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Presence of isomerized aspartic dipeptides in a porcine liver protein hydrolysate and their bioavailability upon ingestion

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ABSTRACT:

Background: Formulated liver protein hydrolysate (LPH) was prepared from porcine liver protein. Animal experiments have demonstrated that oral administration of LPH ameliorates alcohol-induced liver toxicity, as well as exercise- and concanavalin A-induced low locomotor activity in mice. The peptides responsible for the beneficial effect have not yet been identified. Recently, presence of food-derived peptides in human blood has been detected post ingestion of other food protein hydrolysates. The peptides absorbed into blood circulation system have been associated with the biological response post ingestion, as they can reach target organs. The objective of the present study was to identify the food-derived peptides in rat blood after ingestion of LPH.

Results: In the *in vitro* exopeptidase digest of LPH, pyroglutamyl, prolyl, hydroxyprolyl, and aspartic dipeptides were identified. The aspartic peptides (Asp-Val, Asp-Ile, Asp-Leu, and Asp-Phe) showed multiple peaks by LC-MS/MS, indicating the presence of isomers. Four isomers

with L- and D-aspartyl residues, and α and β peptide bonds were present in each sequence. After administration of LPH, the amounts of unusual aspartic dipeptides with β peptide bond and/or D-aspartyl residue significantly increased in rat plasma, while those of the other usual aspartic peptides did not.

Conclusions: Indigestible isomerized aspartic di-peptides are released by *in vitro* exopeptidase digestion of LPH. These peptides are also generated by *in vivo* digestion and absorption process and increase in blood of rat upon ingestion of LPH.

Keywords: Liver protein hydrolysate, isopeptide, bioavailability, β peptide bond, isomerization, D-aspartyl residue.

INTRODUCTION

It has been demonstrated that oral administration of enzymatic hydrolysates of food proteins has some beneficial effects on human health beyond nutritional value, such as moderation of mild hypertension, hyperlipidemia, and inflammation [1, 2] A formulated liver protein hydrolysate (LPH) has been prepared by enzymatic hydrolysis of porcine liver protein. It contains peptides as major its components apart from small amounts of amino acids, saccharides, nucleic acids, and vitamins [3]. This formulated hydrolysate has been used as a nourishing tonic and also a food ingredient in Japan. Animal experiments have demonstrated some beneficial effects of the hydrolysate, such as amelioration of alcohol-induced liver toxicity [4, 5], exercise-induced fatigue [3] and attenuation of the concanavalin A-induced low locomotor activity by ingestion [6]. However, the key bioactive component in LPH has not been elucidated.

It was earlier assumed that orally administered peptides are completely degraded into amino acids during digestion and absorption processes. However, presence of food-derived peptides having biological activities in human and animal blood has been shown [7, 8], which broke the mold. Pro-Hyp and Pro-Gly are present in human blood at 10-50 μM after ingestion of 5-10 g of collagen [9] and elastin hydrolysate [10]. After the identification of peptides in blood, bioactivities of these peptides were also reported [11-13]. In addition to prolyl and hydroxyprolyl peptides, pyroglutamyl-leucine (pyroGlu-Leu), which was first identified as a hepato-protective peptide in wheat gluten hydrolysate, was detected in rat portal blood one hour after the ingestion of pyroGlu-Leu [14]. Recently, it has been reported that glutathione, which has isopeptide bond between γ-carboxyl group and α amino group, in food is directly absorbed

in its electrochemically reduced form in the intestine and then transported in the blood in bound forms [15]. Therefore, these prolyl, hydroxyprolyl, and pyroglutamyl peptides and isopeptides are resistant to gastro intestinal and blood exopeptidases. These findings suggest that food-derived peptides in blood are good candidates for bioactive peptide identification.

The objective of the present study was to identify the food-derived peptides in rat plasma after ingestion of LPH to predict bioactive peptides. In the previous study, we demonstrated that pre-identification of peptides in the exopeptidase digest of protein hydrolysate facilitates the identification of food-derived peptides in the human blood [16]. Employing this approach, the present study shows that aspartic (Asp) dipeptides with unusual β peptide bond and/or D-aspartyl residue are absorbed into the blood circulation upon ingestion of LPH, while usual aspartic peptides with α peptide bond and L-aspartic residue do not significantly increase in blood.

MATERIALS AND METHODS

Porcine liver protein hydrolysate

Porcine liver protein hydrolysate (LPH) was prepared by Zeria Pharmaceutical (Tokyo, Japan) and used as the commercially available formulated liver protein hydrolysate.

