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Effect of Co-Ingestion of Collagen Peptides with Yogurt on Blood Absorption of Short Chain Hydroxyproline Peptides

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Abstract: Collagen peptides (CP) have been used as functional foods for enhancing skin and joint health. Further degradation of CP results in peptide sizes small enough to enter the bloodstream following absorption in the small intestine. We examined the effects of food matrices on CP degradation into short chain peptides and absorption efficiency after ingestion. Changes to hydroxyproline (Hyp)-containing peptide levels in CP after yogurt fermentation and in human plasma by co-ingestion of CP and yogurt, with or without fermentation, were evaluated by liquid chromatography-mass spectrometry (LC-MS). The fermentation of CP with yogurt resulted in the significant degradation of CP into several Hyp-containing peptides such as Ala-Hyp, Leu-Hyp, Phe-Hyp, Ala-Hyp-Gly, and Leu-Hyp-Gly. CP ingestion after yogurt fermentation significantly increased the plasma concentrations of Phe-Hyp, cyclo(Ala-Hyp), and cyclo(Pro-Hyp) compared to water-based CP ingestion. The co-ingestion of CP and yogurt without fermentation significantly increased the plasma levels of Ala-Hyp, Phe-Hyp, Ala-Hyp-Gly, Leu-Hyp-Gly, Pro-Hyp-Gly, cyclo(Ala-Hyp), cyclo(Glu-Hyp), and cyclo(Pro-Hyp). Overall, the co-ingestion of CP and yogurt with or without fermentation significantly enhanced the absorption of CP-derived peptides, represented by the high C_{max} and area under the curve per 1 h (AUC, nmol/h·mL) of Hyp-containing peptides. These results suggest that, in addition to increasing short chain Hyp-containing peptide levels via fermentation, yogurt matrices containing milk-derived peptides and/or lactic acid bacteria-derived peptidases may influence the efficient absorption of CP-derived peptides into human blood.

Keywords: collagen peptides; hydroxyproline-containing peptides; yogurt fermentation; lactic acid bacteria; human plasma

1. Introduction

Collagen is a major protein of the extracellular matrix in various connective tissues. Thermal and enzymatic decomposition products of collagen are referred to as collagen peptides (CP), which have been used as functional foods to improve the health of human skin and joints. Some human trials have demonstrated that oral ingestion of CP has beneficial effects on human health, such as relieving osteoarthritis-associated pain [1,2], improving skin condition in pressure ulcers [3,4], and increasing muscle strength in sarcopenic patients [5]. Iwai et al. and Ohara et al. [6,7] have reported increased levels of hydroxyproline (Hyp)-containing peptides, such as Pro-Hyp, Ile-Hyp, Leu-Hyp, Ala-Hyp-Gly,

Pro-Hyp-Gly, and Ser-Hyp-Gly, in human plasma after 1–2 h of ingesting 10–20 g of CP. In addition, these studies identified Pro-Hyp as a major component among Hyp-containing peptides, reaching micromolar levels in human plasma after the ingestion of CP. The concentration of food-derived CP in human blood is significantly higher than that of other peptides which are absorbed after the ingestion of foods such as sardines [8] and milk [9]. Cell culture studies have demonstrated that Pro-Hyp enhances the proliferation of mouse dermal fibroblasts [10] and the synthesis of hyaluronic acid by human dermal fibroblasts [11] and inhibits the calcification of mouse chondrocytes [12]. We have previously reported the detection of seven types of Hyp-containing cyclic dipeptides in human plasma after CP ingestion [13,14]. We showed that cyclo(Pro-Hyp), a major component of food-derived cyclic peptides, was more effective at enhancing the growth rate of mouse skin fibroblasts than Pro-Hyp [13].

Based on these *in vivo* and *in vitro* studies, linear or cyclic Hyp-containing di- and tripeptides are expected to accumulate in the bloodstream after the ingestion of CP and subsequently regulate the functions of target cells in the skin and joint tissues. Food-derived peptides are absorbed through peptide transporters such as Pept1, an exclusive di- and tripeptide transporter expressed in the intestinal brush-border membrane [15,16]. However, in previous studies, micromolar levels of Hyp-containing peptides that consist of more than four amino acid residues have not been detected in human blood after the ingestion of CP [6,7]. The higher absorption of Hyp-containing di- or tripeptides by the ingestion of CP could promote joint, skin, and muscle health.

Food-derived di- or tripeptides detected in human blood are produced via the degradation of protein and long-chain peptides by gastrointestinal and food-derived enzymes during digestion and fermentation, respectively. The co-ingestion of food or beverage matrices with CP might affect peptide digestion in the gastrointestinal tract, thereby altering the production of small peptides and consequently influencing the absorption of the peptides into the blood. Previous studies have examined the absorption of food-derived collagen peptides in human blood after the ingestion of CP that had been dissolved in water. Walrand et al. have demonstrated that the co-ingestion of fermented milk and CP could increase the plasma concentration of collagen-specific amino acids, especially proline (Pro) [17]. They suggested that milk-derived peptides in fermented milk products might affect the absorption of collagen-derived amino acids in the small intestine. Therefore, it is expected that the co-ingestion of CP and yogurt might enhance the absorption of short chain peptides and amino acids in the blood.

It has been reported that lactic acid bacteria in cheese have proteolytic activities that are accomplished by their cell envelope proteases and intracellular peptidases [18]. A previous study showed that the ingestion of smaller CP cleaved by ginger proteases enhanced the absorption of Hyp-containing peptides in mouse blood after oral administration [19]. These studies suggest that food-derived enzymes can produce small peptides and enhance the absorption of food-derived peptides in human blood after ingestion. Therefore, the fermentation of CP with yogurt might increase the production of short chain Hyp-containing peptides and its ingestion could possibly enhance absorption of Hyp-containing di- and tripeptides in human blood. In this study, we examined the level of Hyp-containing di- and tripeptides in CP which had been prepared with or without yogurt fermentation. We also evaluated the concentration of Hyp-containing di- and tripeptides in human blood after the co-ingestion of CP with or without yogurt fermentation.

