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In vitro protease digestion affects protein-derived bioactive compound content in functional ingredients

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ABSTRACT

Background: Proteins in food are degraded by proteases in the digestive tract, and their products may exhibit physiological activities. *In vitro* protease digestion mimics digestion of food protein in the body. When crude herbal drugs used in Japanese Kampo medicines are subjected to *in vitro* protease digestion, which mimics *in vivo* processes, decarboxylated amino acids (biogenic amines) are detected in the digest. The protease-resistant dipeptide, pyroglutamyl leucine (pyroGlu-Leu), is present in fermented foods. *In vitro* protease digestion of functional foods and ingredients has not yet been studied.

Objective: To identify protein-derived compounds by *in vitro* protease digestion of three functional ingredients that possess anti-inflammatory effects: a standardized extract of *Asparagus officinalis* stem (EAS), a standardized extract of cultured *Lentinula edodes* mycelia (ECLM), and a standardized oligomerized-polyphenol from *Litchi chinensis* fruit extract (OPLFE).

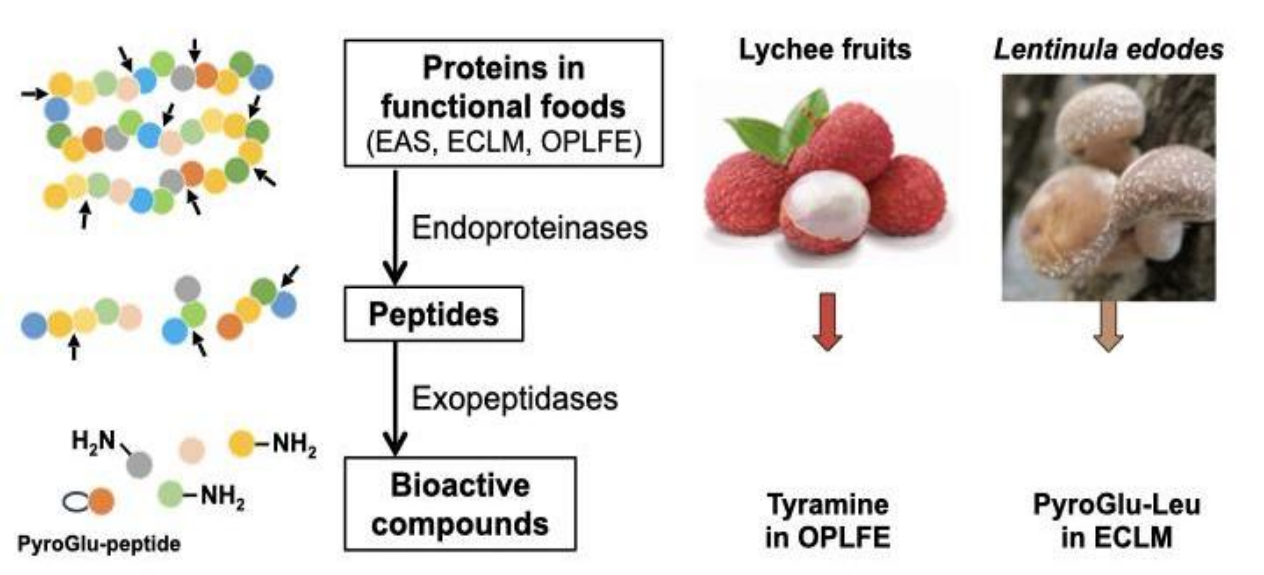
Methods: Functional ingredients were treated with endoproteinases and exopeptidases. To detect decarboxylated amino acids, amino groups were derivatized with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC), and a precursor ion scan was performed using liquid chromatography separation–tandem mass spectrometry (LC–MS/MS) to target the product ions. AQC-derivatized amines were identified, and decarboxylated amino acids and pyroGlu-Leu in

the digests were analyzed and quantified using LC–MS/MS in the multiple reaction monitoring (MRM) mode. Undigested functional ingredients were analyzed by LC–MS/MS and used as the negative control.

Results: Decarboxylated amino acids with anti-inflammatory activity were detected in both undigested functional ingredients and their protease digestion. Tyramine was abundant in OPLFE, but its content was significantly decreased after *in vitro* protease digestion. 2-Methylbutylamine and isoamylamine were present in both undigested ECLM and its digest, whereas trace amounts of isobutylamine were also detected. In addition, pyroGlu-Leu, which also exhibits anti-inflammatory effects, was highly abundant in undigested ECLM, and its content was significantly increased after *in vitro* protease digestion.

Conclusion: We first reported that decarboxylated amino acids and pyroGlu-Leu are present in undigested functional ingredients and/or formed from their proteins by *in vitro* protease digestion. When functional ingredients are ingested, these protein-derived compounds may exert anti-inflammatory and other beneficial effects. This methodology may be applied to other functional foods and ingredients, leading to understanding of their effects in the body.

Keywords: Functional food, bioactive compound, decarboxylated amino acid, trace amine, *in vitro* protease digestion, LC–MS/MS.



Graphical abstract: *In vitro* protease digestion affects protein-derived bioactive compound content in functional ingredients.

