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Presence of Modified Peptides with High Bioavailability and Angiotensin-Converting Enzyme Inhibitory Activity in Japanese Fermented Soybean Paste (*Miso*)

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ABSTRACT: Not only free amino acids and normal short-chain peptides but also modified amino acids, such as *N*-acetyl- and *N*-formyl amino acids, monoamines, polyamines, and modified peptides, such as isomerized aspartyl peptides, pyroglutamyl peptides, and diketopiperazines, were identified in Japanese fermented soy paste (*miso*) prepared using different fungal starters, rice, barley, and soybean-*koji*. One hour after oral administration of water extract of soybean-*koji miso* to rats, the modified peptides increased significantly in the lumen upon the ingestion, while the normal peptides did not. In the blood from the portal vein and abdominal vena cava, 17 and 15 diketopiperazines, 16 and 12 isomerized aspartyl peptides, and 2 and 1 pyroglutamyl peptides significantly increased to approximately 10−400 nM, respectively. The modified peptides, which increased in rat blood, showed angiotensin-converting enzyme (ACE) inhibitory activity in a dose-dependent manner, indicating multiple ACE inhibitory peptides with high bioavailability in *miso*. Among them, L-*β*-Asp-Pro showed the highest ACE inhibitory activity (IC_{50} 4.8 μM)

KEYWORDS: *Miso, Japanese fermented soybean pastes, isomerized aspartyl peptide, racemized aspartyl peptide, diketopiperazine, pyroglutamyl peptide, N-acetyl amino acid, monoamine, polyamine, bioavailability, angiotensin-converting enzyme (ACE)*

INTRODUCTION

Fermented seasonings such as soy sauce (*shoyu*), soy paste (*miso*), and Japanese rice wine (*sake*) are frequently used in traditional Japanese dishes. Among them, *miso* is an important ingredient in Japanese soup.[1] *Miso* soup is made from *miso*, soup stock, and other ingredients such as vegetables, seaweed, fish, and meat. Kuroda et al. demonstrated that the consumption of soup in Japan, mostly *miso* soup, is significantly higher than that in Western and other Asian countries.[2]

Japanese *miso* is manufactured using a fungal starter, referred to as *koji*. Various types of *miso* are manufactured in Japan and are prepared using different ingredients and methods.[1] Generally, Japanese *miso* is classified into rice-*koji*, barley-*koji*, and soybean-*koji miso*.[1] The rice-*koji miso* can be subclassified into short-aging (less than one month) and long-aging types (several months to years). The short-aged type contains a lower salt concentration and has a lighter color (vague) and sweeter taste than the long-aged type. The rice-*koji* and barley-*koji* are prepared by inoculation of spores of *Aspergillus oryzae* or *A. sojae* to steamed rice or barley after cooling and incubated at approximately 35 °C for a few days, which is used as rice-*koji* or barley-*koji*. Rice-*koji* or barely-*koji* is mixed with steamed soybeans and salt and then aged in a tank. To prepare soybean-*koji miso*, steamed soybean is mashed and shaped into a ball (1.9−6.5 cm), referred to as *miso*-ball, or *miso-dama*. Spores of *A. sojae* or *A. oryzae* are inoculated on the surface of the *miso*-ball, incubated for 3 days, and used as soybean-*koji*. The soybean-*koji* is remushed and mixed with salt, placed in a wooded tank, and aged under the pressure of stones for three years.

Miso consumption in Japan has decreased by approximately to half over the last 50 years owing to changes in dietary habits.[3] Dietitians and medical doctors generally recommend patients suffering from hypertension to suppress consumption of *miso* due to the high salt concentration of *miso* (10−15%). However, epidemiological studies have indicated the absence of a positive relationship between the frequency of *miso* soup consumption and blood pressure levels.[4,5] Furthermore, an intervention study demonstrated that consumption of *miso* significantly reduces blood pressure at night.[6] Animal experiments have also shown that the aqueous *miso* extract does not induce hypertension, whereas saltwater at the same concentration as the *miso* extract induces hypertension in Dahl salt-sensitive rats.[7] Epidemiological studies have demonstrated that consumption of *miso* has other health benefits. Kuroda et al. reported that the consumption of *miso* soup is negatively

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correlated with obesity and the waist-to-hip ratio.[2,8] Ikeda et al. reported that women who consumed *miso* soup daily in the Nagahama Cohort showed significantly lower homeostatic model assessment for insulin resistance (HOMA-IR), a risk factor for diabetes.[9] These facts indicate that *miso* contains some specific compounds for its beneficial effects by the consumption of *miso*.

An earlier study demonstrated the presence of peptides in *miso* that did not provide a peptide sequence, possibly because of the presence of so many compounds in *miso*.[10] Shirako et al. recently reported the presence of 12 pyroglutamyl dipeptides in *miso*.[11] In that study, pyroglutamyl peptides in *miso* were specifically detected by using a liquid chromatography tandem mass spectrometer (LC-MS/MS) in the precursor ion scan mode, targeting the immonium ion from the pyroglutamyl residue (mass-to-charge ratio; m/z 84.1). Pyroglutamyl peptides are generated from peptides with a glutaminyl residue at the amino terminus via nonenzymatic cyclization of the glutaminyl residue.[12] Shirako et al. also found that a hydrophobic pyroglutamyl peptide, pyroglutamyl-leucine (pyroGlu-Leu), ameliorated high-fat diet-induced obesity[11] and disturbance of the gut microbiota (dysbiosis) by increasing the secretion of host antimicrobial peptides into the lumen.[13] These findings can explain some of the beneficial effects of the consumption of *miso*.

Numerous studies have reported that some peptides in food protein hydrolysates and fermented foods inhibit angiotensin-converting enzyme (ACE), a key enzyme in the production of the hypertensive peptide hormone, angiotensin II.[14] Based on these facts, ACE inhibitory peptides in food are assumed to be responsible for the antihypertensive activity upon ingestion. To exert antihypertensive effects via ACE inhibition, the ACE inhibitory peptides must reach target organs such as the lungs via blood circulation. However, the concentrations of the food-derived ACE inhibitory peptides in the blood are significantly lower (few nM levels)[15,16] than those used for *in vitro* ACE inhibitory activity (μM levels).[17,18] These facts have raised doubts regarding the proposed antihypertensive effects of the food-derived peptides based on their ACE inhibitory activity. In water extract of *miso*, the ACE inhibitory activity was also found.[19,20] However, *miso*-derived peptides and other compounds with ACE inhibitory activity have not been detected in the blood circulation of animals and humans after the ingestion of realistic doses of *miso*.