2. Materials and Methods

2.1. Chemicals

Collagen peptides (CP) derived from porcine skin were obtained from Q'SAI CO., LTD. Fukuoka, Japan. Yogurt containing *Lactobacillus delbrueckii subsp. Bulgaricus* 2038 and *Streptococcus thermophilus* 1131, as well as milk, were purchased from Meiji Co., Ltd. Tokyo, Japan. Both dairy products are commercially available and contain 3.4 g protein per 100 g or 100 mL.

A standard mixture of amino acids (Type H), Hyp, acetonitrile (liquid chromatography-mass spectrometry: LC-MS grade), trifluoroacetic acid (TFA), and formic acid (LC-MS grade) were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Pro-Hyp, Hyp-Gly, and Gly-Pro-Hyp were purchased from Bachem (Bubendorf, Switzerland), and other peptides were custom synthesized by AnyGen (Gwangju, Korea). All other reagents were of analytical grade or higher.

2.2. Preparation of CP Test Foods

“CP in yogurt” and “CP in water” were prepared by dissolving CP (10 g) in yogurt (50 g) and water (200 mL), respectively. “Yogurt-fermented CP” was prepared by adding 10 g of CP to a mixture of milk (45 g) and yogurt (5 g), followed by incubation at 40 °C overnight. The prepared CP samples that were used as test foods in the human study are shown in Table 1.

Table 1. The composition of collagen peptide (CP) test foods.

Food	Milk (g)	Yogurt (g)	Water (g)	Collagen Peptide (CP, g)	Incubation
Yogurt-fermented CP	45	5	0	10	○
CP in yogurt	0	50	0	10	×
CP in water	0	0	200	10	×

CP test foods prepared by adding CP in yogurt, water, or a mixture of milk and yogurt with incubation (○) and without incubation (×).

2.3. Analysis of Hyp-Containing Di- or Tripeptides in CP Test Foods

The CP test foods were mixed with distilled water and centrifuged at 3000 rpm for 10 min at 10 °C. The supernatants were diluted with 0.1 % formic acid, and the concentration of Hyp-containing di- or tripeptides was quantitated by LC-MS using a 3200 QTRAP hybrid triple quadrupole/linear ion trap mass spectrometer (AB Sciex, Foster City, CA, SUA) coupled to an Agilent 1200 Series HPLC system (Agilent Technologies, Palo Alto, CA, SUA). The Hyp-containing peptides were monitored in multiple reaction monitoring (MRM) mode with chromatographic separation using an Ascentis Express F5 HPLC column (5 µm, 250 mm × 4.6 mm, Supelco, Bellefonte, PA, SUA), as previously described [14]. The concentration of each peptide was determined using external calibration curves that were constructed using standard peptides.

2.4. Human Study Design

The human study was performed according to the guidelines proposed in the Helsinki Declaration, and it was approved by the Ethical Committee of Tokyo Kasei University (certificate number H29-20 Tokyo, Japan). No negative effects were reported for CP ingestion at 5–10 g/day for 12 weeks in human studies [20,21], and the safety of high-dose CP ingestion (1.66 g/kg body weight) was also confirmed by animal experiments [22]. Four healthy female volunteers (aged 20–24 years) were recruited after obtaining informed consent. After 12 h of fasting, the participants ingested the CP test foods. Venous blood was collected in heparin tubes from the cubital veins of the study subjects before and 1, 2, and 4 h after the ingestion. The human studies were conducted with a washout period of 1 week between each ingestion of CP. A schematic representation of the study design is presented in Figure 1.

Isolated plasma was diluted in ethanol (3× volume). Plasma proteins were removed by centrifugation at 3000 rpm for 10 min at 5 °C, and the supernatants were stored at −80 °C until analysis.

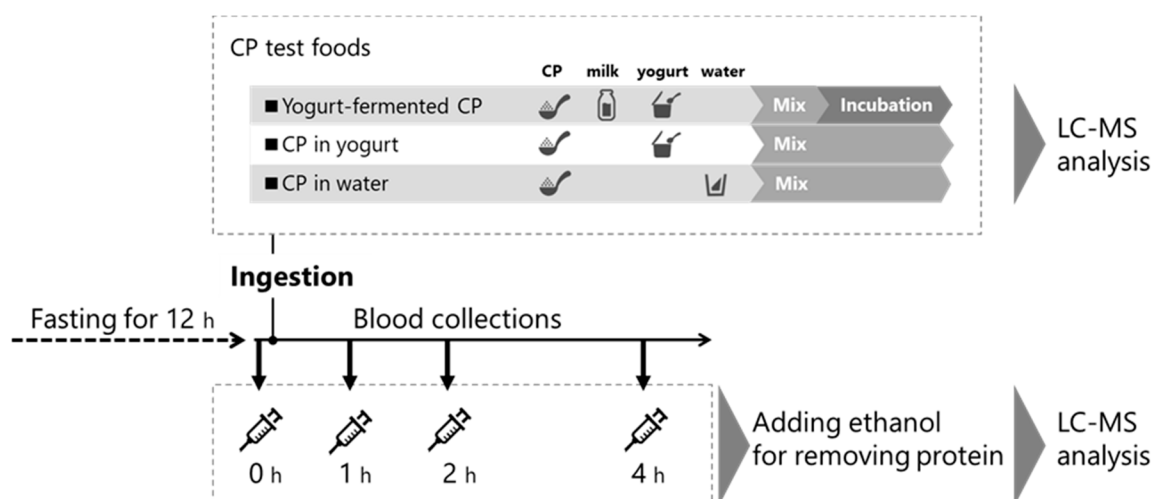


Figure 1. Schematic representation of the study design. The supernatant samples of CP test foods were analyzed by liquid chromatography-mass spectrometry (LC-MS). Plasma samples were collected before and 1, 2, and 4 h after ingestion of CP test foods. Ethanol-soluble plasma samples were analyzed by LC-MS.

2.5. Quantitation of Hyp and Hyp-Containing Di- or Tripeptides in Human Plasma

The concentration of Hyp-containing di- or tripeptides in the plasma of each subject was quantitated using a recently reported LC-MS method with a mixture of stable isotope-labeled internal standard peptides, called SI-oligo [14]. An aliquot of the ethanol-soluble fraction of plasma samples was mixed with SI-oligo and then dried using a CVE-3100 centrifugal evaporator (EYELA, Tokyo, Japan). The samples were reconstituted with 0.1 % formic acid, and the Hyp-containing peptides were analyzed by LC-MS in MRM mode, as described above. The concentration of each peptide was calculated based on the peak area ratio of non-labeled analytes relative to that of the corresponding stable isotopically labeled analytes derived from SI-oligo [14]. The area under the plasma concentration–time curve (AUC) was calculated using the trapezoidal rule.