Regents

Acetonitrile (HPLC-grade) and porcine pancreatin were purchased from Nacalai Tesque (Kyoto, Japan). Porcine leucine aminopeptidase and bovine carboxypeptidase A were purchased from Sigma-Aldrich (St. Louis, MO). Heparin sodium was purchased from NIPRO (Osaka, Japan). For peptide synthesis, piperidine, 4-methylmorpholine, *N,N*-dimethylformamide, and *t*-butyl methyl ether were purchased from Wako Chemical (Osaka, Japan). L-pyroglutamic acid, 9-fluorenylmethoxycarbonyl (Fmoc) amino acid derivatives including Fmoc-L-Asp β-*t*-butyl ester, Fmoc-D-Asp β-*t*-butyl ester, Fmoc-L-Asp α-*t*-butyl ester, and Fmoc-D-Asp α-*t*-butyl ester, Fmoc amino acid-bond *p*-alkoxybenzyl alcohol (Alko) resin, proline-bond 2-chlorotrityl chloride (Barlos) resin, hydroxylproline-bond 2-chlorotrityl chloride (Barlos) resin, 1H-benzotriazol-1-yloxy-tri (pyrrolidino) phosphonium hexafluorophosphate and 1-Hydroxybenzotriazole were purchased from Watanabe Chemical Industries (Hiroshima, Japan).

Exopeptidase digestion of peptides in LPH

In vitro exopeptidase digestion of peptides in LPH was performed by a previously described

protocol [16]. LPH (2.5 mg) was dissolved in 1 mL of 50 mM Tris-HCl buffer (pH 8.0) and added along with pancreatin (100 μg/5 μL in the Tris-HCl buffer), 2.45 U/50 μL of leucine aminopeptidase (sol) and 7.7 U/5 μL of carboxypeptidase A (sol). The reaction mixture was kept at 37°C for 24 h with stirring. To terminate the reaction, enzymes were removed by ultrafiltration using an Amicon Ultra 10K device (Merck Millipore, Burlington, MA). The effluent was collected and used as the exopeptidase digest. To prepare pyroglutamyl peptides fraction, solid phase extraction with a strong cation exchanger (AG50W × 8, hydrogen form, 100-200 mesh, Bio-Rad Laboratories, Hercules, CA) was performed as described previously [16]. The non-adsorbed effluent was collected and used as the pyroglutamyl peptide fraction.

Identification of peptides in the in vitro exopeptidase digest

Peptides in the *in vitro* xopeptidase digest were fractionated by size exclusion chromatography (SEC) using Superdex Peptide 10/300 GL (GE Healthcare, Buckinghamshire, U.K.). The column was equilibrated with 0.1% formic acid containing 10% acetonitrile. Fractions were collected every 1 min at a flow rate of 0.5 mL/min. Aliquots (100 μL) of fractions 35-45 (corresponding to 34-45 min) were dried under vacuum and the amino group of peptides were reacted to 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AccQ) (Toronto Research Chemicals, Ontario, Canada). The residue was dissolved in 20 μL of distilled water and added to 20 μL of 0.3% AccQ acetonitrile solution and 60 μL of 50 mM sodium borate buffer (pH 8.8). The reaction was carried out at 50°C for 10 min. The reactant was mixed with 50 μL of 5 mM sodium phosphate buffer (pH 7.5) containing 5% acetonitrile and clarified by passing through a filter (Cosmonice, 4 mm i.d., 0.45 μm pore size, Nacalai Tesque). The filtrate (30 μL) was injected to liquid chromatography-electronspray ionization-tandem mass spectrometer (LC-MS/MS) using an LCMS 8040 (Shimadzu, Kyoto, Japan). Peptides were resolved by reversed phase high performance liquid chromatography (RP-HPLC) using an Inertsil ODS-3 column (2 mm i.d. × 250 mm, GL Science, Tokyo, Japan). Binary linear gradient was performed with 0.1% formic acid (solvent A) and 0.1% formic acid containing 80% acetonitrile (solvent B) at a flow rate of 0.2 mL/min. The gradient program was as follows: 0-30 min, 0-50 % B; 30-35 min, 50-100% B; 35-40 min, 100% B; 40-40.1 min, 100-0% B; 40.1-50 min, 0% B. The column was maintained at 40°C. AccQ-derivatives were specifically detected in the precursor ion scan mode by selecting the precursor ions, which generated the AccQ-derived product ion (b1, mass to charge ratio (*m/z*) = 171.1) at a positive mode by collision energy -35 eV in the scan range of *m/z* = 290-350, 350-400, 400-450, 450-500, 500-600, 600-800.

The pyroglutamyl peptide fraction (200 μL) was subjected to the SEC under the similar conditions as described above. Aliquot of the SEC fraction (30 μL) was subjected to the LC-MS using the previously described elution conditions. Total ion intensity was monitored at the positive mode in the scan range of *m/z* = 185-700. The *m/z* of AccQ-peptides and pyroglutamyl peptides were recorded. The same samples were re-analyzed by LC-MS/MS in the product ion scan mode by collision energy -15 and -35 eV targeting the precursor ions obtained during the first LC-MS/MS and -MS for the estimation of peptide structure. Peptide sequence was estimated on the basis of *m/z* of precursor ions, amino-terminal (a, b, c ions), carboxy terminal (x, y, z ions), and immonium ions [17].