INTRODUCTION

Identifying the constituents of functional foods and ingredients that affect humans is difficult. Many protein-derived compounds are formed by the digestive processes of food, in which a variety of enzymes are involved. Proteins are digested by proteases in the stomach and pancreatic juice, metabolized, absorbed in the intestine, and then transferred to the blood through

the liver. Enzymes involved in digestion include pepsin from the stomach [1], trypsin, chymotrypsin, and carboxypeptidase A (CPA) in pancreatic juice [2–3], and leucine aminopeptidase (LAP) in the brush border and/or inside enterocytes [4]. Pepsin, trypsin, and chymotrypsin are classified as endo-type proteinases (endoproteinases), whereas CPA and LAP are classified as exo-type peptidases (exopeptidases). Both proteases are required for the degradation of food proteins into peptides and amino acids [5]. To date, various bioactive compounds have been discovered in dietary proteins, and their peptides have been analyzed [6]. The biological activities of protein-derived compounds are crucial to elucidate the beneficial effects of foods, functional foods and ingredients, and crude drugs.

In vitro protease digestion, which includes endoproteases and exopeptidases, simulates the digestive processes in the human gastrointestinal tract. Recent studies demonstrated that most oligopeptides resist degradation by endoproteinases but are degraded by exopeptidases [7–9]. Some peptides harboring specific sequences are resistant to digestion by both exopeptidases and endoproteinases [5,8]. Peptides, their digests, and metabolites formed in the intestine can be detected using liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS). For example, amines in peptides can be detected by using LC–MS/MS via derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC), followed by precursor ion scanning of the target product ion from the AQC moiety [9]. Structural information can be obtained by product ion scanning of the precursor ions detected at different collision energies.

Protease-resistant oligopeptides derived from plants and animals, such as pyroglutamyl peptides, exhibit various biological activities [5,10]. Glutamine is intramolecularly cyclized to form pyroglutamic acid, and a pyroglutamyl peptide with a pyroglutamyl residue at the amino terminus is formed [5,10]. Among them, pyroglutamyl leucine (pyroGlu-Leu), a dipeptide found in hydrolysates of wheat gluten and traditional Japanese fermented foods [10–12], suppresses the induction of both nitric oxide (NO) and inducible nitric oxide synthase (iNOS) in the RAW264.7 macrophages and rat hepatocytes [13–14]. Furthermore, pyroGlu-Leu has been shown to improve high-fat diet-induced disturbances of the microbiota by increasing antimicrobial peptide levels in rat intestines [15].

In previous studies, we used protease digestion *in vitro* on six crude herbal drugs of traditional Japanese (Kampo) medicines [16–17], which are used to clear heat in the body, to identify bioactive compounds [18]. In the protease digests of the *Ziziphus jujuba* var. *inermis* fruit (*Taiso*, in Japanese), we identified three decarboxylated amino acids (isobutylamine, isoamylamine, and 2-methylbutylamine) that may be derived from the valinyl, leucinyl, and isoleucinyl residues of the peptides, respectively [18]. Branched-chain amino acid decarboxylase was not included among the proteases used in this study, because branched chain amino acids alone were not decarboxylated during *in vitro* protease digestion [18]. Tyramine, which may be derived from tyrosyl residues, was also detected in the digests of the six crude drugs, although it was much less abundant than the other decarboxylated amino acids. In a previous study assessing anti-inflammatory effects, the decarboxylated amino acids derived from the crude herbal drugs suppressed the production of both NO and iNOS in interleukin (IL)-1β-treated rat hepatocytes, which are an *ex vivo* liver injury model [19–20]. When crude drugs are orally administered, decarboxylated amino acids may be formed and transferred to the liver to

exhibit anti-inflammatory properties.

In addition, it is speculated that bioactive compounds, such as decarboxylated amino acids and pyroGlu-Leu, are also formed from functional ingredients during digestion *in vivo*. Therefore, we selected three functional ingredients: a standardized oligomerized polyphenol from *Litchi chinensis* fruit extract (OPLFE), a standardized extract of cultured *Lentinula edodes* mycelia (ECLM), and a standardized extract of *Asparagus officinalis* stem (EAS). All three foods inhibited the IL-1β-induced production of iNOS and proinflammatory cytokines in rat hepatocytes [19,21–22]. Several anti-inflammatory compounds in the functional ingredients have been reported: flavanols in OPLFE [21], partially acylated α-1,4-glucan in ECLM [22], and 5-hydroxymethyl-2-furfural and (*S*)-asfural in EAS [19]. Although these molecules are not derived from protein, they exhibit anti-inflammatory effects when added to the medium of rat hepatocytes.

In this study, we focused on protein-derived bioactive compounds in functional ingredients. The above-mentioned functional ingredients were subjected to *in vitro* protease digestion, and the resulting compounds were analyzed using LC–MS/MS to determine whether decarboxylated amino acids and pyroGlu-Leu are present in the protease digests. Undigested functional ingredients were also analyzed to compare the changes in the content of each compound before and after *in vitro* protease digestion.