2.6. Statistical Analysis

The differences between the means were evaluated by analysis of variance, followed by Fisher's protected least significant difference method using Excel-Toukei 2010 (Social Survey Research Information Co., Ltd. Tokyo, Japan).

3. Results

3.1. Detection of Di- and Tripeptides and Cyclic Peptides Derived from CP after Yogurt Fermentation

Peptide analysis using LC-MS revealed that the yogurt and the fermented mixture of milk and yogurt that were used for the preparation of the CP test foods contained negligible amounts of Hyp-containing peptides (data not shown). Therefore, we assumed that all Hyp-containing peptides were collagen-derived peptides. To evaluate the production of Hyp-containing di- and tripeptides and cyclic peptides from CP after yogurt fermentation, the three CP test foods were analyzed using LC-MS (once only) (Figure 2). The total concentration of Hyp-containing peptides in the CP test foods was relatively low compared to that of the CP that was previously prepared by extensive degradation with ginger protease [23]. The highest concentration of peptides detected in the CP test foods was 212.96 µg/g CP for cyclo(Ser-Hyp) from “CP in water,” and the lowest concentration was 0.60 µg/g CP for Pro-Hyp from “CP in yogurt.” Among the 13 linear Hyp-containing peptides tested, all the peptides, except Glu-Hyp-Gly, showed higher concentrations in “yogurt-fermented CP” compared to those in “CP in yogurt” and “CP in water.” In particular, Ala-Hyp, Leu-Hyp,

Phe-Hyp, Ala-Hyp-Gly, and Leu-Hyp-Gly showed a significant (eight-fold or higher) increase in “yogurt-fermented CP” compared to their levels in “CP in yogurt” and “CP in water.” In addition, concentrations of Glu-Hyp-Gly in “yogurt-fermented CP” and “CP in yogurt” were five-fold higher than those in “CP in water.” Moreover, “CP in yogurt” showed a modest (non-significant) increase in the concentrations of Leu-Hyp, Ala-Hyp-Gly, Leu-Hyp-Gly, and Phe-Hyp-Gly compared to those in “CP in water.” There was no considerable change in the concentration of Hyp-containing cyclic peptides in any of the test foods.

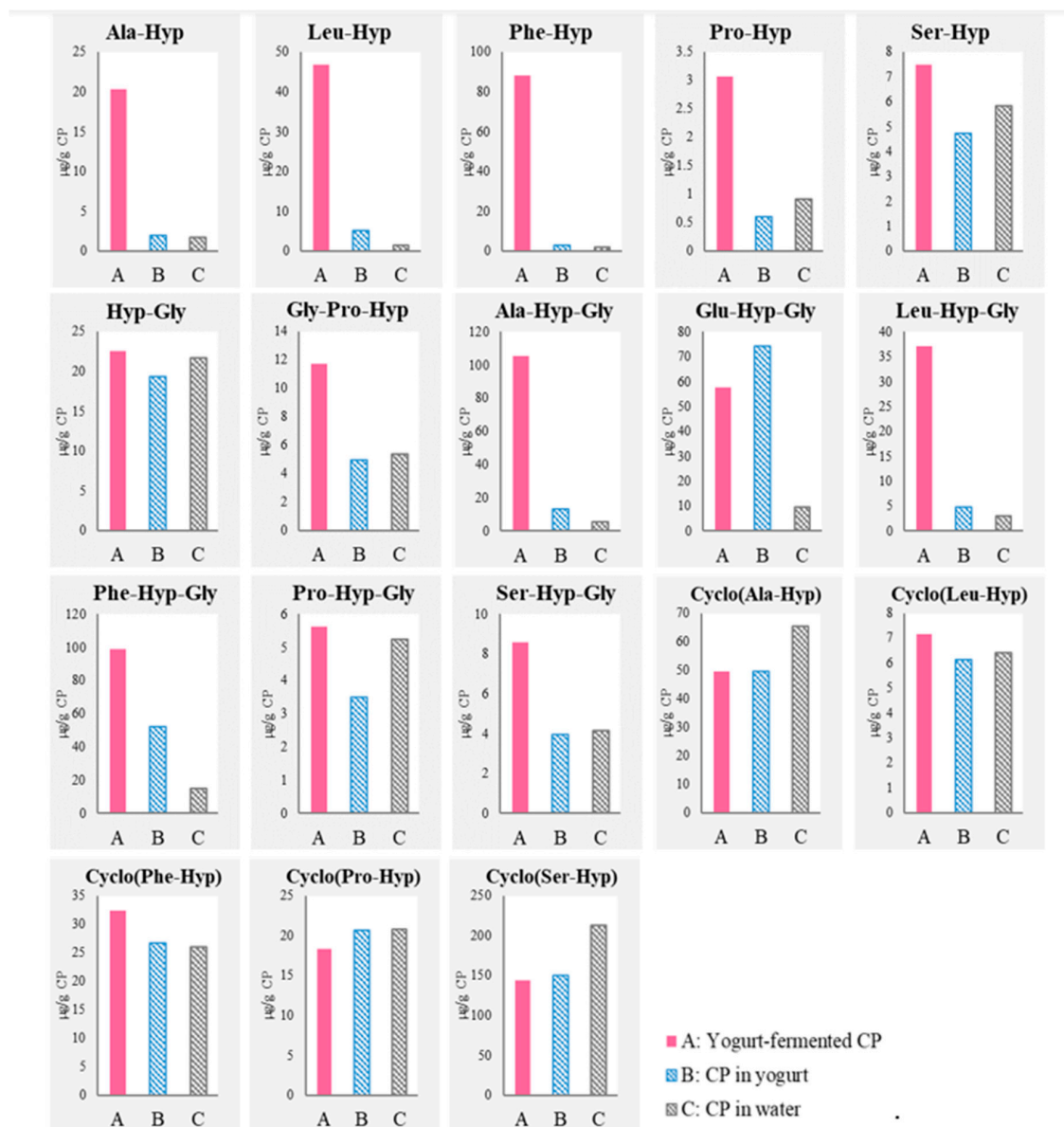


Figure 2. The concentration ($\mu\text{g/g CP}$) of Hyp-containing di- and tripeptides and cyclic peptides in the three CP test foods. Single measurement of the three CP test foods was conducted by LC-MS. The concentrations of Hyp-containing peptides are indicated as: “yogurt-fermented CP” (■), “CP in yogurt” (▨), and “CP in water” (▩).