The objectives of the current study were to comprehensively identify short-chain peptides in the three types of Japanese *miso* and examine their bioavailability using a rat model. Furthermore, the ACE inhibitory activity of *miso* peptides, which were transferred to blood circulation upon ingestion, was examined.

MATERIALS AND METHODS

Materials. Soybean-*koji miso* (A), rice-*koji miso* (B−D), and premixed rice-*koji* and barley-*koji miso* (*miso* E) were purchased from a local market. Rice-*koji miso* (D) is the short-aged type and is referred to as white *miso*. The other *miso* samples are of the long-aged type.

Reagents. HPLC-grade acetonitrile, phosphate buffered saline (PBS), and benzoyl chloride were purchased from Nacalai Tesque (Kyoto, Japan). A mixture of amino acid standards (Type H) was purchased from Wako Chemicals (Osaka, Japan). 9-Fluorenylmethyloxycarboxynyl (Fmoc)-amino acids, pyroglutamic acid (H-Pyr−OH), and Fmoc-amino acid-bound resins (Wang resin, Alko resin, and Trt(2-Cl)-resin) were purchased from Watanabe Chemical Industries (Hiroshima, Japan). 6-Aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AccQ) was purchased from Toronto Research Chemicals (Toronto, ON, Canada). *N*-Formylsaccharin was purchased from Tokyo Chemical Industries (Tokyo, Japan). A long- and short-chain fatty-acid labeling kit was purchased from YMC (Kyoto, Japan). The following reagents were used as the quantification standards: phenethylamine, tyramine, pyrrolidine, γ-aminobutylic acid (GABA), 3,4-dihydroxy-L-phenylalanine (L-DOPA), histamine dihydrochloride, ethanolamine, thymidine, β-alanine, cadaverine, putrescine, and spermidine were purchased from Nacalai Tesque. 2-Methylbutylamine, amylamine, isopentylamine, isobutylamine, tryptamine hydrochloride, uridine, *N*-acetylglycine, *N*-α-acetyl-L-glutamine, *N*-acetyl-L-valine, *N*-acetyl-L-tyrosine, *N*-acetyl-L-phenylalanine, *N*-acetyl-L-glutamic acid, *N*-acetyl-L-leucine, and *N*-α-acetyl-L-lysine were purchased from Tokyo Chemical Industry. D/L-Methionine sulfoxide, *N*-acetyl-L-isoleucine, *N*-acetyl-L-alanine, *N*-acetyl-L-proline, *N*-ε-acetyl-L-lysine, and acetyl putrescine hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). *N*8-acetyl-spermidine dihydrochloride was purchased from Toronto Research Chemicals. *N*1-acetyl-spermidine hydrochloride was purchased from Cayman Chemical (Ann Arbor, MI, USA). *N*1-acetyl-spermine hydrochloride was purchased from Funakoshi (Tokyo, Japan). β-Alanine amide hydrochloride was purchased from Watanabe Chemical Industries (Hiroshima, Japan). Cyclo(L-alanyl-L-proline) was purchased from the Peptide Institute (Osaka, Japan).

Identification of Peptides and Their Modifications in *Miso*. Soybean-*koji miso* paste (1 g) was mixed with four volumes of water and vigorously stirred for several minutes. The resultant suspension was centrifuged at 10,000*g* for 10 min at 5 °C. The supernatant was collected and used as the water extract of soybean-*koji miso* for subsequent experiments.

The primary and secondary amines of the compounds in the water extract were derivatized with AccQ, as described previously.[21] The extract (20 μL) was mixed with 60 μL of 50 mM sodium borate buffer (pH 8.8) and 20 μL of a 0.3% (w/v) AccQ-acetonitrile solution. The reaction was performed 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 it through a filter (Cosmonice, 4 mm i.d., 0.45 μm pore size, Nacalai Tesque). The filtrate (10 μL) was injected into LC-MS/MS using an LCMS-8040 instrument (Shimadzu Kyoto, Japan). AccQ derivatives were resolved by reversed-phase high-performance liquid chromatography (RP-HPLC) using an Inertsil ODS-3 column (2.1 mm i.d. ×250 mm; GL Science, Tokyo, Japan). Binary linear gradient was performed using 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; 40−50 min, 0% B. The column was maintained at 40 °C. To prevent salts in the sample from being delivered to the MS unit, the eluent from the column for 4 min after sample injection was discarded, and then eluent was delivered to the MS unit for 4−40 min. The same was done in the analysis below. AccQ derivatives were specifically detected in the precursor ion scan mode by selecting the precursor ions that generated the AccQ-derived product ion (m/z 171.1) in the positive ion mode at a collision energy of −35 eV in the scan ranges of m/z 250−300, 300−325, 325−350, 350−375, 375−400, 400−450, and 450−500.

To obtain amino group-blocked peptides and amino acids, the water extract was fractionated by solid-phase extraction using a strong cation exchanger (AG50W-×8, hydrogen form, 100−200 mesh, Bio-Rad Laboratories, Hercules, CA, USA) as described previously.[21] The resin was prewashed with 50% methanol and packed into a spin column (15 × 7 mm inner diameter, 5.0 μm pore size, Ultrafree-MC, Merck, Darmstadt, Germany). Two hundred microliters of 50% methanol were further added onto the column and eluted by centrifugation at 815×*g* (five times), and then the column was equilibrated with 200 μL of 10% acetonitrile containing 0.1% formic acid (five times). An aliquot (200 μL) of the water extract was loaded onto the spin column and eluted by centrifugation. The eluent was collected and referred to as the AG50 nonabsorbed fraction. The