Peptide synthesis

Peptides were synthesized by the Fmoc strategy using a PSSM-8 peptide synthesizer (Shimazu). For the syntheses of isomers of aspartic dipeptides, we used Fmoc-L-Asp β-*t*-butyl ester, Fmoc-D-Asp β-*t*-butyl ester, Fmoc-L-Asp α-*t*-butyl ester, and Fmoc-D-Asp α-*t*-butyl ester. Synthetic peptides were purified by RP-HPLC using a Cosmosil 5C18-MS-II column (10 i.d. × 250 mm, Nacalai Tesque). Binary linear gradient was performed with 0.1% formic acid (solvent A) and 0.1% formic acid containing 80% acetonitrile (solvent B) at a flow rate of 2 mL/min. The gradient program was as follows: 0-20 min, 0-50% B; 20-30 min, 50-100% B; 30-35 min, 100% B; 35-35.1 min, 100-0% B; 35.1-45 min, 0% B. The column was maintained at 40°C. Elution of peptides was monitored by absorbance at 214 and 254 nm. Contents of the peptides were evaluated by amino acid analysis following the HCl hydrolysis [18,19].

Animal experiment

All animal experiments were carried out at the Louis Pasteur Center for Medical Research (Kyoto, Japan). Five-week-old male Wistar rats were purchased from Japan SLC (Shizuoka, Japan) and acclimatized to the environmental conditions for 7 days. They were fed the MF diet (Oriental Yeast, Tokyo, Japan) during the acclimatization period. The rats were kept in a room (temperature: 24-26°C, humidity: 40-60%) under a 12 h light-dark cycle and had free access to diet and water. This study was conducted in accordance with the standards established by the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. All experimental procedures were approved by the Animal Care Committee of Louis Pasteur Center for Medical Research. Rats (average body weight:182 g) were divided into two groups; vehicle

(n=3) and LPH (n=3) groups. They were fasted for 18 h before the administration of test sample and vehicle. LPH was dissolved in distilled water and administered by sonde at 167 mg/kg body weight, while distilled water (vehicle) was administered as control. Same dose have been used in some human trials to detect food-derived peptides in blood [7-9]. Blood was collected from the inferior vena cava under isoflurane anesthesia immediately after administration of vehicle and 60 min after administration of LPH, respectively. Plasma was prepared by centrifugation at 800 × *g* for 10 min in the presence of heparin. To precipitate the protein, plasma was mixed with 3 volumes of ethanol and the supernatant was obtained by centrifugation at 13,000 × *g* for 10 min. Subsequently, the supernatant was stored at -20°C until further analysis.

Peptide determination

Aliquots of the *in vitro* exopeptidase digest (50 μL) and ethanol-soluble fraction of rat plasma (250 μL) were dried under vacuum conditions and then derivatized with AccQ as described above. Contents of the AccQ-peptides and pyroglutamyl peptides in the reaction mixture were determined by LC-MS/MS via multiple reaction monitoring (MRM) mode with previously described elution conditions. The gradient program for separation of isomers of aspartic peptides (Asp-Val, Asp-Ile, Asp-Leu, and Asp-Phe) was as follows: 0-100 min, 7-14.5% B; 100-105 min, 14.5-100% B; 105-110 min, 100% B; 110-110.1 min, 100-0% B; 110.1-120 min, 0% B. The synthetic peptides were used for optimization of MRM conditions by LabSolutions LCMS Ver. 5.5 (Shimadzu).

Statistical analyses

The statistical analysis was performed using software GraphPad PRISM version 6.04. (GraphPad software, San Diego, CA). Difference between the plasma peptide contents in vehicle and LPH groups was analyzed using Student's *t* test. $P<0.05$ was considered to indicate a statistically significant result.

RESULTS

Identification of peptides in the in vitro exopeptidase digest

The RP-HPLC-MS/MS chromatograms of AccQ-derivatives in the SEC fractions in the precursor scan mode are shown in Figure 1a. The RP-HPLC-MS chromatograms in the SEC fractions of pyroglutamyl peptide fraction in the total ion scan mode are shown in Figure 1b.