MATERIALS AND METHODS

Materials: EAS, ECLM, and OPLFE manufactured by Amino Up Co., Ltd. (Sapporo, Hokkaido, Japan) were generously provided. Lychee fruits were processed to obtain OPLFE as Oligonol®. *Lentinula edodes* liquid culture was processed to obtain ECLM as AHCC®. The stems of asparagus grown in Hokkaido, Japan, were processed to obtain EAS as ETAS®50. These functional ingredients were prepared without the use of fermentation processes involving bacteria, such as *Lactobacillus*. The protein amounts in the functional ingredients were estimated by the Bradford method using the Protein Assay CBB Solution (Nacalai Tesque Inc., Kyoto, Japan) and bovine serum albumin as a standard. Tyramine, 2-methylbutylamine, isoamylamine, and isobutylamine were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), which were used as standards for the LC–MS/MS analysis.

In vitro protease digestion: Powdered EAS, ECLM, or OPLFE (50 mg each) was dissolved in 7.5 mL of 0.1 M HCl. One aliquot of this solution was subjected to protease digestion using a previously described method [18], and another small aliquot was used for LC–MS/MS analysis as an undigested control. Briefly, 0.5 mg of porcine pepsin (Nacalai Tesque Inc.) was added to each solution in 0.1 M HCl and incubated at 37 °C for 3 h. After neutralization with 500 µL of 1 M Tris-HCl (pH 8.0), 2 mg of porcine pancreatin (Nacalai Tesque Inc.), which is a mixture of pancreatic enzymes (including trypsin), was added to the mixture and further incubated at 37 °C for 24 h. After adjusting the final volume to 10 mL, the mixture was centrifuged at 3000 × *g* at 23 °C for 10 min and ultrafiltered using an Amicon Ultra filter unit (molecular weight cutoff, 10000; Merck, Darmstadt, Germany) at 14000 × *g* at 23 °C for 10 min to terminate the reaction. A 1-mL aliquot of "*endoproteinase digest*" was subjected to digestion by six units of bovine CPA (Sigma-Aldrich Inc., St. Louis, MO, U.S.A.) and five units of porcine LAP (Sigma-Aldrich Inc.) at 37 °C for 24 h. After stopping the reaction by ultrafiltration, the filtrate was collected as the "*exopeptidase digest*."

AQC-derivatization of amines and precursor ion scanning analysis: To detect decarboxylated amino acids,

amine derivatization was performed according to a previously described method [18]. Briefly, samples (150 µL) of endoproteinase digest, exopeptidase digest, or undigested functional ingredient were dried and dissolved in 20 mM HCl, and primary and secondary amines were derivatized by AQC [9]. After the addition of a 0.3% AQC-acetonitrile solution and 50 mM sodium borate buffer (pH 8.8), the mixture was incubated at 50 °C for 10 min. The reactant was then mixed with 5 mM sodium phosphate buffer (pH 7.4) containing 5% acetonitrile. The filtrate was injected into an LC–MS/MS model LCMS8040 (Shimadzu Corporation, Kyoto, Japan) equipped with an Inertsil ODS-3 column (GL Science, Tokyo, Japan). A binary linear gradient was used with solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in 80% acetonitrile), at a flow rate of 0.2 mL/min. The gradient program was as follows: 0–30 min, 0–30% B; 30–40 min, 30–100% B; 40–50 min, 100% B; 50.0–50.1 min, 100–0% B; and 50.1–60 min, 0% B. The column was maintained at 40 °C. AQC derivatives were specifically detected by selecting precursor ions, which generated the AQC-derived ions [b1 ion, the ion mass to charge ratio (*m*/*z*) of 171.1] in positive mode (collision energy, −35 V) in the scan range of *m*/*z* 240–275, 275–300, 300–325, 325–350, 350–375, 375–400, 400–450, 450–500, and 500–1000 (precursor ion scan). The *m*/*z* values of the AQC-peptides were recorded and incorporated to the LabSolutions version 5.80(Shimadzu Corporation).

A product ion scan targeting of the detected AQC-derivatized precursor ions was carried out to estimate the structures of the compounds in the peaks by product ion scanning at different collision energies. For example, the MS spectrum of the AQC-derivatized precursor (*m*/*z* 308.1) showed a shift of 137.2 from the AQC fragment ion (*m*/*z* 171.0), suggesting that this precursor ion is derived from tyramine. Similarly, it has been suggested that the AQC-derivatized precursor ions (*m*/*z* 244.2, 258.2, and 258.2) are derived from isobutylamine, 2-methylbutylamine, and isoamylamine, respectively [18].

Quantification of compounds by multiple reaction monitoring (MRM): The content of AQC-decarboxylated amino acids in the reaction mixture was measured using LC–MS/MS in the MRM mode, as previously described [18]. The elution conditions were the same as those used for the precursor and product ion scanning. Decarboxylated amino acids were dissolved in water and used as standards for optimization of the MRM conditions. The calibration equations and correlation coefficients of the standard compounds are as follows: tyramine, $y = 30919227x + 52983$ ($R^2 = 0.9993$); 2-methylbutylamine, $y = 21305297x + 79556$ ($R^2 = 0.9936$); isoamylamine, $y = 20966656x + 88784$ ($R^2 = 0.9940$); and isobutylamine, $y = 15638803x + 47845$ ($R^2 = 0.9962$). The peak areas of the decarboxylated amino acids in the sample solution were fitted to the calibration curve. The content was calculated by dividing the amount of each decarboxylated amino acid by the dried weight of the functional ingredient (mg).