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AG50 nonabsorbed fraction was clarified by passing it through a Cosmonice Filter. The filtrate (10 μL) was analyzed directly by LC-MS/MS. The gradient program was as follows: 0−30 min, 0−30% B; 30−40 min, 30−100% B; 40−45 min, 100% B; 45−55 min, 0% B. Other elution conditions were the same as those described above. Pyroglutamyl peptides were specifically detected in the precursor ion scan mode by selecting the precursor ions that generated the immonium ion of the pyroglutamyl residue (m/z 84.1) in the positive mode with a collision energy of −35 eV. Other amino group-blocked peptides and amino acids in the AG50 nonabsorbed fraction were detected in total ion monitoring mode. The scan ranges were as follows: m/z 100−200, 200−225, 225−250, 250−275, 275−300, and 300−350. The m/z values of the precursor ions in the major peaks were also recorded. The same samples were reanalyzed by LC-MS/MS in the product ion scan mode at collision energies of −15, −25, and −35 eV to obtain product ions to estimate the structure of the peptides and amino acid derivatives. The peptide sequence was determined based on the precursor and product ions according to definition by Papayannopoulos.[22]

Synthesis of Peptides and *N*-Acetyl Amino Acids. Peptides were synthesized using the Fmoc strategy using a PSSM-8 solid-phase peptide synthesizer (Shimadzu). The synthesis and cleavage reactions were performed according to protocols provided by the manufacture's instructions. Pyroglutamic acid was used instead of Fmoc-amino acids to synthesize the pyroglutamyl peptides. *N*-Acetyl amino acids were synthesized similarly using acetic anhydride instead of Fmoc-amino acid. Fmoc-amino acid-bound Alko resin or Wang resin was used for the synthesis of nonmodified peptides and *N*-acetyl amino acids, except for peptides with proline at the carboxy-terminal. For the synthesis of linear peptides with proline at the carboxy terminus, proline-bound Trt(2-Cl)-resin, whose linker has high steric hindrance, was used, as diketopiperazines are frequently generated using the Fmoc-proline-Wang resin.

Four isomers of dipeptides with aspartyl residues at the amino terminus (L-α-aspartyl peptides, L-β-aspartyl peptides, D-α-aspartyl peptides, and D-β-aspartyl peptides) were synthesized using the method described by Ejima et al.[23] Fmoc-L-Asp(OtBu)−OH, Fmoc-L-Asp-OtBu, Fmoc-D-Asp-(OtBu)−OH, and Fmoc-D-Asp-OtBu were used for L-α, L-β, D-α, and D-β aspartyl peptides, respectively. For Asp-Gly, we synthesized only L-α and L-β Asp-Gly.

For the direct synthesis of diketopiperazines such as cyclo-(Ser-Pro), cyclo-(Thr-Pro), cyclo-(Pro-Pro), cyclo-(Val-Pro), cyclo-(Ile-Pro), cyclo-(Leu-Pro), and cyclo-(Phe-Pro), Fmoc-proline-bound Wang resin was used. Synthesis and cleavage reactions were performed as previously described. However, cyclo-(Asn-Pro), cyclo-(Asp-Pro), cyclo-(Glu-Pro), and cyclo-(Tyr-Pro) could not be synthesized by using this method. These diketopiperazines were synthesized by the conversion of linear peptides via heat treatment.[24] Linear Asn-Pro, Glu-Pro, and Tyr-Pro were dissolved in 0.1 M sodium phosphate buffer (pH 6.9) and heated to 100 °C for 3 h. Diketopiperazines without prolyl residues, such as cyclo-(Ser-Ile), were synthesized from linear dipeptide methyl esters using the method of Niteck et al.[25] with some modifications. Linear dipeptides were esterified with methanol in the presence of a sulfonated polystyrene resin (AG50W×8, hydrogen form). The resin was packed in a spin column, as described above. The resin was equilibrated with a 50% methanol−water solution. Peptide solution (approximately 0.1 mg/200 μL) in 0.1% formic acid containing acetonitrile at 50% (v/v) was loaded onto a column and left to stand for 90 min at room temperature. The resultant peptide methyl ester was eluted with a 7.5 M ammonium water solution (200 μL five times). The effluent was dried under vacuum, dissolved in *tert*-butanol containing 0.1% acetic acid (1 mL), and heated at 98 °C for 3 h. The solvent level was maintained during the reaction by adding an *tert*-butanol solution. The final product was dried under vacuum.

Synthesis of *N*-Formyl Isoleucine and *N*-Formyl Leucine. *N*-Formyl isoleucine and *N*-formyl leucine were synthesized by the method of Cochet et al.[26] One millimole of *N*-formyl saccharin was suspended in THF (1 mL). One millimole of Ile or Leu was added, and the reaction was performed for 15 min at room temperature with stirring.

Purification of Synthetic Peptides and Amino Acid Derivatives. Synthetic peptides and amino acid derivatives were purified by RP-HPLC using a Cosmosil 5C18-MS-II column (10 mm inner diameter × 250 mm, Nacalai Tesque). A binary linear gradient was set up by using 0.1% formic acid (solvent A) and 0.1% formic acid containing 80% acetonitrile (solvent B) at a flow rate of 2.0 mL/min. The gradient program was 0−20 min, 0−50% B; 20−30 min, 50−100% B; 30−35 min, 100% B; 35−35.1 min, 100−0% B; and 35.1−45 min, 0% B. The column temperature was maintained at 40 °C. Peptide elution was monitored at 214 and 254 nm. The purity of the synthetic peptides was determined using the LC-MS. The contents of the synthetic compounds were evaluated by amino acid analysis of their HCl hydrolysates using the method of Bidlingmeyer et al.[27] However, the nonprolyl diketopiperazines examined were not completely hydrolyzed using the vapor HCl hydrolysis conditions. Intact diketopiperazine, linear dipeptides, and constituent amino acids from diketopiperazine were present in the hydrolysate. The intact diketopiperazine before and after hydrolysis were detected using LC-MS/MS in the multiple reaction (MRM) mode. A decrease in the diketopiperazine peak area was observed after hydrolysis. Next, increases in the sum of linear dipeptides and constituent amino acids in the hydrolysate were determined by amino acid analysis, which corresponded to a decrease in the level of diketopiperazine. Based on these data, a standard curve for nonprolyl diketopiperazines was constructed.

