The presence of food-derived collagen peptides in human body-structure and biological activity

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It has been demonstrated that the ingestion of some protein hydrolysates exerts health-promoting effects. For understanding the underlying mechanisms responsible for these effects, the identification of bioactive peptides in the target organ is crucial. For this purpose, in vitro activity-guided fractionation for peptides in the protein hydrolysate has been performed. However, the peptides in the hydrolysate may be further degraded during digestion. The concentration of the active peptides, which were identified by in vitro activity-guided fractionation, in human blood is frequently very low (nanomolar levels). In contrast, micromolar levels of food-derived collagen peptides are present in human blood. Pro-Hyp, one of the major food-derived collagen peptides, enhances the growth of fibroblasts and synthesis of hyaluronic acid. These observations partially explain the beneficial effects of collagen hydrolysate ingestion on the enhancement of wound healing and improvement in the skin condition. The recent advancement involving liquid chromatography and mass spectrometry coupled with a pre-column derivatization technique has enabled the identification of food-derived peptides at nanomolar levels in the body post-ingestion of protein hydrolysates. Thus, this technique can be used for the identification of bioactive food-derived peptides in the body.

Introduction

Yoshikawa and his coworkers, pioneers in the field of functional food science, have demonstrated that the ingestion of enzymatic hydrolysates of protein containing numerous peptides can moderate hypertension not only in animal models but also in humans. In addition to the moderation of mild hypertension, numerous beneficial properties of protein hydrolysates upon their oral administration have been reported. Protein hydrolysates with beneficial activities can be produced via the enzymatic hydrolysis of the underutilized proteins. Sufficient amounts of starting materials are available for the production of protein hydrolysates for food ingredients. A variety of protein hydrolysates have been prepared commercially in Japan and some of them have been approved by the Japanese government as Food for Specified Health Uses (FOSHU) in lieu of their health-promoting effects.

Identification of the active peptide is critical for understanding its underlying mechanism of action and controlling the quality of the protein hydrolysate. It is a challenging task to identify the active peptide from a highly complex mixture of peptides. Conventional, in vitro activity-guided fractionation, based on enzyme reactions and cell culture systems, has been used to identify the active peptide from a protein hydrolysate. However, the peptides in the hydrolysate may be further degraded into smaller peptides and amino acids by carboxypeptidases and amino peptidases in the digestive tract and blood. In fact, serum amino acid level increases after the ingestion of enzymatic hydrolysates of food proteins, which indicates that most peptides are degraded into amino acids during the digestion and absorption processes. Furthermore, it has been demonstrated that the concentration of some “bio-active peptides” in the plasma, which were identified using conventional in vitro activity-guided fractionation, was considerably lower (nM level) than that necessary for exerting their biological activities in vitro (μM–mM levels). Thus, their proposed mechanism of action is highly questionable. To address this problem, the present author and coworkers have proposed to first identify food-derived peptides present in the blood or target organs for the assay of bioactivities. However, blood and organs generally contain a number of biological compounds. When the deproteinized fraction of human blood was directly injected into reversed phase-high performance liquid chromatography (RP-HPLC), a powerful HPLC separation mode for peptide fractionation, several large peaks were observed especially in the non-adsorbed fractions (Fig. 1A). It is nearly impossible to detect food-derived peptides in such a highly complex matrix by using direct RP-HPLC analysis. To facilitate the detection of food-derived peptides in such a
complex matrix, we have used sub-fractionation and precolumn derivatization techniques. By using these techniques, we have successfully identified some food-derived peptides in human blood. The present review highlights the bioactive peptides detected in human blood post-ingestion of collagen and other protein hydrolysates. A representative example of the health-promoting biological activities of these food-derived peptides in the body has been illustrated by elaborating the beneficial effects of collagen hydrolysate ingestion.

Identification of the food-derived collagen peptides in human blood

In the past few decades, several studies have suggested that the ingestion of collagen hydrolysate may improve skin and joint conditions. However, most of the peptides present in the collagen hydrolysate were believed to be degraded into amino acids during the digestion and absorption processes. Thus, the potential beneficial effects of collagen hydrolysate ingestion were considered to be questionable. In contrast, recent human clinical trials using a placebo control have demonstrated that the ingestion of collagen hydrolysate improves the objective and subjective skin conditions and enhances the healing of pressure ulcers. These observations suggested the presence of food-derived bioactive peptides in the body post-ingestion of collagen hydrolysate. Since collagen specifically consists of hydroxyproline (Hyp), Hyp-containing peptides can be considered to be collagen-derived peptides. As shown in Fig. 2, the collagen-derived peptide content in human plasma increased to approximately 20 μM 60 min post-ingestion of the collagen hydrolysate (10 g) and remained high at 180 min post-ingestion. This finding clearly indicated the presence of food-derived collagen peptides in human blood. Furthermore, these peptides were present at a concentration 10,000 times higher compared to the previously reported values (nM).