Detection and quantification of pyroGlu-Leu: The pyroGlu-Leu content was measured using aliquots of endoproteinase digest, exopeptidase digest, and undigested functional ingredients, according to a previously described method [11]. Briefly, the samples were analyzed using a model LCMS-8040 equipped with an Inertsil ODS-3 column. A binary linear gradient was

established using solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in 80% acetonitrile) at a flow rate of 0.2 mL/min. The gradient program was as follows: 0–30 min, 0–30% B; 30–40 min, 30–100% B; 40–50 min, 100% B; 50–50.1 min, 100–0% B; and 50.1–60 min, 0% B. The column was maintained at 40 °C. The pyroGlu-Leu concentrations in the digests were measured in the MRM mode. The calibration equation and correlation coefficient of the standard pyroGlu-Leu [11] were $y = 38992.0x + 2319.6$ ($R^2 = 0.9998$). The peak areas of pyroGlu-Leu in the sample solution were fitted to the calibration curve, and the content was calculated as pyroGlu-Leu (pmol) divided by the dried functional ingredient (mg).

Statistical analysis: All experiments were measured in triplicate, and the values are presented as the mean ± standard deviation (SD). Differences were analyzed using the Student's *t*-test followed by Bonferroni correction. Statistical significance was set at $P < 0.05$ and $P < 0.01$.

RESULTS

In vitro protease digestion of functional ingredients: The presence of protein in the functional ingredients was confirmed. The protein content in the dried functional ingredients was 34.62 µg/mg dried powder (EAS), 17.69 µg/mg (ECLM), and 1.623 µg/mg (OPLFE). Then, these functional ingredients were subjected to *in vitro* protease digestion, and the resulting products were analyzed using LC–MS/MS (Figure 1).

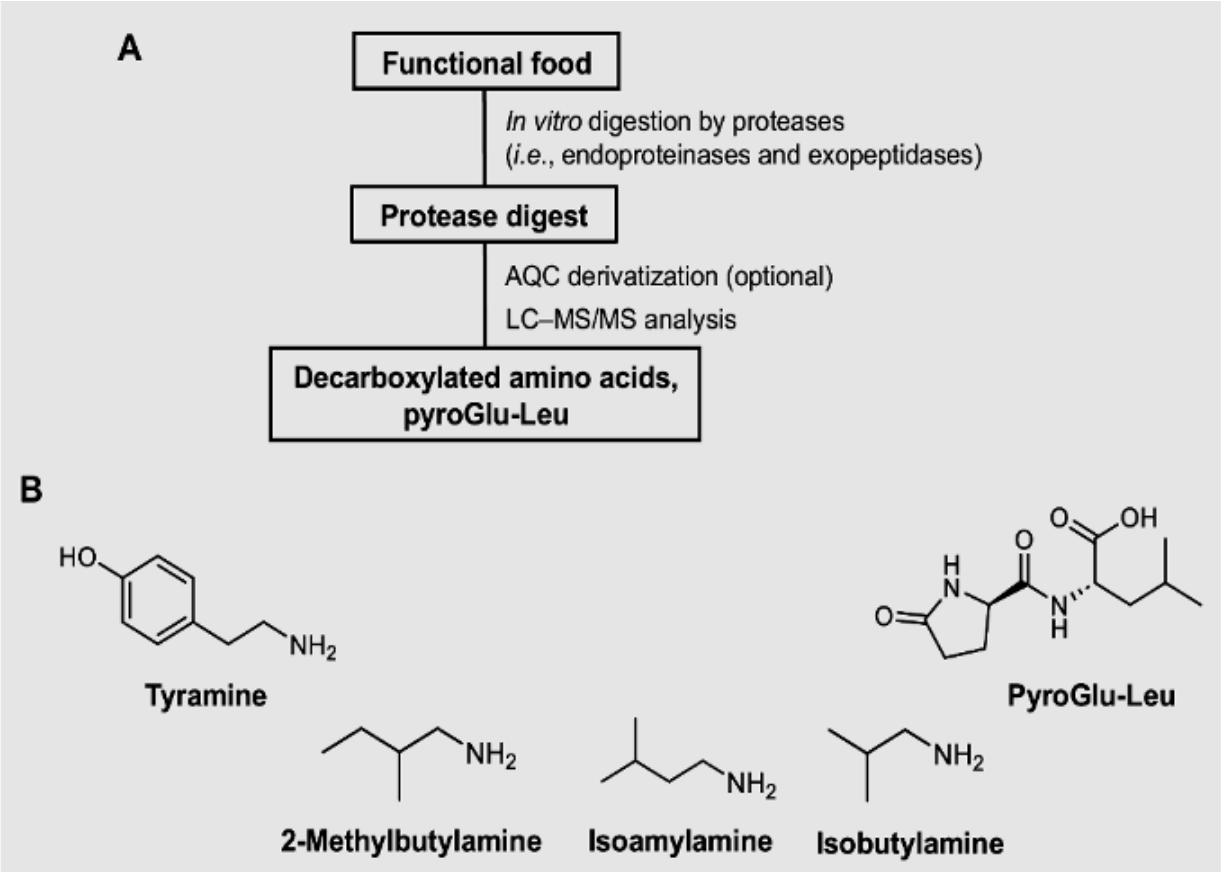


Figure 1. A flowchart of the procedures used to identify amines in functional ingredients for LC–MS/MS analysis. (A) The powder of each functional ingredient was subjected to *in vitro* protease digestion. The resulting digest was AQC-derivatized and subjected to LC–MS/MS analysis. (B) The chemical structures of the identified protein-derived compounds