Determination of Peptides and Amino Acid Derivatives in *Miso*. Peptides and amino acid derivatives in the water extracts were quantified using LC-MS/MS in the MRM mode. Synthetic and commercial peptides and amino acid derivatives were used to optimize multireaction monitoring (MRM) conditions using LabSolutions LC-MS ver. 5.5 (Shimadzu). These compounds were used as external standards for quantification. Compounds with primary and secondary amines and peptides were derivatized with AccQ, and the AccQ derivatives were resolved using the elution conditions described above. For separation of four isomers of aspartyl dipeptides consisting of D/L-aspartyl residues with alpha and beta peptide bonds, other three elution programs using the same solvents were used as follows; 0−20 min, 4% B; 20−20.1 min, 4−9% B; 20.1−36 min, 9% B; 36−40 min, 9−30% B; 40−45 min, 30−100% B; 45−55 min, 100% B; 55−65 min, 0% B (separation of isomers of Asp-Asn, Asp-Gln, Asp-Asp, Asp-Glu, and Asp-Pro), 0−45 min, 2−5% B; 45−50 min, 5−100% B; 50−55 min, 100% B; 55−65 min, 2% B (Asp-Ser, Asp-Gly, Asp-Thr, and Asp-Ala); and 0−100 min, 7−14.5% B; 100−105 min, 14.5−100% B; 105−110 min, 100% B; and 110−120 min, 7% B (Asp-Val, Asp-Ile, Asp-Leu, and Asp-Phe), respectively.

Pyroglutamyl peptides and diketopiperazines in the aqueous extract were directly determined using LC-MS/MS in MRM mode without derivatization. The elution conditions the same as those used for the precursor ion scan of the AG50-nonabsorbed fraction were used.

N-Terminally acetyl and formyl amino acids were derivatized from their carboxyl group with 2-nitrophenylhydrazine using a long-chain and short-chain fatty-acid labeling kit. An aliquot (60 μL) of water extract was mixed with 40 μL of reagent A (2-nitrophenylhydrazine, 2-NPH) and B [1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide, hydrochloride] and reacted at 60 °C for 20 min. The reactant was mixed with 40 μL of regent C (KOH) and kept at 60 °C for 15 min. To neutralize the reactant, 20 μL of 10% formic acid was added. For separating the 2-NPH derivatives, a binary linear gradient was set up using 0.1% formic acid (solvent A) and 0.1% formic acid containing 80% acetonitrile (solvent B). The gradient program was 0−20 min, 0−40% B; 20−40 min, 40−100% B; 40−50 min, 100% B; and 50−60 min, 0% B.

Cadaverine, putrescine, spermidine, spermine, acetyl putrescine, *N*-acetyl spermidine, *N*-acetyl spermine, and *Nα*- and *Nε*-acetyl lysine were derivatized with benzoyl chloride as previously described.[28,29] An aliquot (10 μL) of water extract was mixed with 45 μL of 100 mM sodium borate buffer (pH 8.8) and 45 μL of 2% benzoyl chloride solution (v/v in acetonitrile) and kept at room temperature for 10

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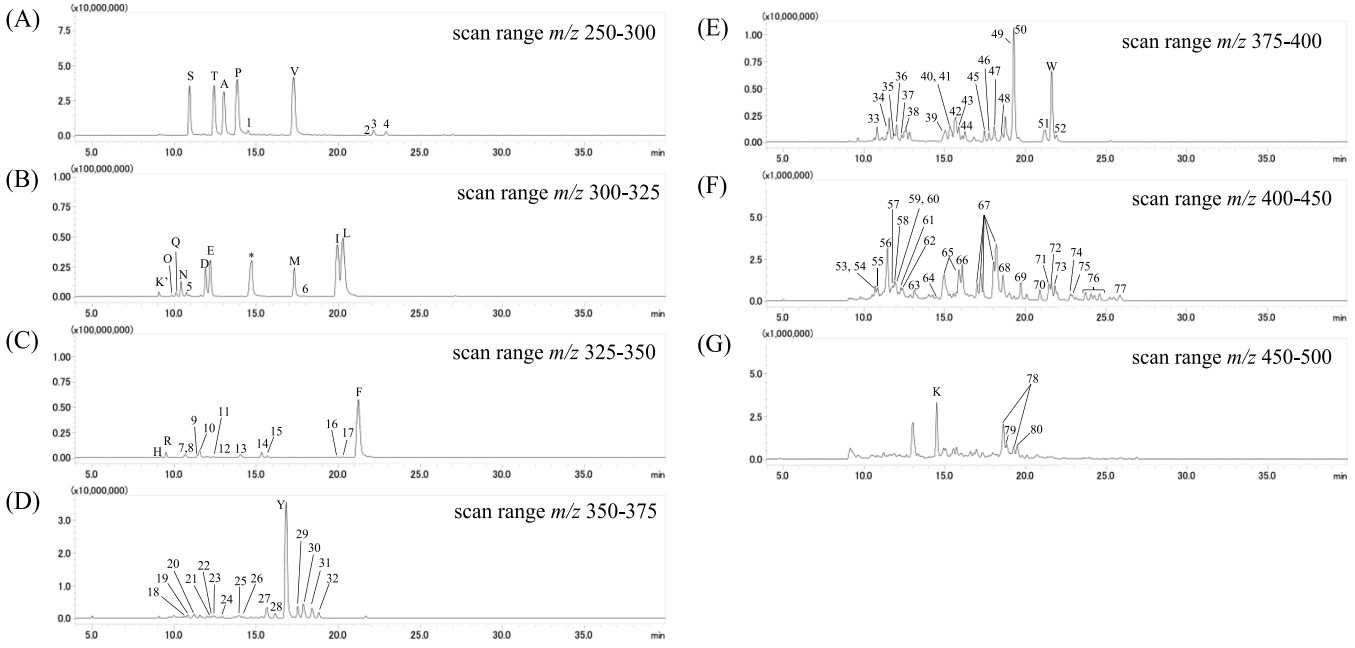

Figure 1. Mass spectrometry (MS) chromatograms of AccQ derivatives of amino compounds in aqueous extract of soybean-*koji miso*. AccQ derivatives were detected by the precursor ion scan targeting the AccQ-derived b1 ion in the scan ranges of *m*/*z* 250−300 (A), 300−325 (B), 325−350 (C), 350−375 (D), 375−400 (E), 400−450 (F), and 450−500 (G). Amino acid peaks are indicated with one letter abbreviation. O and K′ represent hydroxyproline and lysine of which one amino group is reacted with AccQ, respectively. Asterisk (*) indicates the reagent peak. Peaks marked with a number (peak 1−80) were subjected to product ion scan analysis to estimate the structure. Different peaks with the same number, such as peaks 65, 67,76, and 78, shared the same *m*/*z* value.