3.2. Detection of Food-Derived Hyp-Containing Peptides in Human Blood after Ingestion of CP with or without Yogurt Fermentation

Figure 3 shows the average concentrations of Hyp and Hyp-containing peptides in the plasma of the four subjects after the ingestion of the test foods, “yogurt-fermented CP,” “CP in yogurt,” or “CP

in water.” Only negligible amounts of free Hyp and Hyp-containing peptides were observed in the plasma before the ingestion of the test foods. The concentrations of free Hyp and Hyp-containing peptides increased and reached their maximum levels 1–2 h after ingestion of each CP test food. The maximum concentrations ($C_{\max}$) of free Hyp were 68.15, 78.13, and 61.80 nmol/mL after ingestion of “yogurt-fermented CP,” “CP in yogurt,” and “CP in water,” respectively, and these levels were higher than those of Hyp-containing peptides (Table 2). Among the Hyp-containing peptides, the highest concentrations were observed for Pro-Hyp, which were 15.53, 20.86, and 16.36 nmol/mL after ingestion of “yogurt-fermented CP,” “CP in yogurt,” and “CP in water,” respectively, and there was no significant difference among their $C_{\max}$ values. Compared to “CP in water,” “yogurt-fermented CP” significantly increased the plasma $C_{\max}$ of Phe-Hyp, cyclo(Ala-Hyp), and cyclo(Pro-Hyp). Moreover, ingestion of “yogurt-fermented CP” did not result in a significant increase in the $C_{\max}$ of any peptide compared to that after ingestion of “CP in yogurt.” However, the ingestion of “CP in yogurt” significantly increased the $C_{\max}$ of eight types of Hyp-containing peptides, namely Ala-Hyp, Phe-Hyp, Ala-Hyp-Gly, Leu-Hyp-Gly, Pro-Hyp-Gly, cyclo(Ala-Hyp), cyclo(Glu-Hyp), and cyclo(Pro-Hyp), relative to their $C_{\max}$ values after the ingestion of “CP in water.” In addition, ingestion of “CP in yogurt” significantly increased the $C_{\max}$ of Pro-Hyp-Gly in the plasma relative to that observed after ingestion of “yogurt-fermented CP.”

Table 2. $C_{\max}$ (nmol/mL) and area under the curve per 1 h (AUC, nmol/h·mL) of free Hyp and Hyp-containing peptides after ingestion of three CP test foods.

Free and Peptide Form of Hyp	$C_{\max}$ (nmol/mL)			AUC (nmol/h·mL)		
	Yogurt-Fermented CP	CP in Yogurt	CP in Water	Yogurt-Fermented CP	CP in Yogurt	CP in Water
Hyp	68.15 ± 8.37	78.13 ± 19.39	61.80 ± 9.31	55.00 ± 6.82	51.54 ± 12.41	48.68 ± 8.95
Ala-Hyp	1.12 ± 0.29	1.60 ± 0.19 **	0.90 ± 0.36	0.69 ± 0.12	0.75 ± 0.12 *	0.56 ± 0.08
Glu-Hyp	1.06 ± 0.21	1.22 ± 0.44	0.98 ± 0.25	0.78 ± 0.10	0.81 ± 0.22	0.63 ± 0.21
Ile-Hyp	1.31 ± 0.32	1.42 ± 0.23	0.98 ± 0.26	0.52 ± 0.10	0.51 ± 0.85	0.42 ± 0.07
Leu-Hyp	2.88 ± 0.87	3.30 ± 0.67	2.35 ± 0.76	1.03 ± 0.27	1.09 ± 0.23	0.92 ± 0.24
Phe-Hyp	0.93 ± 0.15 *	1.01 ± 0.13 **	0.71 ± 0.08	0.37 ± 0.06	0.38 ± 0.04	0.32 ± 0.07
Pro-Hyp	15.53 ± 2.96	20.86 ± 7.70	16.36 ± 2.43	10.75 ± 1.45	12.47 ± 4.31	11.06 ± 3.10
Ser-Hyp	0.84 ± 0.23	1.02 ± 0.22	0.76 ± 0.02	0.57 ± 0.08	0.58 ± 0.11	0.51 ± 0.12
Hyp-Gly	1.77 ± 0.40	2.28 ± 0.59	1.56 ± 0.35	0.88 ± 0.16	1.01 ± 0.25	0.83 ± 0.09
Gly-Pro-Hyp	0.27 ± 0.14	0.39 ± 0.11	0.24 ± 0.14	0.18 ± 0.07	0.20 ± 0.06	0.14 ± 0.06
Ala-Hyp-Gly	1.29 ± 0.55	1.84 ± 0.31 *	0.90 ± 0.35	0.52 ± 0.18	0.62 ± 0.11 *	0.40 ± 0.04
Glu-Hyp-Gly	0.82 ± 0.30	1.14 ± 0.32	0.84 ± 0.45	0.50 ± 0.12	0.58 ± 0.13	0.47 ± 0.24
Leu-Hyp-Gly	0.11 ± 0.05	0.14 ± 0.09 *	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.02	0.02 ± 0.01
Phe-Hyp-Gly	0.025 ± 0.011	0.032 ± 0.020	0.011 ± 0.005	0.01 ± 0.003	0.01 ± 0.01	0.01 ± 0.002
Pro-Hyp-Gly	1.51 ± 0.41 †	2.26 ± 0.57 **	1.10 ± 0.40	0.64 ± 0.05 †	0.89 ± 0.18 **	0.54 ± 0.14
Ser-Hyp-Gly	1.00 ± 0.48	1.99 ± 0.47	2.19 ± 1.79	0.62 ± 0.32	0.85 ± 0.12	0.81 ± 0.45
Cyclo(Ala-Hyp)	0.13 ± 0.04 *	0.15 ± 0.02 **	0.09 ± 0.02	0.09 ± 0.01	0.09 ± 0.02	0.08 ± 0.01
Cyclo(Glu-Hyp)	0.046 ± 0.006	0.052 ± 0.007 *	0.031 ± 0.012	0.04 ± 0.004 **	0.03 ± 0.004 *	0.03 ± 0.01
Cyclo(Leu-Hyp)	0.05 ± 0.52	0.06 ± 0.02	0.04 ± 0.00	0.03 ± 0.003	0.03 ± 0.01	0.03 ± 0.004
Cyclo(Phe-Hyp)	0.030 ± 0.02	0.028 ± 0.002	0.027 ± 0.05	0.02 ± 0.002	0.02 ± 0.004	0.02 ± 0.002
Cyclo(Pro-Hyp)	2.69 ± 0.69 *	2.97 ± 0.97 **	0.09 ± 0.03	1.84 ± 0.28 **	1.76 ± 0.52 *	1.00 ± 0.21
Cyclo(Ser-Hyp)	0.11 ± 0.02	0.13 ± 0.03	0.11 ± 0.01	0.09 ± 0.01 *	0.08 ± 0.01	0.07 ± 0.02