Spectral analysis of functional ingredient digests was performed as described in the Materials and Methods section. The total ion chromatograms of the protease digests revealed peaks in the *m/z* range 200–325. Most peaks corresponded to the *m/z* of the AQC-derivatives of decarboxylated amino acids The presence of decarboxylated amino acids, which have been identified in crude herbal drugs [18], was confirmed by detecting the retention times of the standard substances using LC–MS/MS in the MRM mode, followed by product ion scan targeting of the detected precursor ions to predict the structure of the compounds in the peaks. pyroGlu-Leu was identified without derivatization.

The contents of decarboxylated amino acids and pyroGlu-Leu in the exopeptidase digests were measured, calculated. They are summarized with half-maximal inhibitory concentration (IC_{50}) for NO production in hepatocytes in Table 1.

Table 1. The contents of compounds detected after *in vitro* protease digestion.

Compound identified	Content [pmol/mg dried powder] [a]			IC_{50} [mM] [b]
	EAS	ECLM	OPLFE	
Tyramine	4.34 ± 0.202	23.1 ± 1.48	236 ± 10.1	0.833 ± 0.142
2-Methylbutylamine	1. 47 ± 0.139	11.7 ± 0.987	0.501 ± 0.0425	2.98 ± 0.339
Isoamylamine	3.29 ± 0.120	16.5 ± 1.10	0.619 ± 0.377	2.42 ± 0.245
Isobutylamine	0.00143 ± 0.00006	0.00193 ± 0.00030	0.00075 ± 0.00008	4.23 ± 0.308
PyroGlu-Leu	78.7 ± 56.2	475 ± 12.3	4.30 ± 2.81	Not calculated [c]

[a] The content in the exopeptidase digests was estimated using LC–MS/MS as the mean ± standard deviation (SD) (n = 3). [b] The IC_{50} value of NO production in IL-1β-treated hepatocytes as the mean ± SD (n = 3) [18]. [c] PyroGlu-Leu suppressed NO production at concentrations up to 1.6 mg/mL (6.6 mM) in IL-1β-treated hepatocytes (the IC_{50} value was not calculated in this instance) [14].

The contents of the identified compounds varied widely across the functional ingredients used. The LC–MS/MS results indicated that the tyramine content in the OPLFE digest was the second most abundant among the five compounds examined, whereas a much lower content was detected in EAS and ECLM. The other decarboxylated amino acids were much less abundant in the protease digest, and isobutylamine was present in trace amounts in all digests. In contrast, pyroGlu-Leu was highly abundant in the protease digest of ECLM, and its content was the highest among the five compounds. When pyroGlu-Leu was analyzed in the protease digests, its content in ECLM was 6.1- and 111-fold higher than that in EAS and OPLFE, respectively.

Changes in product contents before and after in vitro protease digestion: Because functional ingredients are generally processed from original materials, it is possible that the above-mentioned compounds in the protease digests are present in undigested functional ingredients. Therefore, *in vitro* digestion by endoproteinases and exopeptidases was monitored using LC–MS/MS, and the functional ingredients were analyzed directly as undigested controls. To detect decarboxylated amino acids, AQC-derivatization was performed prior to the analysis. The chromatograms of these compounds are shown in Figure 2.