min with continual stirring. The reactant was mixed with 50 μL of 5 mM sodium phosphate buffer (pH 7.5) containing 5% acetonitrile and clarified by centrifugation (12,000*g*, 4 °C, 10 min) and passing through the Cosmonice filter. The derivatives were determined using LC-MS/MS in MRM mode using another reversed-phase column (Cosmosil 5C18 MS-II, 2.0 mm i.d. ×150 mm, Nacalai Tesque). A binary linear gradient was set up using 0.1% formic acid (solvent A) and 0.1% formic acid in 100% acetonitrile (solvent B). The column was equilibrated using 15% B for 10 min. The gradient program was 0−10 min, 15−25% B; 10−25 min, 25−100% B; and 25−30 min, 100% B, and re-equilibration for 10 min.

Animal Experiments. All of the animal experiments were performed at the Louis Pasteur Center for Medical Research (Kyoto, Japan). This study was conducted in accordance with 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 the Louis Pasteur Center for Medical Research (approval number: 20211). Five-week-old male Wistar/ST rats (120 and 140 g) were purchased from Japan SLC (Shizuoka, Japan) and acclimatized to environmental conditions for 1 week. They were fed a rodent diet (MF 12 mm pellets, 359 kcal/100 g; Oriental Yeast, Tokyo, Japan) during the acclimatization period. The rats were allowed free access to water and food at 24−26 °C and a humidity of 40−60% under a 12 h light/dark cycle.

The rats (average body weight: 148 g) were fasted for 16 h at night. Distilled water (vehicle) was administered as control (n = 3). Aqueous extract of *miso* (2 mL) prepared from 1.0 g of soybean-*koji miso* per kg of body weight was administered to the rats (n = 3). The average body weight of a Japanese person is approximately 60 kg (men 70.4 and women 55.6 kg), and 17−20 g of *miso* is used per serving of miso soup, so 1 g/kg is approximately equivalent to an intake of 3 bowls of *miso* soup per day. All rats were sacrificed under isoflurane anesthesia immediately 60 min after administration of the vehicle or aqueous extract of soybean-*koji miso*, as our preliminary study indicated that orally administered peptides were absorbed into the blood and also remained in the intestinal lumen 60 min after administration. Blood was collected from the portal vein and abdominal vena cava using a heparinized syringe. Portal and abdominal blood were centrifuged (800*g*, 4 °C, 10 min) to obtain plasma. After collecting the small intestine, the inner contents were immediately flushed out with 10 mL of cold PBS. The blood plasma and inner contents of small intestines suspended in the PBS were mixed with three volumes of ethanol, and the supernatants were obtained by centrifugation (12,000*g*, 5 °C, 10 min). Supernatants were stored at −80 °C until use.

Peptides and amino acid derivatives identified in *miso* were quantified in rat plasmas (portal and peripheral blood) and inner contents of the small intestines by LC-MS/MS in the MRM mode, as described above. Aliquots of ethanol supernatant of plasma (100 μL) and inner contents of the small intestine (50 μL) were dried and used as samples for quantification analyses, as described above.

ACE Inhibitory Activity. Each synthetic peptide was dissolved in 0.1% formic acid, yielding a 10 mM solution, and was used as a stock solution. Stock solutions were mixed and further diluted with distilled water to obtain appropriate concentrations and used to measure the ACE inhibitory activity. The ACE inhibitory activities of peptide mixtures and single peptide solutions were determined using an ACE Kit-WST (Dojindo Laboratories, Kumamoto, Japan), according to the manufacture's protocol. The dose-inhibitory data were fitted using the four-parameter log−logistic function in ImageJ software to obtain the half-maximal inhibitory concentration (IC_{50} value).

Statistical Analysis. The results of the experiments were presented as the mean ± standard deviation (SD). Statistical analyses were performed using R version 4.1.0. Differences in the average values between the two groups (control group vs soybean-*koji miso* group) were analyzed using Welch's *t*-test. The ACE inhibitory activity of the control and samples at different concentrations was evaluated by using the Steel−Dwass *t*-test. The significance level was set at 5%.

RESULTS

Identification of Compounds with Primary and Secondary Amines. Compounds with primary and secondary amines in the aqueous extract of soybean-*koji miso* were derivatized with AccQ and analyzed by LC-MS/MS in