The peaks marked in uppercase in Figure 1a were identified as amino acids on the basis of their *m/z* and retention time. The peaks marked with asterisks could not be assigned to *m/z* of peptides with any combination of amino acids. The peaks marked in lowercase (Figure 1a and 1b) were assigned to precursor ions of peptides and then subjected to MS/MS analysis. Table 1 summarizes the estimated sequences of the peptides in the *in vitro* exopeptidase digest of LPH based on precursor and product ions. Peak k1 and k2, p1 and p2, q1 and q2, and r1 to r4 were estimated to be the same sequence due to exact same precursor and product ion patterns. The estimated peptides were first synthesized with all the L-amino acids. Presence of the estimated peptides in the *in vitro* exopeptidase digest of LPH was confirmed by comparison of the retention times of the synthetic peptides by LC-MS/MS in MRM mode. Consequently, presence of nine pyroglutamyl peptides and twenty peptides with amino group in the *in vitro* exopeptidase digest were confirmed (Figure 2). Pyroglutamyl peptides were found to be the major constituent in the digests. In addition to the pyroglutamyl peptides, prolyl, hydroxyprolyl, and aspartic dipeptides were also detected. The LC-MS/MS analysis in MRM mode revealed that some aspartic peptides; Asp-Val, Asp-Ile, Asp-Leu, and Asp-Phe showed multiple peaks (data not shown). The compositions of these aspartic dipeptides having the same retention time with standard from all L-amino acids are present in Figure 2. To sum up the results with the data on the product scan mode (Table 1), we speculated the presence of isomers of the aspartic dipeptides (Asp-Val, Asp-Ile, Asp-Leu, and Asp-Phe). Isomerized aspratic residues have been detected in proteins of aged animal and human, which has been associated with the loss of function of proteins by aging [20, 21]. However, presence of isomerized asparatic dipeptides in the food protein hydrolysates has not been reported. Thus, we decided to elucidate structure of these asparatic dipeptides. Therefore, the D-α and -β aspartic peptides, and L-β aspartic peptides were synthesized in addition to the usual L-α aspartic peptides. The four isomers of the aspartic peptides were resolved by RP-HPLC in different gradient conditions and detected by MS/MS in the MRM mode. As shown in Figure 3a, except for L-α Asp-Ile and L-α Asp-Leu, D-α Asp-Ile and D-α Asp-Leu, all isomers were resolved. Contents of these unresolved peptides are expressed as sum of two peptides. Interestingly, unusual forms of Asp-Val, Asp-Ile, Asp-Leu, and Asp-Phe; D-α and -β aspartic peptides and L-β aspartic peptides were detected in the *in vitro* exopeptidase digest in addition to the usual forms. Nonetheless, in all the cases, contents of the peptides with β peptide bond (isopeptide) were higher than those of the α peptide bond carrying peptides (Figure 3b).