Data are shown as mean ± SD (n = 4). * Significant increase compared with “CP in water” ($p < 0.05$). ** Significant increase compared with “CP in water” ($p < 0.01$). † Significant differences compared with “CP in yogurt” ($p < 0.05$). The values highlighted in bold showed significant differences. Among 21 types of Hyp-containing peptides, Pro-Hyp was the one detected in the highest concentration after ingestion of each CP test food.

Ingestion of “yogurt-fermented CP” significantly increased the area under the curve per 1 h (AUC (nmol/h·mL)) of cyclo(Glu-Hyp), cyclo(Pro-Hyp), and cyclo(Ser-Hyp) compared to that after ingestion of “CP in water.” Moreover, ingestion of “CP in yogurt” significantly increased the AUC of Ala-Hyp, Ala-Hyp-Gly, Pro-Hyp-Gly, cyclo(Glu-Hyp), and cyclo(Pro-Hyp) compared to “CP in water” (Table 2). The AUC of Pro-Hyp-Gly after ingestion of “CP in yogurt” was significantly higher than that after ingestion of “yogurt-fermented CP.” These results indicate that the CP test foods containing yogurt significantly enhanced the absorption of Hyp-containing peptides compared to ingestion of CP without yogurt, while “CP in yogurt” without fermentation most effectively increased the absorption of Hyp-containing peptides.

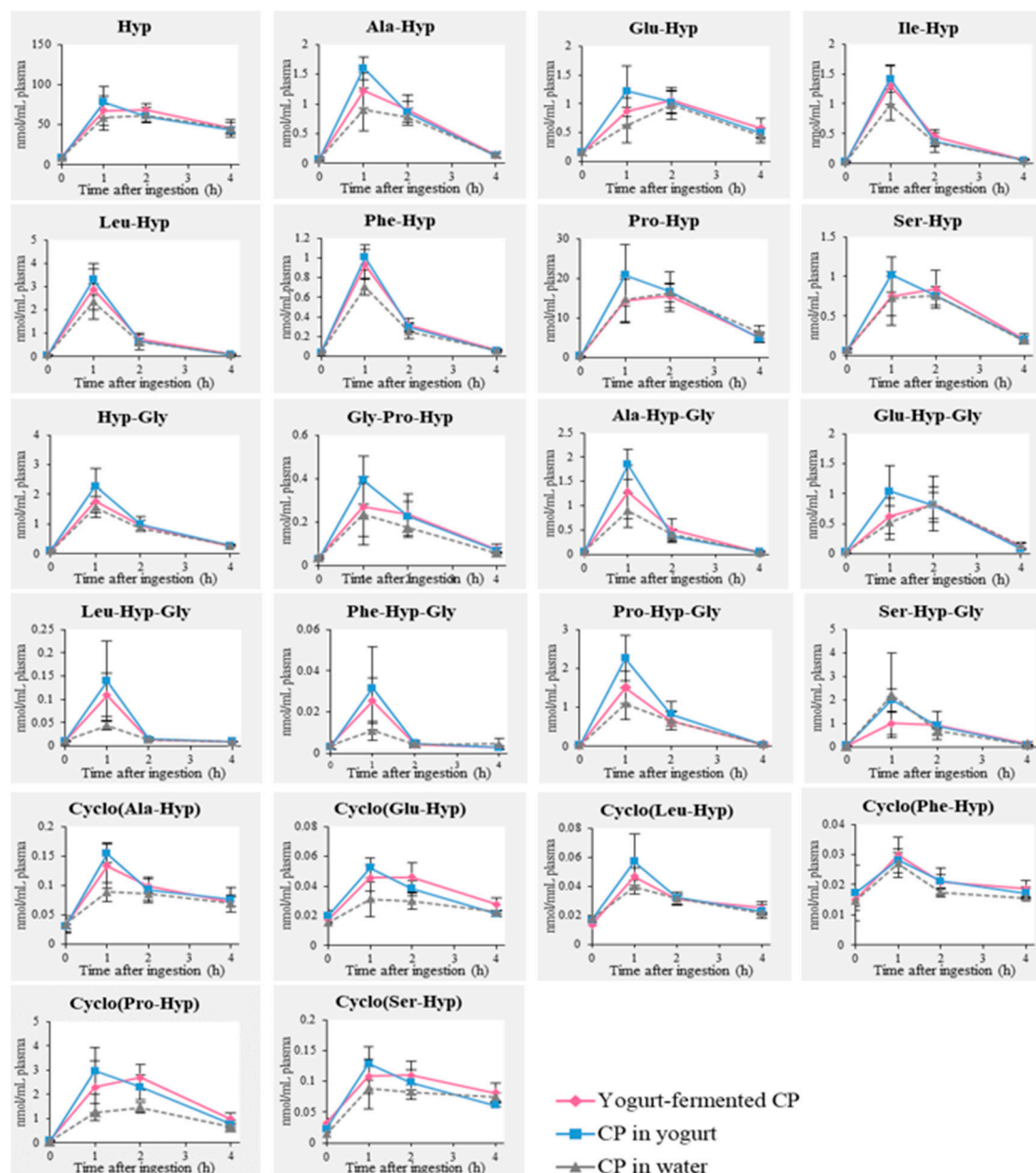


Figure 3. The average concentration (nmol/mL) of Hyp and Hyp-containing peptides in the plasma of four subjects before and after the ingestion of three CP test foods. Measurement was conducted by LC-MS. Plasma samples were collected before and 1, 2, and 4 h after ingestion of “yogurt-fermented CP” (◆), “CP in yogurt” (■), and “CP in water” (▲), respectively.