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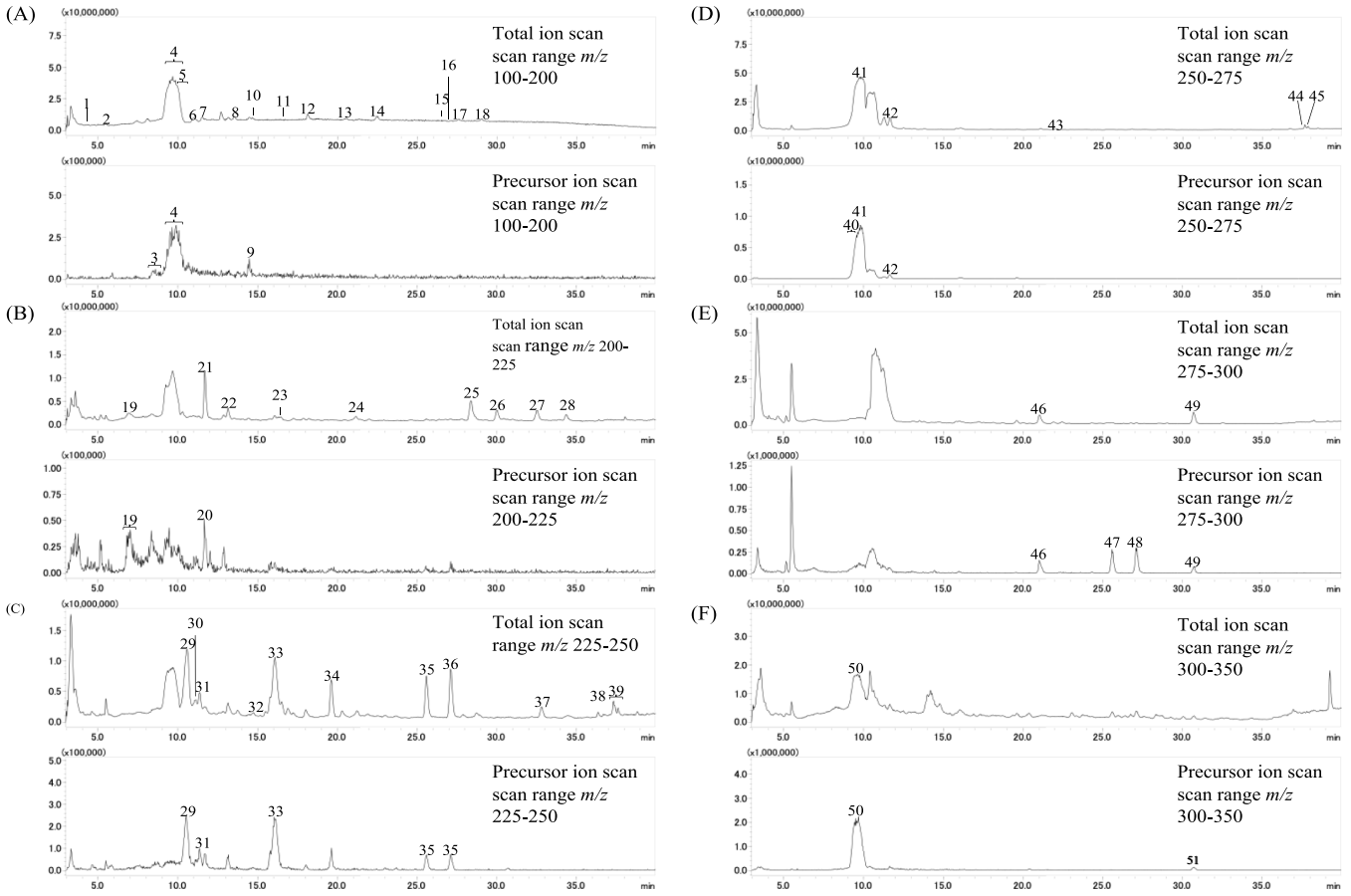

Figure 2. MS chromatograms of compounds in the AG50 nonabsorbed fraction of aqueous extract of soybean-*koji miso*. Compounds were detected by the total ion scan (upper chromatograms), and pyroglutamyl peptides were detected by the precursor ion scan targeting immonium ion of pyroglutamyl residue (lower chromatograms) in the scan ranges of *m*/*z* 100−200 (A), 200−225 (B), 225−250 (C), 250−275 (D), 275−300 (E), and 300−350 (F). Peaks marked with a number (peak 1−51) were subjected to product ion scan analysis.

precursor ion scan mode, targeting the b1 ion generated from the AccQ moiety (*m*/*z* 171.1). Mass chromatograms (Figure 1A−G) were obtained by scanning across different *m*/*z* ranges (250−300, 300−325, 325−350, 350−375, 375−400, 400−450, and 450−500). Amino acids, monoamines, and polyamines were identified by molecular weight based on the *m*/*z* of the precursor ions and the retention time of the AccQ derivatives (Supporting Information, Table S1). All proteins constituting amino acids, except for cysteine, were detected and are indicated with one letter abbreviation for amino acids on each peak (Figure 1). Lysine coupled with one and two AccQs are indicated by K′ and K, respectively. Peaks that could not be assigned to the protonated ions of proteins constituting amino acids were numbered (Figure 1). The precursor ions in the numbered peaks were further analyzed by LC-MS/MS in product ion scan mode to estimate their structures. The precursors and products ions of the numbered peaks are presented in Table S1. Adduct formation with acetonitrile was observed for AccQ-Ile (peak 16), AccQ-Leu (peak 17), and AccQ-Phe (peak 51). The presence of four mono amines, 2-methyl-buthylamine (peak 2), isopentylamine (peak 3), phenethylamine (peak 4), and tyramine (peak 6), and one polyamine, acetyl spermidine (peak 1), was estimated (Figure 1 and Table S1). Peaks 10 and 26 are attributed to AccQ-methionine sulfoxide and AccQ-one-*N*-acetyl lysine, respectively. Peaks with other numbers were assigned to peptides based on the immonium ions and a, b, c, and x, y, and z series ions of the peptides, as shown in Table S1. Different peaks with the same number, such as peak numbers 65, 66, 76, and 78, shared compounds with the same *m*/*z* values of the precursor and product ions. Eighty-two dipeptides and one tripeptide were identified (Table S1 and Figure 3). Dipeptides

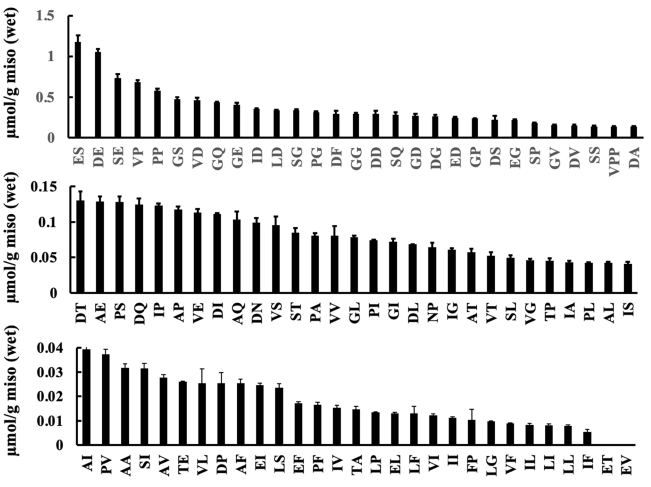

Figure 3. Contents of short-chain linear peptides in aqueous extract of soybean-*koji miso*. Peptide sequences are indicated by one letter abbreviation of amino acid residues. Data are presented as mean ± SD (*n* = 3).