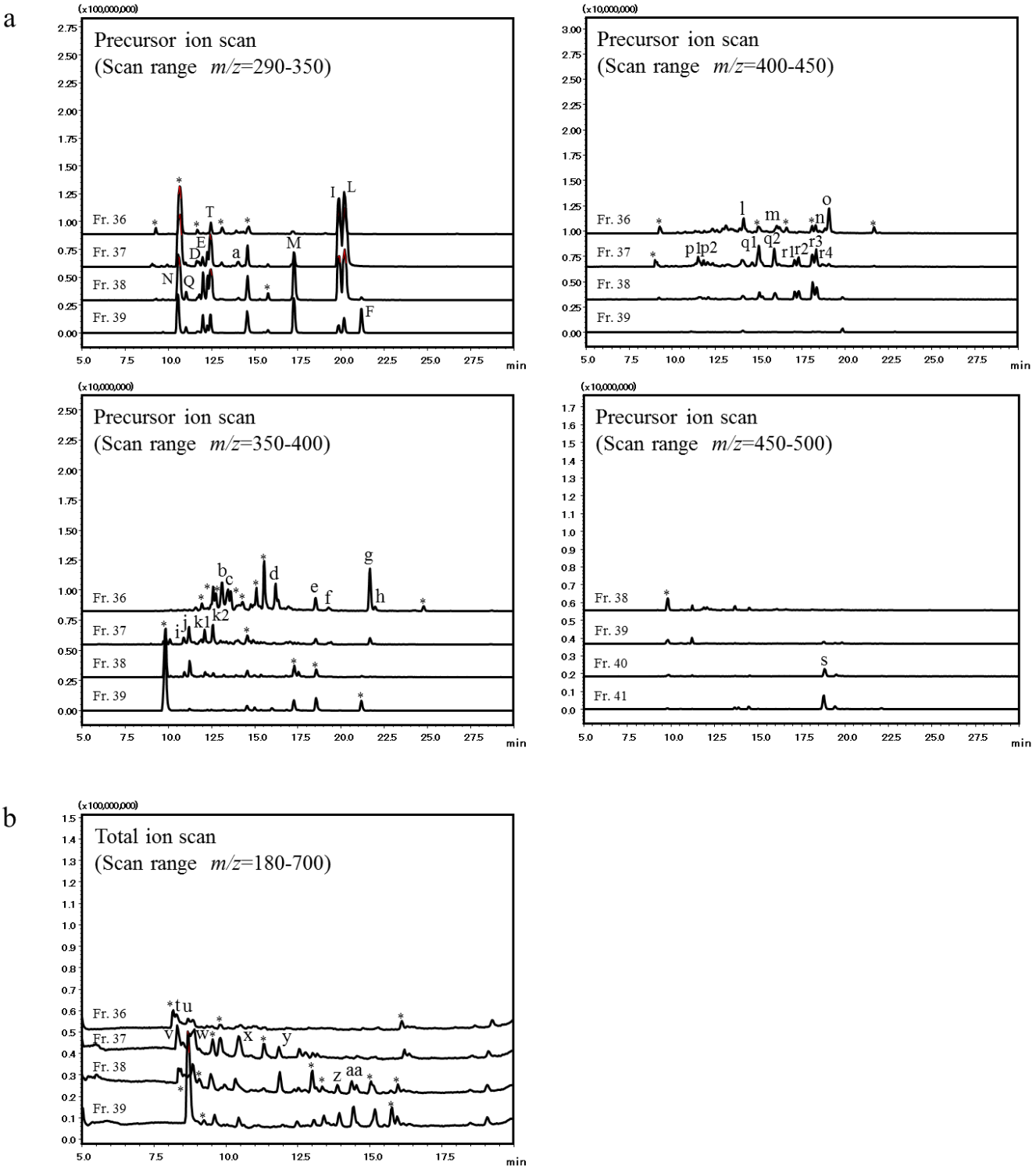

Figure 1. RP-HPLC-MS/MS and -MS chromatograms in SEC fractions

RP-HPLC-MS/MS chromatograms of AccQ-derivatives (a) and RP-HPLC-MS chromatograms of non-derivatives of pyroglutamyl peptide fraction (b). Peaks marked in uppercase represent amino acid derivatives. Lowercase, AccQ-peptides (a-s) and pyroglutamyl peptides (t-aa) were assigned to precursor ions of AccQ-peptides and pyroglutamyl peptides, respectively. Peaks marked with asterisks could not be assigned to peptides with any combination of amino acids.

Table 1. Estimated sequences of peptides in size-exclusion chromatographic (SEC) fractions of *in vitro* exopeptidase digests of liver protein hydrolysate

SEC Fr.	Peak	Sequence	Precursor ions (*m/z*)	Product ions (*m/z*)
37	a	Gly-Pro	343.2	116 (y1), 228.1 (b2)
36	b	Ala-Hyp	373.1	131.9 (y1), 171 (AccQ, b1), 203.1 ($[y2\text{-}NH_3]^+$), 242.2 (b2)
36	c	Pro-Hyp	399.2	70 (Pro*), 132 (y1), 229 ($[y\text{-}NH3]^+$)
36	d	Pro-Val	385.2	70 (Pro*), 215 ($[y\text{-}NH_3]^+$), 239.9 (a2), 268 (b2)
36-37	e	Pro-Ile	399.2	70 (Pro*), 229 ($[y\text{-}NH_3]^+$), 240 (a2), 268 (b2)
36	f	Val-Pro	385.2	72 (Val*), 116.2 (y1), 215 ($[y\text{-}NH3]^+$), 241.7 (a2), 269.9 (b2)
36-37	g	Ile-Pro	399.2	86 (Leu*), 116 (y1), 229 ($[y\text{-}NH_3]^+$), 256 (a2), 284 (b2)
36	h	Leu-Pro	399.2	86 (Leu*), 116 (y1), 229 ($[y\text{-}NH_3]^+$), 256 (a2), 284 (b2)
37-38	i	Asp-Ser	391.1	106.2 (y1), 202.9 ($[y\text{-}NH_3]^+$), 285.9 (b2)
37-38	j	Asp-Gly	361.1	76 (y1), 286 (b2)
37	k1	Asp-Ala	375.1	90 (y1), 171 (AccQ, b1), 205 ($[y\text{-}NH_3]^+$), 286 (b2)
37	k2	Asp-Ala	375.1	90.1 (y1), 115.9 (Asp*), 171 (AccQ, b1), 204.9 ($[y\text{-}NH3]^+$), 286 (b2)
36-38	l	Asp-Pro	401.2	69.9 (Pro*, Asp*), 115.8 (y1), 231 ($[y\text{-}NH_3]^+$), 258.1 (a2), 285.9 (b2)
36	m	Val-Hyp	401.2	72.1 (Val*), 132.1 (y1), 171 (AccQ, b1), 231.1 ($[y2\text{-}NH_3]^+$), 242 (a2), 270.2 (b2)
36	n	Ile-Hyp	415.2	86 (Leu*), 132.2 (y1), 244.9 ($[y2\text{-}NH_3]^+$), 256 (a2), 284 (b2)
36	o	Leu-Hyp	415.2	86 (Leu*), 132 (y1), 245 ($[y2\text{-}NH_3]^+$), 256 (a2), 284 (b2)
37	p1	Asp-Thr	405.1	69.9 (Asp*), 234.9 ($[y\text{-}NH_3]^+$), 286 (b2)
37	p2	Asp-Thr	405.1	120 (y1), 235 ($[y\text{-}NH_3]^+$), 286 (b2)
37-38	q1	Asp-Val	403.1	72.1 (Val*), 232.9 ($[y\text{-}NH_3]^+$), 285.8 (b2)
37-38	q2	Asp-Val	403.2	72.1 (Val*), 233 ($[y\text{-}NH_3]^+$), 285.8 (b2)
37-38	r1	Asp-Ile	417.1	86 (Ile*), 247 ($[y\text{-}NH_3]^+$), 285.9 (b2)
37-38	r2	Asp-Leu	417.1	86 (Leu*), 246.9 ($[y\text{-}NH_3]^+$), 286 (b2)
37-38	r3	Asp-Ile	417.1	86 (Ile*), 247.1 ($[y\text{-}NH_3]^+$), 285.9 (b2)
37-38	r4	Asp-Leu	417.1	69.9 (Asp*), 86.1 (Leu*), 247.1 ($[y\text{-}NH_3]^+$), 286 (b2)
40-41	s	Asp-Phe	451.1	70 (Asp*), 120 (Phe*), 280.9 ($[y\text{-}NH_3]^+$), 286.1 (b2)
36-38	t	pyroGlu-Gln	258.1	84 (pyroGlu*)
36-37	u	pyroGlu-Thr	231	74 (Thr*), 84 (pyroGlu*), 119.9 (y1)
37-38	v	pyroGlu-Asp	245	84 (pyroGlu*), 88 (Asp*), 115.9 (Asp*), 133.8 (y1)
37-38	w	pyroGlu-Glu	259.1	84 (pyroGlu*), 102 (Glu*), 148 (y1)
37	w	pyroGlu-Ala	201.1	44.1 (Ala*), 84 (pyroGlu*), 90 (y1)
37-39	x	pyroGlu-Pro	227	70 (Pro*), 84 (pyroGlu*), 116 (y1)
37-38	y	pyroGlu-Val	229.1	72 (Val*), 84 (pyroGlu*), 117.9 (y1)
38-39	z	pyroGlu-Ile	243.1	84 (pyroGlu*), 86 (Ile*)
38-39	aa	pyroGlu-Leu	243.1	84 (pyroGlu*), 86 (Leu*)