4. Discussion

We evaluated the production of short chain peptides in CP test foods and reported the changes in 19 types of di- and tripeptides including cyclic peptides, following the ingestion of the three CP test foods. Our results revealed that “yogurt-fermented CP” increased the concentration of 12 linear and two cyclic short chain Hyp-containing peptides in CP. However, we did not observe significant differences in the concentration of Hyp-containing cyclic peptides in any test food. Lactic acid bacteria are known to degrade peptides using their proteolytic systems, including cell envelope proteases and intracellular peptidases [18]. Aguirre et al. reported the proteolytic effects of four strains of lactic acid bacteria, *Lactobacillus delbrueckii*, on soybean proteins β -conglycinin and glycinin, and they demonstrated that the lactic acid bacteria could degrade proteins that were not milk-derived [24].

For the preparation of our CP test foods, yogurt—containing *Lactobacillus delbrueckii subsp. Bulgaricus* 2038 and *Streptococcus thermophiles* 1131—and CP with an average molecular weight of 3000 Da were used in this study. The lactic acid bacteria used in our yogurt sample contained X-prolyl dipeptidyl peptidase (PepX) that might degrade CP into dipeptides [25,26]. Based on the results of previous studies and the present results, it can be concluded that yogurt fermentation by lactic acid bacteria could result in the degradation of CP into Hyp-containing di- and tripeptides. These short chain peptides are small enough to enter the bloodstream following absorption in the small intestine. Moreover, mixing CP and yogurt could increase the levels of some CP-derived peptides, especially Glu-Hyp-Gly, shortly following CP test food preparation and prior to ingestion. Although the mechanism underlying the increase in the levels of CP-derived short chained peptides just by mixing CP with yogurt is unclear, lactic acid bacteria proteases in yogurt possibly contributed to this process. Both the “yogurt-fermented CP” and “CP in yogurt” test foods contained 50 g of yogurt. However, they (and the yogurt) were not exactly the same. This difference in the yogurt content of each test food may have affected the amounts of milk-derived peptides and peptidases and, consequently, altered the absorption of Hyp-containing peptides into the bloodstream after the ingestion of “yogurt-fermented CP” or “CP in yogurt.” Short chain peptides may be absorbed into the bloodstream more efficiently than the long-chain peptides; this might be due to the characteristic features of the peptide absorption system that transports di- or tripeptides from the intestinal lumen [15,16]. Based on the results of CP degradation by yogurt fermentation in vitro, “yogurt-fermented CP” was expected to be the best enhancer of Hyp-containing peptide absorption into human blood among the three CP test foods.

However, while the ingestion of “yogurt-fermented CP” significantly increased Hyp-containing peptide absorption represented by $C_{\max}$ and AUC compared to the ingestion of “CP in water,” the values were not significantly higher than those with “CP in yogurt” (Table 2). In fact, the $C_{\max}$ and AUC values for Pro-Hyp-Gly were significantly enhanced upon ingestion of “CP in yogurt,” compared to those observed upon ingestion of “yogurt-fermented CP.” Thus, the increase in the levels of short chain Hyp-containing peptides in the CP test foods due to yogurt fermentation could not be the only the reason for the enhancement of Hyp-containing peptide absorption in human blood.

Studies have shown that the co-ingestion of foods affects the absorption of food components in the small intestine. Walrand et al. demonstrated that the co-ingestion of fermented milk and CP could increase the plasma concentration of collagen-specific amino acids, and they suggested that the absorption of CP-derived amino acids might be controlled by yogurt-derived components in the human intestine [17]. β -casomorphin (BCM) is a representative bioactive peptide in milk products. BCM exhibits an opioid type effect on gastrointestinal motility [27] and the absorption of electrolytes [28] due to its interaction with the opioid receptors expressed in the intestine. Meisel et al. revealed that BCM promotes the intestinal absorption of nutrients via the opioid receptor or via specific binding sites in the brush border membrane [29]. Based on these studies, BCM in yogurt could promote the transport of amino acids and peptides derived from CP and consequently enhance the levels of amino acids as well as those of the eight Hyp-containing peptides in human blood (observed in the present study) (Table 2). However, the *Lactobacillus delbrueckii subsp. Bulgaricus* and *Streptococcus thermophiles* used in present study may degrade BCM via PepX [25,26] and consequently suppress the absorption of CP-derived short chain peptides. Although the mechanisms are still not completely clear, we suggest that PepX’s presence in the yogurt and BCM degradation in the milk that was used for the preparation of the CP test foods might differentially affect the absorption of Hyp-containing peptides in our study. This may explain the unexpected observation that the ingestion of “yogurt-fermented CP” could not significantly enhance the intestinal absorption of Hyp-containing peptides such as Ala-Hyp, Ala-Hyp-Gly, Leu-Hyp-Gly, and cyclo(Glu-Hyp) compared to the ingestion of “CP in yogurt.” Moreover, milk contains dipeptidyl peptidase-IV (DPP-IV) inhibitors such as LKPTPEGDL, LPYPY, IPIQY, and IPI [30]. DPP-IV is expressed in the gastrointestinal tract and possibly enables the degradation of CP, as well as PepX. Thus, the short incubation time during the preparation of our CP test foods might have decreased the amount of these milk-derived DPP-IV inhibitory peptides, and

consequently the activity of DPP-IV was retained in the “CP in yogurt” preparation. Therefore, the intestinal absorption of Ala-Hyp, Ala-Hyp-Gly, Leu-Hyp-Gly, and cyclo(Glu-Hyp) after the ingestion of “yogurt-fermented CP” was not more than that observed after ingestion of “CP in yogurt.”