To isolate the food-derived collagen peptides, firstly, the oligo-peptide fraction was obtained by performing size exclusion chromatography (SEC). The peptides in the oligo-peptide fraction were resolved using RP-HPLC. As shown in Fig. 1B, in this case, fewer peaks were observed compared to those observed after direct injection of the whole deproteinized plasma fraction (Fig. 1A). All peaks were subjected to...
sequence analysis using Edman degradation. While some peaks did not display any peptide sequence, a few Hyp-containing peptides were identified as shown in Fig. 1B.\textsuperscript{11} However, resolution of the short-chain peptides, especially the peptides that were weakly absorbed onto the RP-HPLC column, was not satisfactory even after subfractionation via SEC. To improve the resolution and detection of such peptides, a pre-column labeling technique using phenyl isothiocyanate (PITC) was employed (Fig. 3, top panel).\textsuperscript{6} The peptide derivatives labeled with PITC were strongly retained on the RP-HPLC column due to the presence of a hydrophobic moiety in PITC. These peptides could be specifically detected by monitoring the absorbance at 254 nm.\textsuperscript{6} In addition, since the phenyl thiocarbamoyl (PTC)-peptide is an intermediate product of the Edman degradation reaction, the sequence of these peptides can be directly analyzed. The peptides present in the SEC fractions of human plasma were collected before and 60 min post-ingestion of the collagen hydrolysate, derivatized with PITC, and resolved using RP-HPLC. As shown in Fig. 3 (bottom panel), better peptide resolution was obtained after derivatization compared to that without derivatization (Fig. 1B).\textsuperscript{12} The areas of peaks marked 1, 2, and 3, and some minor peaks increased after ingestion. Sequence analysis revealed that peaks 1, 2, and 3 corresponded to Pro-Hyp, Hyp-Gly, and free Hyp, respectively.\textsuperscript{12} Other collagen peptides present in minor amounts in human blood were also identified using this technique.\textsuperscript{13} The identity of these peptides was confirmed using liquid chromatography tandem mass spectrometry (LC-MS/MS) in the multi-reaction monitoring (MRM) mode.\textsuperscript{14,15} In all samples, Pro-Hyp was observed to be present most abundantly. Collagen peptides containing more than three amino acids (tripeptides) were not significantly observed in human blood. It has been demonstrated that Pro-Hyp and Hyp-Gly show resistance to blood peptidase.\textsuperscript{11,12} The Gly-Pro-Hyp-Gly motif is abundantly present in type I collagens. In addition, collagen hydrolysate has a less bitter taste compared to other hydrolysates. Then, relatively high doses (2.5–10 g) of collagen hydrolysate are consumed as the supplement. These facts can at least partially explain why Pro-Hyp and Hyp-Gly present abundantly in human plasma after the ingestion of collagen hydrolysate compared to other peptides after the ingestion of non-collagen hydrolysates.

The mechanism for the intestinal absorption of collagen peptides has been examined by in vitro assays. Pro-Hyp is incorporated into porcine brush-border membrane vesicles in a pH dependent manner, suggesting that Pro-Hyp passes through proton coupling peptide transporters such as peptide transporter 1 on enterocytes.\textsuperscript{16} Furthermore, it has been demonstrated that collagen peptides larger than Pro-Hyp such as Gly-Ala-Hyp-Gly-Leu-Hyp-Gly-Pro pass through the Caco-2 cell monolayer via the paracellular pathway.\textsuperscript{17} However, such
larger peptides have not been found in blood after the ingestion of collagen hydrolysate. Then, data obtained by the Caco-2 cell layer system cannot be directly used to predict peptide bioavailability. The resistance of peptides to peptidases in cells and blood should also be considered along with the results obtained by the Caco-2 assay.

Pro-Hyp, Hyp-Gly, and other minor collagen peptides are potentially generated by the degradation of endogenous collagen. In fact, it has been demonstrated that Pro-Hyp is also present in mice ears showing inflammation18 and in skin wound healing sites19 without ingestion of the collagen hydrolysate.