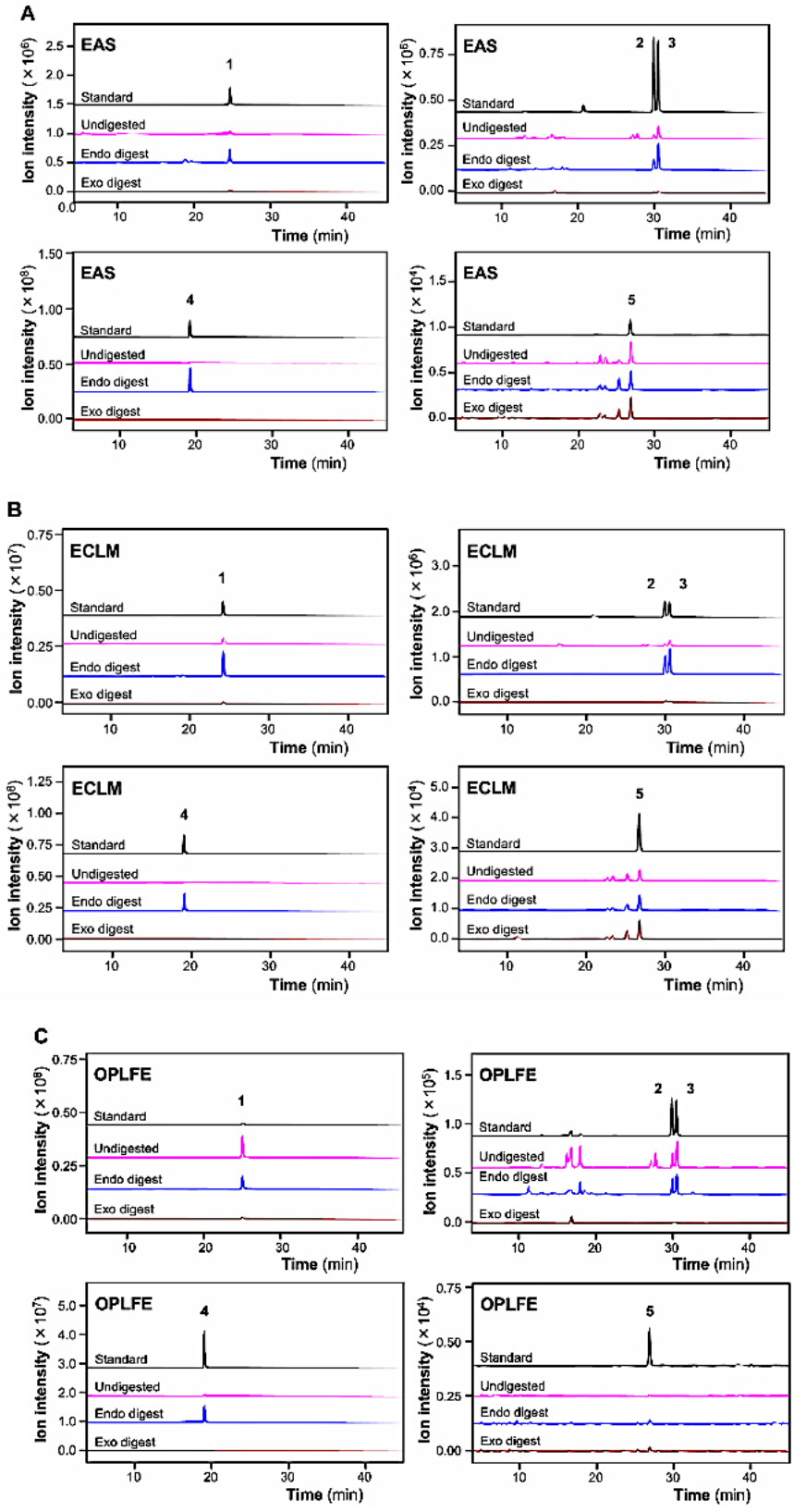


Figure 2. Detection of decarboxylated amino acids and pyroGlu-Leu. Each functional ingredient was digested *in vitro*, and the resulting products were detected using LC–MS/MS in MRM mode. Total chromatograms of the compounds detected in EAS (**A**), ECLM (B), and OPLFE (C). Tyramine (peak 1), 2-methylamine (2), isoamylamine (3), isobutylamine (4), and pyroGlu-Leu (5). Each standard compound (Standard) was used for measuring the content in undigested functional ingredients (Undigested), the endoproteinase digests (Endo digest), and the exopeptidase digests (Exo digest). *x-axis, elution time (min); y-axis*, ion intensity.

The content of decarboxylated amino acids and pyroGlu-Leu in the undigested functional ingredients (*Before*) was compared with that in the digest of functional ingredients after *in vitro* digestion by both endoproteinases and exopeptidases (*After*). The values obtained after *in vitro* digestion are the same as those listed in Table 1. The changes in their contents after *in vitro* protease digestion (Before versus after) are shown in Figure 3.

