entering the systemic circulation. The advantage of the perfusion method is to use artificial blood. As a result, endogenous circulating peptides and proteolytic enzymes are suppressed to an extremely low content, leading to the detection of absorbed peptides with high sensitivity. The fact that the peptide GEAGPAGPAGPAGPR was validated as absorbed peptide in vivo illustrates the utility of increasing the detection sensitivity by filtering out contaminants and factors affecting the stability of CH-derived peptides during circulation.

An unexpected finding was that HCPs larger than tripeptides could be transported from intestinal tract to portal vein. To our knowledge, 30 kinds of di- and tri-peptides were identified in blood after oral administration of CH (9, 19, 22, 25, 26, 31, 34). Thus, these results are in good agreement with the general understanding about intestinal absorption of food-derived peptides. Practically, the absorption of peptides with sizes larger than tripeptide is rarely

| Peptide | Concentration (nmol/mL) | Portal vein blood | Carotid artery blood |
|---------|-------------------------|-------------------|---------------------|
| GP      | 3.19 ± 0.25*            | 0.12 ± 0.01       | 0.43 ± 0.04*        |
| OG      | 11.52 ± 1.28            | N.D.              | 1.17 ± 0.07         |
| AO      | 2.03 ± 0.17*            | 0.09 ± 0.01       | 0.32 ± 0.05*        |
| SO      | 2.72 ± 0.30*            | 0.15 ± 0.02       | 0.79 ± 0.13*        |
| PO      | 37.95 ± 3.56*           | 2.31 ± 0.18       | 16.73 ± 0.52*       |
| GPA     | 0.56 ± 0.08*            | 0.02 ± 0.01       | 0.0107 ± 0.0004     |
| AOG     | 8.27 ± 1.81*            | 0.15 ± 0.01       | 0.37 ± 0.03*        |
| SOG     | 6.79 ± 0.44*            | 0.19 ± 0.01       | 1.09 ± 0.15*        |
| POG     | 18.72 ± 2.68*           | 0.34 ± 0.03       | 1.04 ± 0.09*        |
| GPO     | 1.49 ± 0.05*            | 0.23 ± 0.01       | 0.46 ± 0.03         |
| OGP     | 0.0124 ± 0.0003*        | 0.0040 ± 0.0002   | 0.0094 ± 0.0010     |
| LOG     | 3.07 ± 0.48*            | 0.04 ± 0.01       | 0.08 ± 0.01         |
| EOG     | 8.79 ± 2.07*            | 0.34 ± 0.03       | 1.96 ± 0.19*        |
| Total   | 105.11 ± 8.29*          | 3.98 ± 0.32       | 24.47 ± 0.95*       |

| Peptide | Concentration (fmol/mL) | Portal vein blood | Carotid artery blood |
|---------|-------------------------|-------------------|---------------------|
| POGP    | 7393 ± 1149             | 4988 ± 260        | 8875 ± 514          |
| OGPO    | 2098 ± 576              | 1490 ± 97         | 2159 ± 63           |
| GETGPAGROG | 105.2 ± 28.2     | 72.5 ± 8.6        | N.D.               |
| DGEAGKPGROGE | 49.5 ± 5.7     | (20.3, N.D., N.D.) | N.D.               |
| GEAGPAGPAGPR | 75.0 ± 11.7* | 9.4 ± 6.0         | N.D.               |
| Total   | 9720 ± 1649             | 6567 ± 218        | 11034 ± 570         |

The amount of each peptide and the method of evaluation was described in Table 2. Table A represents the amount of di- and tri-peptides. Table B represents the amount of the peptides larger than tripeptide.
Absorption of collagen peptide

have demonstrated that the analytical methods, as well as physical conditions of the intestinal cells, could largely affect the intestinal transport of PO. Together, the mechanisms for PO transport have variously ascribed to intracellular or intercellular transport systems, suggesting the requirement of careful evaluation of transport mechanisms of PO, as well as other HCPs. Currently, the molecular mechanism of transport of HCPs larger than tripeptide is absolutely unknown. The intestinal absorption of larger HCPs is assumed to be due to passive diffusion in the cell gap junction, based on the previous studies reporting the transport of specific peptides and proteins. Detailed mechanisms of intestinal transport of larger HCPs should be analyzed in future studies, which could shed some light into the molecular mechanisms of peptide hormone delivery or food allergy. Together, this study could provide additive information about more common diseases or physiological phenomena related to intestinal absorption of peptides.