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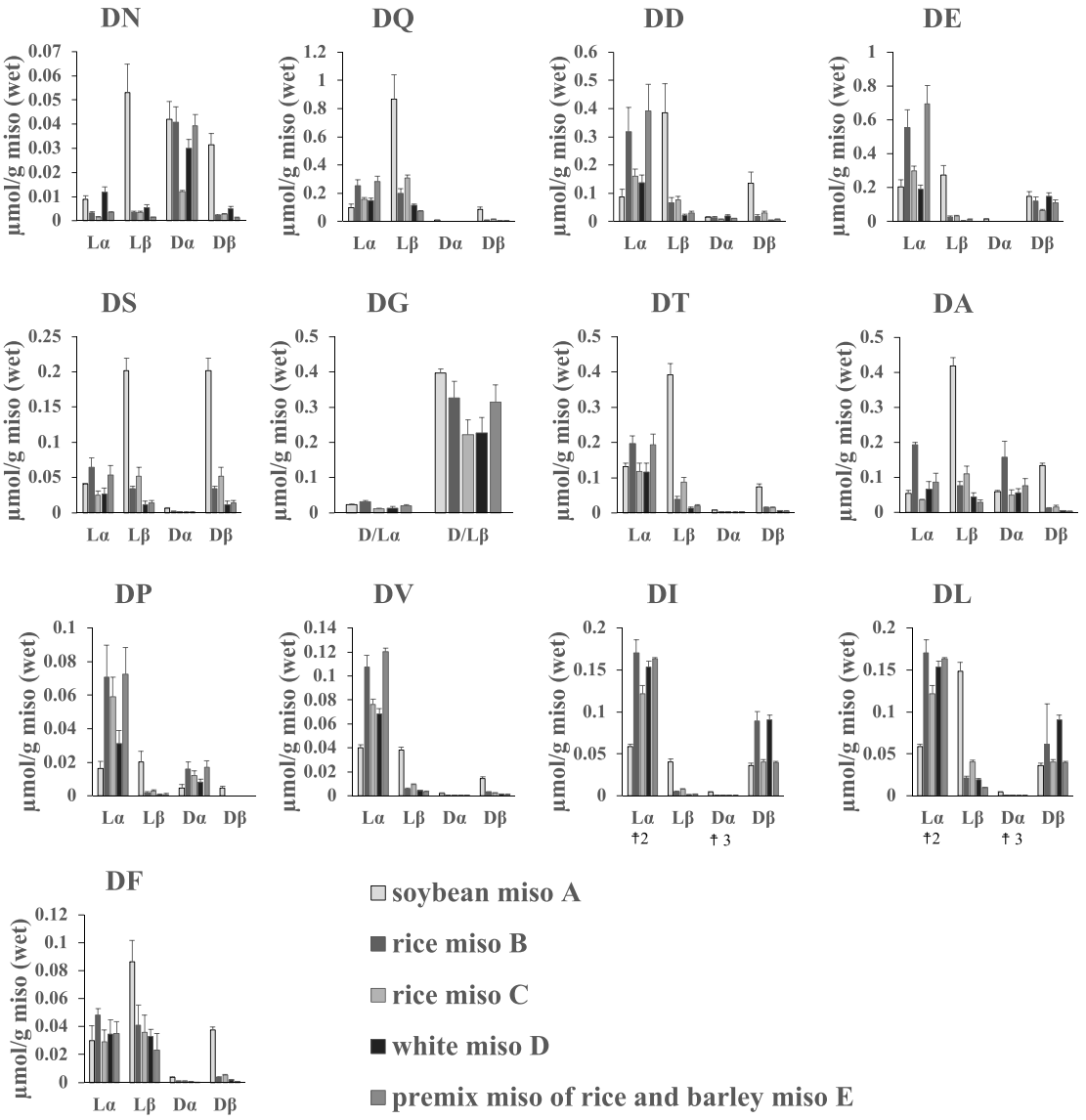

Figure 4. Contents of four isomers of aspartyl dipeptides in five commercially available *miso*. Data are presented as mean ± SD (n = 3). L-β-Asp-L-Ser and D-β-Asp-L-Ser, L-α-Asp-L-Ile and L-α-Asp-L-Leu, and D-α-Asp-L-Ile and D-α-Asp-L-Leu could not be separated using the present method. Contents of these unresolved peptides are expressed as the sum of two peptides.

containing prolyl (18 molecules), seryl (16 molecules), glycyl (15 molecules), aspartyl (14 molecules), or branched-chain amino acids residues (42 molecules) were frequently observed. Because Ile and Leu have the same molecular mass, all possible structures of the peptides containing Ile and Leu are listed in Table S1. The structures of the Ile- and Leu-containing peptides were identified by comparing the retention times of the synthetic peptides to all possible structures in subsequent experiments. AccQ-Ile-Pro/Leu-Pro was appeared in two scan ranges (peak 52: monoisotopic mass ion, m/z 399.1; peak 73: isotopomer ion, m/z 400.1). Some aspartyl peptides exhibited different peaks corresponding to the same precursor and product ions (peaks 65, 67, and 78). The structures of these aspartyl peptides were identified by comparing the retention times of the synthetic peptides with those of all of the possible structures in subsequent experiments.

Identification of Amino Group-Blocked Peptides and Amino Acids. Compounds in the AG50 nonabsorbed fraction were detected in the total ion monitoring mode (Figure 2A–F upper). Pyroglutamyl peptides in the AG50 nonabsorbed fraction were also detected in precursor ion scan mode by selecting the precursor ions that generated the immonium ion of the pyroglutamyl residue (m/z 84.1) (Figure 2A–F lower). Peaks containing the same compound detected in the total ion and precursor ion scanning modes shared the same number. The precursor and product ions and estimated structures of the compounds in the AG50 nonabsorbed fraction are summarized in Table S2. Thirteen pyroglutamyl dipeptides were identified (peaks 3, 19, 20, 29, 31, 33, 34, 35, 36, 40, 42, 46, and 49). Adduct formation with acetonitrile was observed for pyroGlu-Ile (peak 47), pyroGlu-Leu (peak 48), and pyroGlu-Phe (peak 51). In addition, adduct ions generated from two molecules of pyroglutamic acid with a proton or sodium ion and acetonitrile were observed in peaks 41 and 50, respectively. Diketopiperazines were identified based on the presence of product ions derived from the diketopiperazine ring (m/z 98, 113, 125, and 154). Twenty-two diketopiperazines, indicated with cyclo before the sequence of dipeptides, were estimated, as shown in Table S2 (peaks 7, 8, 10, 12, 14, 21, 22, 23, 25, 26, 28, 37, 38, 39, 43, 44, and 45). The diketopiperazines contained prolyl