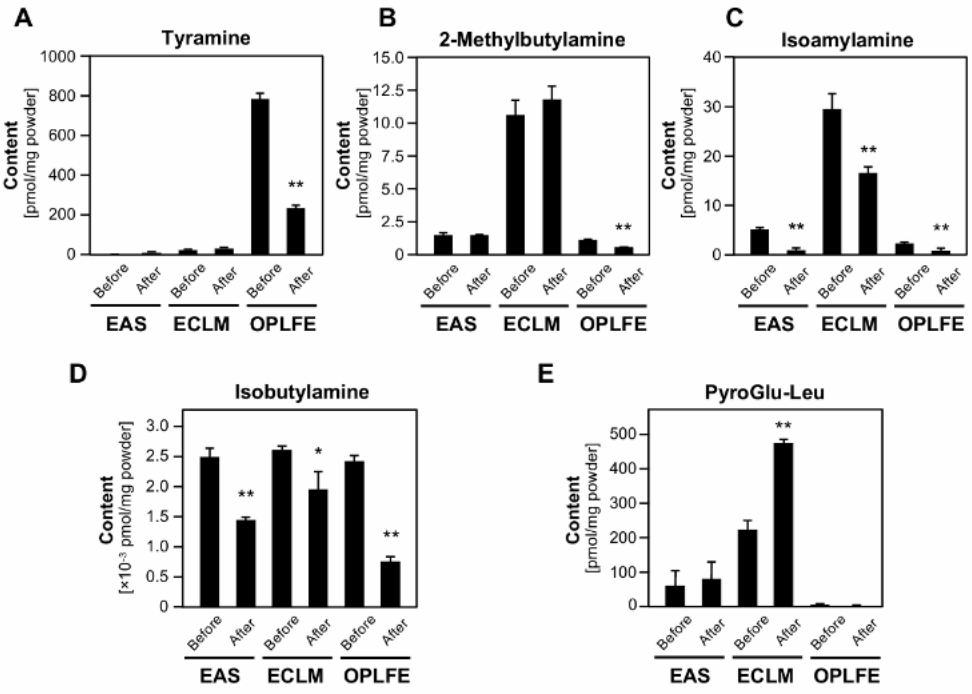


Figure 3. Changes in the contents of the compounds before and after *in vitro* protease digestion. Compounds in functional ingredients with and without protease digestion were analyzed. The content of each compound in an undigested functional ingredient (*Before*) and its exopeptidase digest (*After*) was estimated using LC–MS/MS. The values represent the content (pmol/mg dried powder) as the mean ± standard deviation (SD) (n = 3). (A) Tyramine, (B) 2-methylbutyramine, (C) isoamylamine, (D) isobutylamine, and (E) pyroGlu-Leu. Asterisks indicate significant differences at $^{*}P < 0.05$ and $^{**}P < 0.01$ *versus* the undigested sample (*Before*).

Interestingly, the tyramine content after *in vitro* protease digestion of OPLFE was 3.3-fold lower than that of undigested OPLFE (*Before*), as shown in Figure 3. These data suggest that the tyramine in OPLFE is degraded during digestion. Conversely, pyroGlu-Leu was significantly increased by *in vitro* protease digestion; the pyroGlu-Leu content after ECLM digestion was 2.1-fold higher than that in undigested ECLM (*Before*). 2-Methylbutylamine and isoamylamine were present in both the digested and undigested ECLM, whereas they were less abundant in the digested and undigested EAS and OPLFE. The isobutylamine content was low and comparable to that found in the undigested functional ingredients. *In vitro* protease digestion significantly decreased isobutylamine content of all functional ingredients.

DISCUSSION

To our knowledge, this is the first report to apply *in vitro* protease digestion (endoproteinase and exopeptidase) to functional foods and ingredients. The methodology used in this study was designed to mimic the digestion of food in the body, and various bioactive compounds formed during digestion were identified. These

compounds are crucial to clarify the beneficial effects of functional ingredients. Similar to our previous study using crude herbal drugs used in Kampo medicines [18], decarboxylated amino acids (tyramine, 2-methylbutylamine, isoamylamine, and isobutylamine) were found in the protease digests of the three functional ingredients (EAS, ECLM, and OPLFE). These decarboxylated amino acids inhibit NO production in IL-1β-treated rat hepatocytes [18]. The findings obtained in this study reveal novel properties of the three functional ingredients.

The four decarboxylated amino acids detected in this study are biogenic amines present in grape juice and Port wine [23]. In a previous study on the storage of red wines made with the Merlot grapes in oak barrels, isobutylamine was degraded during the storage of Merlot red wine, while tyramine produced at the beginning of the aging process did not accumulate in the wine, likely because it was degraded [24]. Isobutylamine and 2-methylbutylamine are also used as food additives for flavoring; there are no safety concerns regarding these compounds [25]. The isobutylamine content is 64.2 pmol/mg dried extract of *Ziziphus jujuba* Miller var. *inermis* fruits when calculated from both the content and yield of the hydrophilic fraction [18], which was much higher than that of the OPLFE digest (0.00143 pmol/mg dried powder). Collectively, the decarboxylated amino acids formed by *in vitro* protease digestion may depend on proteins and/or peptides, as well as the processing of functional ingredients.

Unexpectedly, decarboxylated amino acids were also detected in undigested functional ingredients (Figure 3). Tyramine was much less abundant in the protease digests of the crude herbal drugs we examined [18]. The tyramine content was low ranging from 0.0952 pmol/mg dried extract (*Angelica acutiloba* roots) to 0.812 pmol/mg dried extract (*Atractylodes macrocephala* rhizomes) when calculated from both the content and yield of the hydrophilic fraction [18]. In contrast, although the tyramine content was high in undigested OPLFE, it prominently decreased after *in vitro* protease digestion (Figure 3).

Tyramine is formed from tyrosine and tyrosyl residues and subsequently metabolized. Tyramine has been detected in the feces of newborns and breast milk of mothers after childbirth [26]. Decarboxylated amino acids are also produced by bacteria in the intestine. Tyramine has been detected in the colon and cecum of equines, and the concentrations of isoamylamine and isobutylamine in the colon were several times greater than the concentration of tyramine [27]. Some foods rich in tyramine, such as ripened cheese, may increase blood pressure by vasoconstriction [28–30]. Two types of monoamine oxidases (MAO-A and B) catalyze the degradation of biogenic amines, such as tyramine and phenylethylamine derivatives (*e.g*., dopamine) and play important roles in the intestine and central nervous system [30]. Because bacteria are not used to prepare OPLFE, a trace of MAO, which contaminates exopeptidases (CPA and LAP), might degrade tyramine in OPLFE, as well as other decarboxylated amino acids in functional ingredients (Figure 3). It cannot be ruled out that a MAO activity in OPLFE may have induced the observed decrease in tyramine.