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Table 4  *The stability of CH-derived peptides toward serum peptidases*

| Remaining ratio (%) | Hyp containing peptides | Non Hyp containing peptides |
|--------------------|------------------------|-----------------------------|
| Peptide            | Residual ratio (%)     | Peptide                     | Residual ratio (%)     |
| AOG                | 89.8 ± 4.5             | GPO                         | 87.1 ± 2.7             |
| OGPO               | 87.1 ± 1.8             | OG                          | 86.7 ± 1.8             |
| OG                 | 86.0 ± 1.8             | (POG)₅                      | 86.0 ± 1.8             |
| 100–70             |                        | GPO                         | 79.9 ± 28.4            |
| POG                | 84.1 ± 1.1             | GPO                         | 73.2 ± 4.6             |
| PO                 | 81.3 ± 2.1             | SO                          | 71.0 ± 7.9             |
| DGEAGKPGROGE       | 71.0 ± 2.1             | GGG                         | 69.7 ± 31.2            |
| 70–30              |                        | GPA                         | 34.3 ± 2.9             |
| LOG                | 59.0 ± 4.2             | GEAGPAGPAGPGPR              | 12.3 ± 1.4             |
| AO                 | 41.8 ± 3.6             | GP                          | 1.59 ± 0.40            |
| GETGPAGROG         | 35.7 ± 1.5             | (PPG)₅                      | 0.99 ± 0.14            |
| 30–0               |                        | POGP                        | 0.11 ± 0.07            |
| GETGPAGPAG         | 12.3 ± 1.4             | (PPG)₅                      | 0.99 ± 0.14            |
| GPA                | 34.3 ± 2.9             | LGG                         | 0.04 ± 0.02            |

Percentage of intact peptides remained after 2 h incubation with serum peptidases was determined by LC-MS/MS as described in Materials and Methods. Each value is expressed as the mean ± SEM (n = 3).
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Absorption of collagen peptide

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SUPPLEMENTAL METHOD

Analysis of the perfusate by RP-HPLC and MALDI-TOF MS/MS. The intestinal perfusate treated with ethanol was dried under vacuum and dissolved in water. Then, the sample was applied to reversed-phase (RP)-HPLC. RP-HPLC was carried out by using TSK gel ODS-80 column (4.6 mm x 25 cm, 5 μm, Tosoh, Tokyo, Japan). Elution was performed with 0.1% TFA for 10 min followed by a linear gradient to 40% acetonitrile in the presence of 0.1% TFA over 80 min. The flow rate and monitoring wavelength were adjusted to 0.5 mL/min and 215 nm, respectively. Fractions were collected every 2 min and concentrated under vacuum to obtain partially purified peptide samples with as high concentration as possible.

One μL of 50% acetonitrile solution containing 0.1% TFA was added to each sample. Then the sample was spotted to sample plate, which was overlaid by a matrix solution (CHCA: α-Cyano-4-hydroxycinnamic acid) saturated in 50% acetonitrile solution containing 0.1% TFA. Finally, the sample spot was dried in air for the MALDI-TOF MS/MS (Matrix Assisted Laser Desorption/Ionization – time of flight tandem mass spectrometry) analysis. All mass spectra were acquired with an Autoflex TOF/TOF mass spectrometer (Bruker Daltonics, Billerica, MA, USA) equipped with a 337-nm pulsed nitrogen laser. The ion was accelerated at 2 kV for TOF/TOF measurements. MS/MS experiments were carried out using argon as the collision gas. Amino acid sequences were determined by de novo peptide sequencing. Peptides identified by this analysis were described in Table 1 with elution times. Identified sequences were confirmed as the sequences in porcine type I collagen by using the public data base (National Center for Biotechnology Information Reference Sequence: XM_021067153.1 for collagen type I alpha 1 and NM_001243655.1 for collagen type I, alpha 2).

Fig. S1 Peptide sequencing of CH-derived peptides larger than tripeptides. Tandem mass spectra of CH-derived peptides in intestinal perfusate were obtained by MALDI-TOF MS/MS analysis. A, B and C represent the product ion spectrums for the peptides of m/z 913.4, 1184.5 and 1260.6, respectively.