Among all the short chain Hyp-containing peptides that were analyzed in this study, Phe-Hyp was the only peptide whose levels were significantly increased in either “yogurt-fermented CP” and plasma. This result might be attributed to the production of over thirty-fold higher levels of Phe-Hyp in “yogurt-fermented CP” compared to that in “CP in yogurt,” a level that was potentially high enough to invalidate the suppression of peptide transport by PepX or DPP-IV. In order to clarify the effects of PepX, BCM, and DPP-IV inhibitors, as well as different lactic acid bacteria on peptide absorption, we plan to quantify them in yogurt in follow-up studies. Additionally, we also intend to increase our sample size (use a higher number of subjects) in order to accurately and undoubtedly disclose the mechanisms behind the effects of food matrices on peptide absorption in humans.

Takeda et al. demonstrated that theaflavins inhibited the peptide transport system via the Pept1 that is expressed in human intestinal Caco-2 cells and that Pept1 expression in the small intestine was controlled by AMP-activated protein kinase [31]. Park et al. reported that theaflavins could enhance the intestinal barrier function through the expression of claudin-1, occludin, and ZO-1 via AMP-activated protein kinase [32]. These reports indicate that the co-ingestion of CP and functional foods such as black tea may suppress the absorption of CP-derived peptides in human blood and consequently alter their beneficial effects.

5. Conclusions

Clearly, the present study has revealed that the co-ingestion of CP and yogurt can significantly increase the absorption of Hyp-containing peptides into plasma. Therefore, the supplementation of food sources with CP should be considered in order to take advantage of the beneficial effects of CP byproducts on human health, particularly in the context of skin, joint, and muscle disorders. We demonstrated that lactic acid bacteria in yogurt could produce short chain Hyp-containing peptides from CP in “yogurt-fermented CP” samples and that yogurt enhanced the levels of CP-derived peptides in human blood. However, peptide absorption after the ingestion of the CP test foods was more affected by the bioactive peptides present in yogurt than by their molecular size. Follow-up studies are required in order to clarify the effects of PepX, BCM, and DPP-IV inhibitors in yogurt, as well as different lactic acid bacteria on peptide absorption. Additionally, an advanced human study with a large number of subjects is also needed for an accurate investigation regarding the mechanisms behind the effects of food matrices on peptide absorption in humans. Further studies on the detection of bioactive peptides in yogurt that promote peptide absorption into human blood are in progress.

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References

1. Schauss, A.G.; Stenehjem, J.; Park, J.; Endres, J.R.; Clewell, A.E. Effect of the Novel Low Molecular Weight Hydrolyzed Chicken Sternal Cartilage Extract, BioCell Collagen, on Improving Osteoarthritis-Related Symptoms: A Randomized, Double-Blind, Placebo-Controlled Trial. *J. Agric. Food Chem.* **2012**, *60*, 4096–4101. [\[CrossRef\]](#) [\[PubMed\]](#)
2. Kumar, S.; Sugihara, F.; Suzuki, K.; Inoue, N.; Venkateswarathirukumara, S. A double-blind, placebo-controlled, randomised, clinical study on the effectiveness of collagen peptide on osteoarthritis. *J. Sci. Food Agric.* **2014**, *95*, 702–707. [\[CrossRef\]](#)
3. Yamanaka, H.; Okada, S.; Sanada, H. A multicenter, randomized, controlled study of the use of nutritional supplements containing collagen peptides to facilitate the healing of pressure ulcers. *J. Nutr. Intermed. Metab.* **2017**, *8*, 51–59. [\[CrossRef\]](#)
4. Sugihara, F.; Inoue, N.; Venkateswarathirukumara, S. Ingestion of bioactive collagen hydrolysates enhanced pressure ulcer healing in a randomized double-blind placebo-controlled clinical study. *Sci. Rep.* **2018**, *8*, 11403. [\[CrossRef\]](#)
5. Zdzieblik, D.; Oesser, S.; Baumstark, M.W.; Gollhofer, A.; Koenig, D. Collagen peptide supplementation in combination with resistance training improves body composition and increases muscle strength in elderly sarcopenic men: A randomised controlled trial. *Br. J. Nutr.* **2015**, *114*, 1237–1245. [\[CrossRef\]](#)
6. Iwai, K.; Hasegawa, T.; Taguchi, Y.; Morimatsu, F.; Sato, K.; Nakamura, Y.; Higashi, A.; Kido, Y.; Nakabo, Y.; Ohtsuki, K. Identification of Food-Derived Collagen Peptides in Human Blood after Oral Ingestion of Gelatin Hydrolysates. *J. Agric. Food Chem.* **2005**, *53*, 6531–6536. [\[CrossRef\]](#)
7. Ohara, H.; Matsumoto, H.; Ito, 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. [\[CrossRef\]](#)
8. Matsui, T.; Tamaya, K.; Seki, E.; Osajima, K.; Matsumoto, K.; Kawasaki, T. Absorption of Val-Tyr with in Vitro Angiotensin I-Converting Enzyme Inhibitory Activity into the Circulating Blood System of Mild Hypertensive Subjects. *Boil. Pharm. Bull.* **2002**, *25*, 1228–1230. [\[CrossRef\]](#) [\[PubMed\]](#)
9. Foltz, M.; Meynen, E.E.; Bianco, V.; Van Platerink, C.; Koning, T.M.M.G.; 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. [\[CrossRef\]](#)
10. 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. [\[CrossRef\]](#)
11. 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. [\[CrossRef\]](#) [\[PubMed\]](#)
12. Nakatani, S.; Mano, H.; Sampei, C.; Shimizu, J.; Wada, M. Chondroprotective effect of the bioactive peptide prolyl-hydroxyproline in mouse articular cartilage in vitro and in vivo. *Osteoarthritis Cartil.* **2009**, *17*, 1620–1627. [\[CrossRef\]](#) [\[PubMed\]](#)
13. Shigemura, Y.; Iwasaki, Y.; Tateno, M.; Suzuki, A.; Kurokawa, M.; Sato, Y.; Sato, K. A Pilot Study for the Detection of Cyclic Prolyl-Hydroxyproline (Pro-Hyp) in Human Blood after Ingestion of Collagen Hydrolysate. *Nutrients* **2018**, *10*, 1356. [\[CrossRef\]](#)
14. Taga, Y.; Iwasaki, Y.; Shigemura, Y.; Mizuno, K. Improved in Vivo Tracking of Orally Administered Collagen Hydrolysate Using Stable Isotope Labeling and LC-MS Techniques. *J. Agric. Food Chem.* **2019**, *67*, 4671–4678. [\[CrossRef\]](#)
15. Adibi, S.A.; Soleimanpour, M.R. Functional Characterization of Dipeptide Transport System in Human Jejunum. *J. Clin. Investig.* **1974**, *53*, 1368–1374. [\[CrossRef\]](#)
16. Adibi, S.A.; Morse, E.L.; Masilamani, S.S.; Amin, P.M. Evidence for two different modes of tripeptide disappearance in human intestine. Uptake by peptide carrier systems and hydrolysis by peptide hydrolases. *J. Clin. Investig.* **1975**, *56*, 1355–1363. [\[CrossRef\]](#)
17. Walrand, S.; Chiotelli, E.; Noirt, F.; Mwewa, S.; Lassel, T. Consumption of a Functional Fermented Milk Containing Collagen Hydrolysate Improves the Concentration of Collagen-Specific Amino Acids in Plasma. *J. Agric. Food Chem.* **2008**, *56*, 7790–7795. [\[CrossRef\]](#)