Amino acids are produced from dietary proteins during digestion. Dietary tryptophan is catabolized via the kynurenine pathway to synthesize NAD^+ and is also metabolized by the microbiota via the indole–pyruvate pathway [31–32]. Some metabolites formed in these pathways affect blood pressure and cause vascular inflammation during hypertension and sepsis [31–32]. Tryptophan, which may be present in protease digests, suppresses NO production in hepatocytes [20]. Although tyramine has the greatest ability to suppress NO production (IC_{50} value = 0.833 mM) in IL-1β-treated

hepatocytes [18], the branched-chain amino acids and tyrosine did not significantly inhibit NO production in hepatocytes [20]. Collectively, many factors, such as digestion, metabolism, microbiota, and absorption, are involved in the complex mechanisms that regulate blood pressure and inflammatory responses. Further studies are required to elucidate how functional ingredients affect blood pressure and inflammation.

PyroGlu-Leu, which was first identified in ECLM in the present study, is another anti-inflammatory compound that is newly formed during *in vitro* protease digestion. PyroGlu-Leu is found in Japanese rice wine (*sake*) and traditional Japanese fermented foods, such as *miso* [10–12]. PyroGlu-Leu suppresses NO production in mouse RAW264.7 cells and rat hepatocytes [13–14] and exhibits beneficial effects by downregulating the *iNOS* gene in rat hepatocytes, suggesting that this dipeptide exhibits hepatoprotective activity [14]. Additionally, pyroGlu-Leu attenuated hepatitis and colitis in mouse models [33] and increased the level of host antimicrobial peptides in the intestinal microbiota [15]. A prominent increase in pyroGlu-Leu content in ECLM by *in vitro* protease digestion (Figure 3) was likely caused by proteases present in the ECLM and may be favorable for humans.

Oligopeptides possess other pharmacological activities and function as inhibitors of angiotensin-converting enzyme (ACE), which plays a major role in blood pressure regulation [34]. Trypsin- and papain-resistant tripeptide (Ile-Glu-Arg) isolated from the fruit of *Ziziphus jujuba* var. *inermis* competitively inhibited ACE [35]. Three other ACE inhibitors (including Ser-Ala-Pro) have been identified in oyster proteins via *in vitro* digestion with chymotrypsin and proline-specific endopeptidases [36]. Lactotripeptides (Val-Pro-Pro and Ile-Pro-Pro) are formed during the fermentation of milk by *Lactobacillus helveticus* after casein degradation to produce natural ACE inhibitors [37–38]. Tryptophan-containing dipeptides (Ile-Trp and Val-Trp) in food protein hydrolysates are selective competitive ACE inhibitors [39]. Several reports have demonstrated that oligopeptides derived from milk and other foods have bioactive properties, such as antimicrobial activity, immunopotentiation, and ileal contraction [40–41].

Many bioactive compounds have been identified in functional ingredients, such as rice-derived glucosylceramides [42] and compounds in *Moringa oleifera* leaf extract [43]. Some functional ingredients improved glucose metabolism in hyperglycemic model mice [44] and gut microbiome in diabetes patients [45]. Protein-derived compounds identified in this study (decarboxylated amino acids and pyroGlu-Leu) are classified to a new type of bioactive compounds [46] in functional ingredients. Therefore, our findings indicate that *in vitro* protease digestion can be applied to pharmacological studies on protein-derived compounds of functional foods and ingredients. The research using *in vitro* protease digestion facilitate our understanding of how protein-derived compounds work in the cell and how functional foods and ingredients manifest their beneficial effects during digestion in the body.

CONCLUSION

To identify protein-derived compounds that may exhibit pharmacological activities in the digestive tract, we performed *in vitro* protease digestion of three functional ingredients (EAS, ECLM, and OPLFE) followed by LC–MS/MS analysis. We first reported that decarboxylated amino acids and pyroGlu-Leu were detected in both the undigested functional ingredients and their protease digestion. Although tyramine was abundant in the undigested OPLFE, it was significantly decreased by *in vitro* protease digestion. In contrast, pyroGlu-Leu was present at high levels in the undigested ECLM and significantly increased during digestion. This study on protein-derived bioactive compounds revealed novel properties of functional ingredients. When the

methodology is applied to other functional foods and ingredients, effects and mechanisms of their action may be elucidated.

List of Abbreviations: OPLFE: a standardized oligomerized-polyphenol from *Litchi chinensis* fruit extract, ECLM: a standardized extract of cultured *Lentinula edodes* mycelia, EAS: a standardized extract of *Asparagus officinalis* stem, AQC: 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate, LC–MS/MS: liquid chromatography separation–tandem mass spectrometry, pyroGlu-Leu: pyroglutamyl leucine, MRM: multiple reaction monitoring.

Competing Interests: M. Nishizawa and K. Sato received research grants from Amino Up Co., Ltd. (Sapporo, Japan). M. Nishizawa and K. Sato are vice presidents of the International Congress on Nutrition and Integrative Medicine (Sapporo, Japan). This institution investigates the effects of functional foods and ingredients by conducting basic and clinical research in integrative medicine, thereby contributing to the development of disease prevention and treatment using integrative medicine and functional foods and ingredients.

Author's Contributions: S. Shirako and S. Miyauchi performed the experiments, data collection, and data analysis. S. Shirako and M. Nishizawa participated in the design of the study. M. Nishizawa and K. Sato supervised the study and the drafting of the manuscript. All authors were involved in performing experiments and preparing the manuscript.

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