Biological activities of the food-derived collagen peptides

It has been demonstrated that Pro-Hyp is generated by the degradation of endogenous collagen and post-ingestion of the collagen hydrolysate. The next question was whether Pro-Hyp is an inactive metabolite or a bioactive peptide that indicates degradation of the extracellular matrix. Since fibroblasts are mainly responsible for the secretion of extracellular matrix components and play a significant role in wound healing, the effects of Pro-Hyp on the growth of fibroblasts were examined. As shown in Fig. 4A, fibroblasts displayed rapid growth on plastic plates in the presence of fetal bovine serum (FBS), which is rich in growth factors. However, the growth of fibroblasts on collagen gels was inhibited even in the presence of FBS, which mimicked the fibroblasts in the body.20 However, the addition of Pro-Hyp (200 μM) into the medium triggered the growth of fibroblasts on collagen gels; however, free Pro and Hyp displayed no such effect (Fig. 4B).21 As shown in Fig. 5, Pro-Hyp increased the growth rate of fibroblasts on collagen gels in a dose dependent manner.21 It has been demonstrated that Hyp-Gly, the second major food-derived collagen peptide in human blood, also enhances the growth of fibroblasts on collagen.12 However, even in the presence of Pro-Hyp, the fibroblasts did not grow, when FBS was absent. These observations indicated that the growth of fibroblasts is controlled by growth factors, attachment onto the extracellular matrix, and the presence of the collagen dipeptides (such as Pro-Hyp and Hyp-Gly). This clearly indicates that these peptides are important bioactive peptides rather than inactive metabolites. Recently, we observed that the direct application of Pro-Hyp in the damaged tissues enhances cutaneous wound healing in mice. Thus, it can be inferred that both food-derived and endogenous Pro-Hyp triggers wound healing by stimulating fibroblast growth. This also partially explains the beneficial effects of collagen hydrolysate ingestion in patients suffering from pressure ulcers.9,10 In addition, it has been demonstrated that Pro-Hyp increases hyaluronic acid production in fibroblasts22 and decreases the diameter of lipid droplets in adipocytes.23 Thus, Pro-Hyp demonstrates multiple functions. These functions may be associated with the other beneficial effects upon collagen hydrolysate ingestion. Due to the presence and function of food-derived and endogenous Pro-Hyp in the body, the health-promoting effects of collagen hydrolysate are being actively explored.

Food-derived peptides post-ingestion of other protein hydrolysates

The concentration of food-derived collagen peptides in human blood is considerably higher compared to the previously
reported values. These collagen-derived peptides consist of the post-translationally modified amino acid hydroxyproline (Hyp). This suggested that the presence of post-translationally modified amino acids in peptides might be responsible for their elevated concentrations in human plasma. However, as shown in Fig. 6, the micromolar levels of Pro-Gly, which consists of unmodified amino acids, were present in human plasma after ingestion of 10 g of elastin hydrolysate.24 Pro-Gly increases the production of elastin by fibroblasts.24 These observations suggest that higher levels of food-derived bioactive peptides may be potentially present in the body upon ingestion of other food protein hydrolysates compared to the previously hypothesized levels.5 For the identification of food-derived peptides in the body, subfractionation and pre-column labeling techniques are useful. As mentioned above, derivatization of the peptides with PITC provides better resolution and specific detection of these peptides using RP-HPLC. Additionally, the derivatized peptides (PTC-peptides) can be sequenced using the Edman degradation reaction. However, the PTC-peptides are weakly ionized using electron spray ionization-mass spectrometry (ESI-MS). Thus, it is difficult to identify the PTC-peptides using ESI-LC-MS/MS. Alternatively, the peptides derivatized using another reagent, namely 6-aminoquinolyl-N-hydroxysuccinimidyl-carbamate (AQC) can be detected at nanomolar levels and sequenced using LC-MS/MS. As shown in Fig. 7, Val-Ala was identified in human blood 30 min post-ingestion of the globulin hydrolysate.13

**Conclusion**

Identification of the bioactive peptide, which exerts beneficial effects on the target organ, is necessary for understanding its mechanism of action. Conventionally, the bioavailability of the peptides identified in protein hydrolysates and displaying *in vitro* bioactivity has been examined. However, performing isolation and activity assays for all peptides present in a protein hydrolysate is tedious and time-consuming. In addition, the bioavailability of peptides displaying bioactivity *in vitro* is frequently low (in nanomolar levels in blood). The present author and coworkers have proposed an alternate approach involving the evaluation of bioactivities of food-derived peptides in the body (blood and organs). Recent advances in HPLC and LC-MS/MS coupled with pre-column derivatization techniques...
enable the identification of food-derived peptides in the body. It has been observed that very few numbers but highly specific food-derived peptides are present in the body. Thus, the biological activities of food-derived peptides in the body can be assayed much more easily compared to the activity of those present in a protein hydrolysate. If the food-derived peptides present in the body display some bioactivities on target cells, this outcome can be associated with the biological response upon ingestion of the protein hydrolysate.

Conflicts of interest
The author declares no competing financial interest.

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References