18. Griffiths, M.W.; Tellez, A.M. *Lactobacillus helveticus*: The proteolytic system. *Front. Microbiol.* **2013**, *4*, 30. [CrossRef]
19. Taga, Y.; Kusubata, M.; Ogawa-Goto, K.; Hattori, S. Efficient Absorption of X-Hydroxyproline (Hyp)-Gly after Oral Administration of a Novel Gelatin Hydrolysate Prepared Using Ginger Protease. *J. Agric. Food Chem.* **2016**, *64*, 2962–2970. [CrossRef]
20. Ohara, H.; Iida, H.; Ito, K.; Takeuchi, Y.; Nomura, Y. Effects of Pro-Hyp, a Collagen Hydrolysate-Derived Peptide, on Hyaluronic Acid Synthesis Using in Vitro Cultured Synovium Cells and Oral Ingestion of Collagen Hydrolysates in a Guinea Pig Model of Osteoarthritis. *Biosci. Biotechnol. Biochem.* **2010**, *74*, 2096–2099. [CrossRef]
21. Trč, T.; Bohmova, J. Efficacy and tolerance of enzymatic hydrolysed collagen (EHC) vs. glucosamine sulphate (GS) in the treatment of knee osteoarthritis (KOA). *Int. Orthop.* **2010**, *35*, 341–348. [CrossRef] [PubMed]
22. Wu, J.; Fujioka, M.; Sugimoto, K.; Mu, G.; Ishimi, Y. Assessment of effectiveness of oral administration of collagen peptide on bone metabolism in growing and mature rats. *J. Bone Miner. Metab.* **2004**, *22*, 547–553. [CrossRef] [PubMed]
23. Taga, Y.; Kusubata, M.; Ogawa-Goto, K.; Hattori, S.; Funato, N. Collagen-derived X-Hyp-Gly type tripeptides promote differentiation of MC3T3-E1 pre osteoblasts. *J. Funct. Foods* **2018**, *46*, 456–462. [CrossRef]
24. Aguirre, L.; Hebert, E.M.; Garro, M.S.; De Giori, G.S. Proteolytic activity of *Lactobacillus* strains on soybean proteins. *LWT* **2014**, *59*, 780–785. [CrossRef]
25. Hafeez, Z.; Cakir-Kiefer, C.; Girardet, J.M.; Jardin, J.; Perrin, C.; Dary, A.; Miclo, L. Hydrolysis of milk-derived bioactive peptides by cell-associated extracellular peptidases of *Streptococcus thermophilus*. *Appl. Microbiol. Biotechnol.* **2013**, *97*, 9787–9799. [CrossRef]
26. Atlan, D.; Laloi, P.; Portalier, R. X-Prolyl-Dipeptidyl Aminopeptidase of *Lactobacillus delbrueckii* subsp. *bulgaricus*: Characterization of the Enzyme and Isolation of Deficient Mutants. *Appl. Environ. Microbiol.* **1990**, *56*, 2174–2179. [CrossRef]
27. Daniel, H.; Vohwinkel, M.; Rehner, G. Effect of Casein and β -Casomorphins on Gastrointestinal Motility in Rats. *J. Nutr.* **1990**, *120*, 252–257. [CrossRef]
28. Tomé, D.A.; Dumontier, A.M.; Hautefeuille, M.A.; Desjeux, J.F. Opiate activity and transepithelial passage of intact β -casomorphins in rabbit ileum. *Am. J. Physiol.* **1987**, *253*, 737–744. [CrossRef]
29. Meisel, H.; Fitzgerald, R.J. Opioid peptides encrypted in intact milk protein sequences. *Br. J. Nutr.* **2000**, *84*, 27–31. [CrossRef]
30. Lacroix, I.M.E.; Chen, X.-M.; Kitts, D.D.; Li-Chan, E.C. Investigation into the bioavailability of milk protein-derived peptides with dipeptidyl-peptidase IV inhibitory activity using Caco-2 cell monolayers. *Food Funct.* **2017**, *8*, 701–709. [CrossRef]
31. Takeda, J.; Park, H.-Y.; Kunitake, Y.; Yoshiura, K.; Matsui, T. Theaflavins, dimeric catechins, inhibit peptide transport across Caco-2 cell monolayers via down-regulation of AMP-activated protein kinase-mediated peptide transporter PEPT1. *Food Chem.* **2013**, *138*, 2140–2145. [CrossRef] [PubMed]
32. Park, H.-Y.; Kunitake, Y.; Hirasaki, N.; Tanaka, M.; Matsui, T. Theaflavins enhance intestinal barrier of Caco-2 Cell monolayers through the expression of AMP-activated protein kinase-mediated Occludin, Claudin-1, and ZO-1. *Biosci. Biotechnol. Biochem.* **2014**, *79*, 130–137. [CrossRef] [PubMed]