*means immonium ion.

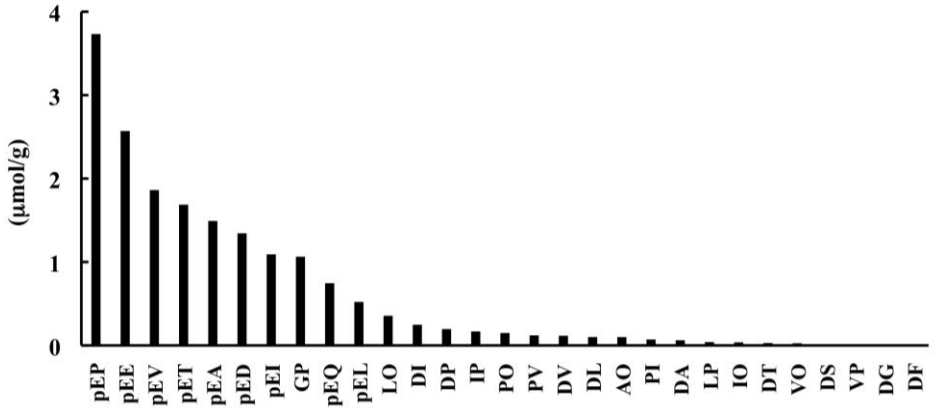

Figure 2. Contents of the peptides in the *in vitro* exopeptidase digest of LPH.

Peptides with retention time same as the standard peptides were determined by LC-MS/MS in MRM mode. Standard peptides with α peptide bond were synthesized from L-amino acids. One-letter abbreviations are used for amino acid residues while pE represents pyroglutamyl (pyroGlu) residue.

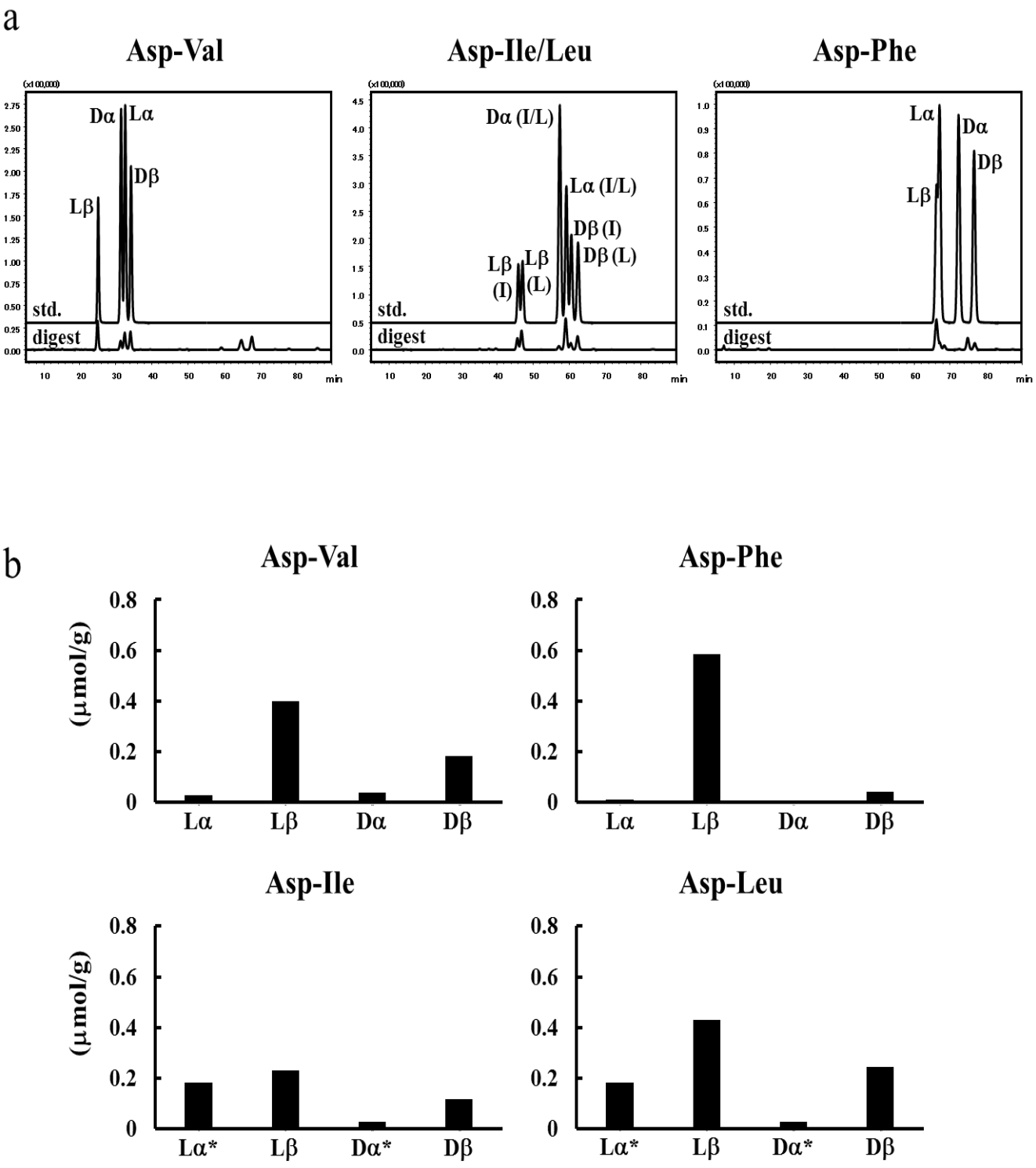

Figure 3. Presence of isomers of aspartic dipeptides in exopeptidase digest of LPH.

Resolution of isomers of Asp-Val, Asp-Ile, Asp-Leu, and Asp-Phe by RP-HPLC-MS/MS in MRM mode (a) and contents of the isomers in the exopeptidase digest (b). Std. and digest represent the chemically synthesized peptides and peptides in the *in vitro* exopeptidase digest, respectively. Lα, Lβ, Dα, and Dβ represent peptides with L-α Asp, L-β Asp, D-α Asp, and D-β Asp residues, respectively. I and L in parenthesis represent peptides with Ile and Leu residues. L-α Asp-Ile and L-α Asp-Leu, D-α Asp-Ile and D-α Asp-Leu could not be resolved. Values with * of the lower panel are sum of Asp-Ile and Asp-Leu.

Food-derived peptides in rat blood

We also checked the presence of the peptides as well as isomers of aspartic dipeptides, as shown in Figure 2 and Figure 3b, respectively, in the rat blood plasma upon ingestion of the LPH (167 mg/kg body weight) and vehicle. No significant increase in the levels of prolyl, hydroxyprolyl, and pyroglutamyl peptides was observed after ingestion of LPH. In contrast, levels of unusual forms of aspartic dipeptides with β peptide bond and/or D aspartyl residue significantly increased in the blood plasma after ingestion of LPH compared to vehicle, while no remarkable change in the levels of usual form of aspartic dipeptides was observed.

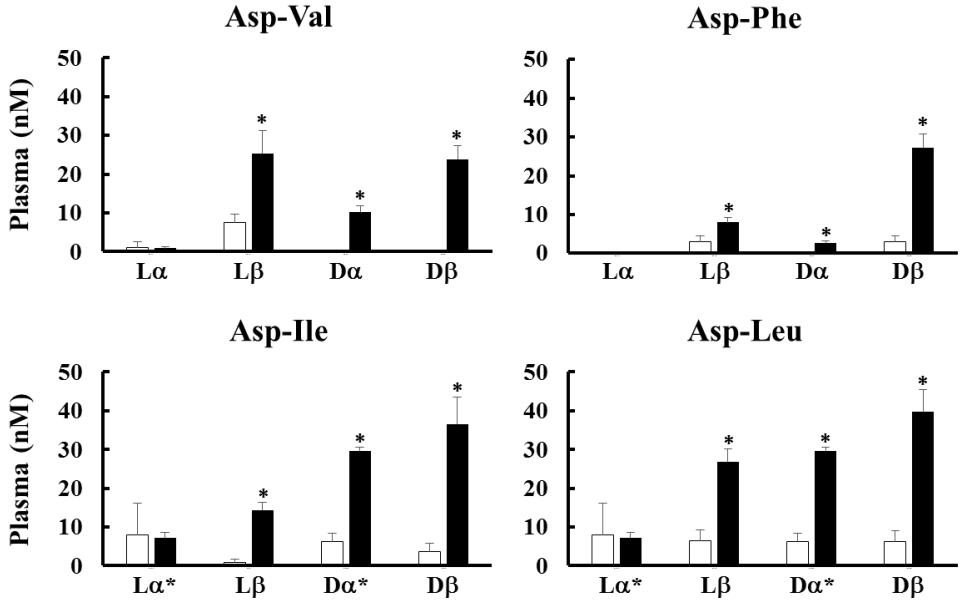

Figure 4. Contents of aspartic dipeptides in rat plasma after ingestion of LPH.

Data are shown as the mean ± standard deviation (n = 3). White and black bars represent contents in the plasma of rats, which received vehicle and LPH, respectively. Asterisks indicate significant differences between values of vehicle and after ingestion of LPH ($p<0.01$). Refer to the legend for Figure 3 for abbreviations.

DISCUSSION:

After ingestion of the sardine meat hydrolysate and lacto tripeptide (Ile-Pro-Pro)-enriched fermented milk, peptides with inhibitory activity against angiotensin converting enzyme have been detected in human blood [22, 23]. However, these products were detected at low nM levels. Recently, after ingestion of collagen and elastin hydrolysates, some food-derived peptides were identified in human blood at μM levels [7-10]. More recently, it was demonstrated that concentrations of some peptides significantly increase to 10-100 nM levels in human plasma after ingestion of corn and wheat gluten hydrolysates [16]. These peptides are prolyl,

hydroxyprolyl, and pyroglutamyl di- and tri-peptides. These food-derived peptides, produced after ingestion of the protein hydrolysate, exert significant *in vitro* and *in vivo* biological activities [10-14]. The present study showcases the presence of novel food-derived peptides in rat blood after oral administration of LPH. These peptides are Asp-Val, Asp-Ile, Asp-Leu, and Asp-Phe with β peptide bond and/or D-aspartyl residue. These unusual dipeptides resist *in vitro* exopeptidase digestion and show elevated levels in rat plasma after the ingestion. Generally, these unusual peptides are not encrypted in the parent protein. On the other hand, it has been reported that L-aspartyl residues in proteins convert to D-form while the α peptide bond between carboxyl group of aspartyl residue and amino group of adjutant residue convert to β peptide bond in human tissues during aging [20, 21]. The racemization and isomerization of aspartyl residues in proteins are induced via formation of succinimide as a reaction result of β carboxyl group of aspartyl residue and nitrogen in the peptide bond [20, 21]. These findings, perhaps, suggest that porcine liver proteins might possess such unusual peptide motives. More likely, these unusual aspartic peptides might be generated during the industrial preparation of LPH or following *in vitro* digestions, though there are no reports demonstrating the racemization and isomerization of aspartyl residues in short chain peptides during protease digestion, heat process, etc. In the previous study however, these peptides were not detected in the exopeptidase digest of corn and wheat gluten hydrolysates [16]. One possible explanation is the relatively lower Asp-Val, Asp-Ile, Asp-Leu, and Asp-Phe motives in the plant proteins. Alternatively, presence of some minor components, such as minerals, in the hydrolysate might enhance racemization and isomerization of aspartyl residues in peptides. Further studies probing the mechanism underlying racemization and isomerization of aspartyl residues in peptides during protease reaction using model peptides is in progress. The present study also demonstrates that the unusual aspartic dipeptides are absorbed into blood system by oral administration. However, biological activity of the aspartic dipeptides with β peptide bond and/or D-aspartyl residue remains to be solved. Further study on the effect of the unusual aspartic dipeptides on high fat diet-induced hepatitis in mice is under progress.

List of Abbreviations: LPH, Liver protein hydrolysate; HPLC, high performance liquid chromatography; LC-MS, liquid chromatography-mass spectrometry; LC-MS/MS, liquid chromatography tandem mass spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl. Three-letters abbreviations are used for amino acid residues in peptides, while Hyp and pyroGlu represent hydroxyprolyl and pyroglutamyl residues, respectively.

Competing Interests: There are no conflicts of interest to declare.

Authors' contributions: KS and KY designed the research. EA conducted the research. EA and KS prepared the manuscript.

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