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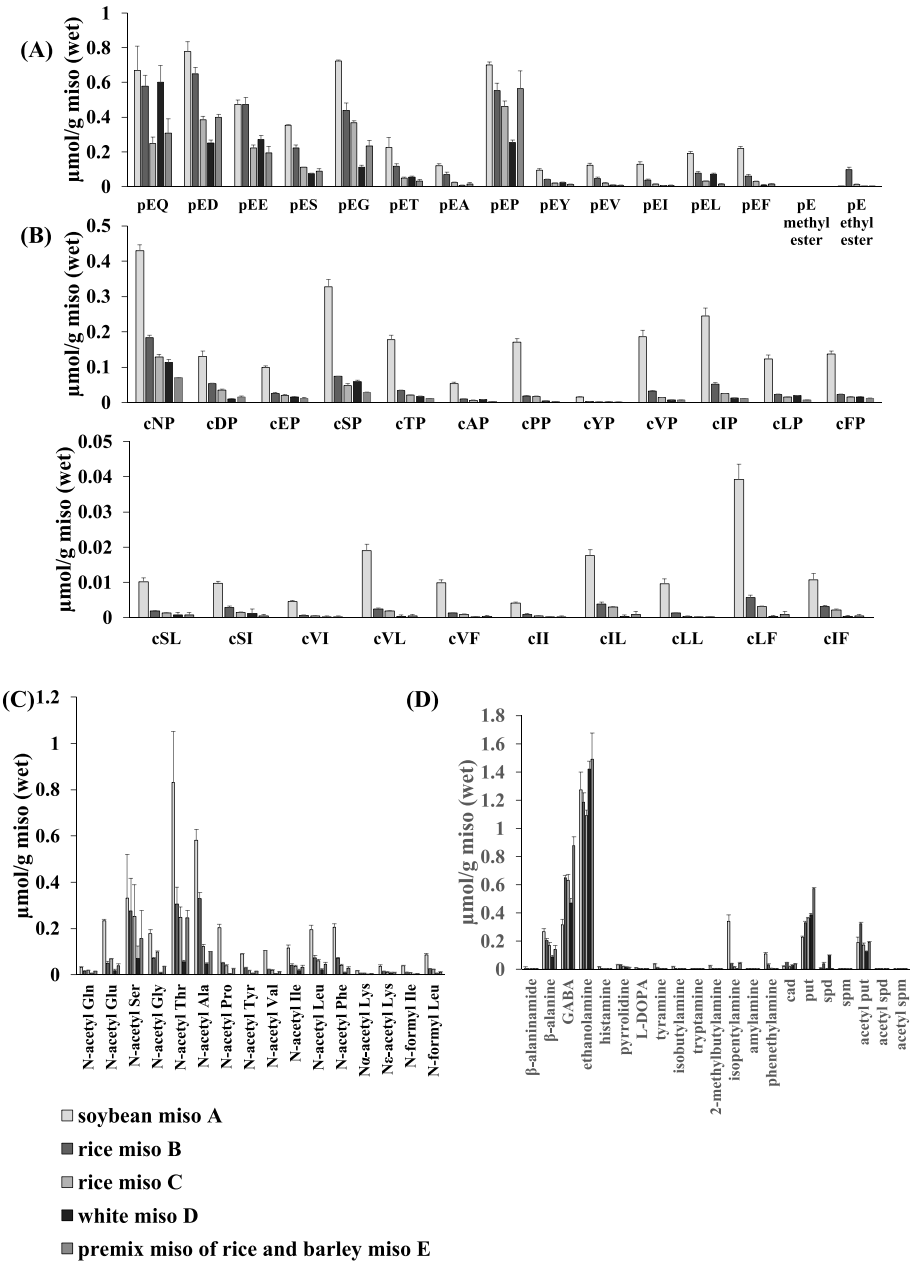

Figure 5. Contents of pyroglutamyl peptides (A), diketopiperazines (B), *N*-acetyl and *N*-formyl amino acids (C), and mono- and polyamine (D) in five commercially available *miso*. pE represents pyroglutamyl residue. Diketopiperazines are indicated by adding c in front of the dipeptide sequence presented by one letter abbreviation of amino acid residue. Data are presented as mean ± SD (n = 3).

(11 molecules) and branched-chain amino acid residues (13 molecules).

Acetyl and formyl amino acids were detected in the presence of b1 ions from the acetyl and formyl groups (m/z = 43 and 29, respectively). Ten acetyl amino acids (peaks 1, 2, 5, 6, 11, 13, 17, 18, 24, and 27) and two formyl amino acids (peaks 15 and 16) were detected. Precursor ions corresponding to propionyl and butyl amino acids were not detected. Peaks 30 and 32 in Figure 2 were identified as thymidine and uridines, respectively, by matching the precursor and product ions by using a database (MassBank).

Quantification of Peptides in *Miso*. The peptides listed in Tables S1 and Table S2 were either obtained commercially or synthesized. Using these compounds as standards, the peptides in the aqueous extract of *miso* were quantified by LC-MS/MS in MRM mode. All peptides with the amino groups listed in Table S1 were detected in the aqueous extracts of soybean-*koji miso*. Contents of these peptides were distributed between 0.01 and 1.2 μmol/g of soybean-*koji miso* (Figure 3). Glu-Ser and Asp-Glu were abundant in *miso* (>1 μmol/g). Asp-Ser, Asp-Ala, Asp-Asn, Asp-Gln, Asp-Thr, Asp-Glu, Asp-Asp, Asp-Pro, Asp-Val, Asp-Ile, Asp-Leu, and Asp-Phe showed multiple peaks by LC-MS/MS in the MRM mode (Figure S1), indicating the presence of isomers with L- and D-aspartyl residues and α- and β-peptide bonds. The isomers of aspartyl peptides in five commercially available *miso* samples were determined using synthetic peptides with 4 possible isomers as standards. Unfortunately, L-β-Asp-L-Ser and D-β-Asp-L-Ser, L-α-Asp-L-Ile and L-α-Asp-L-Leu, and D-α-Asp-L-Ile and D-α-Asp-L-Leu could not be separated using the present method.

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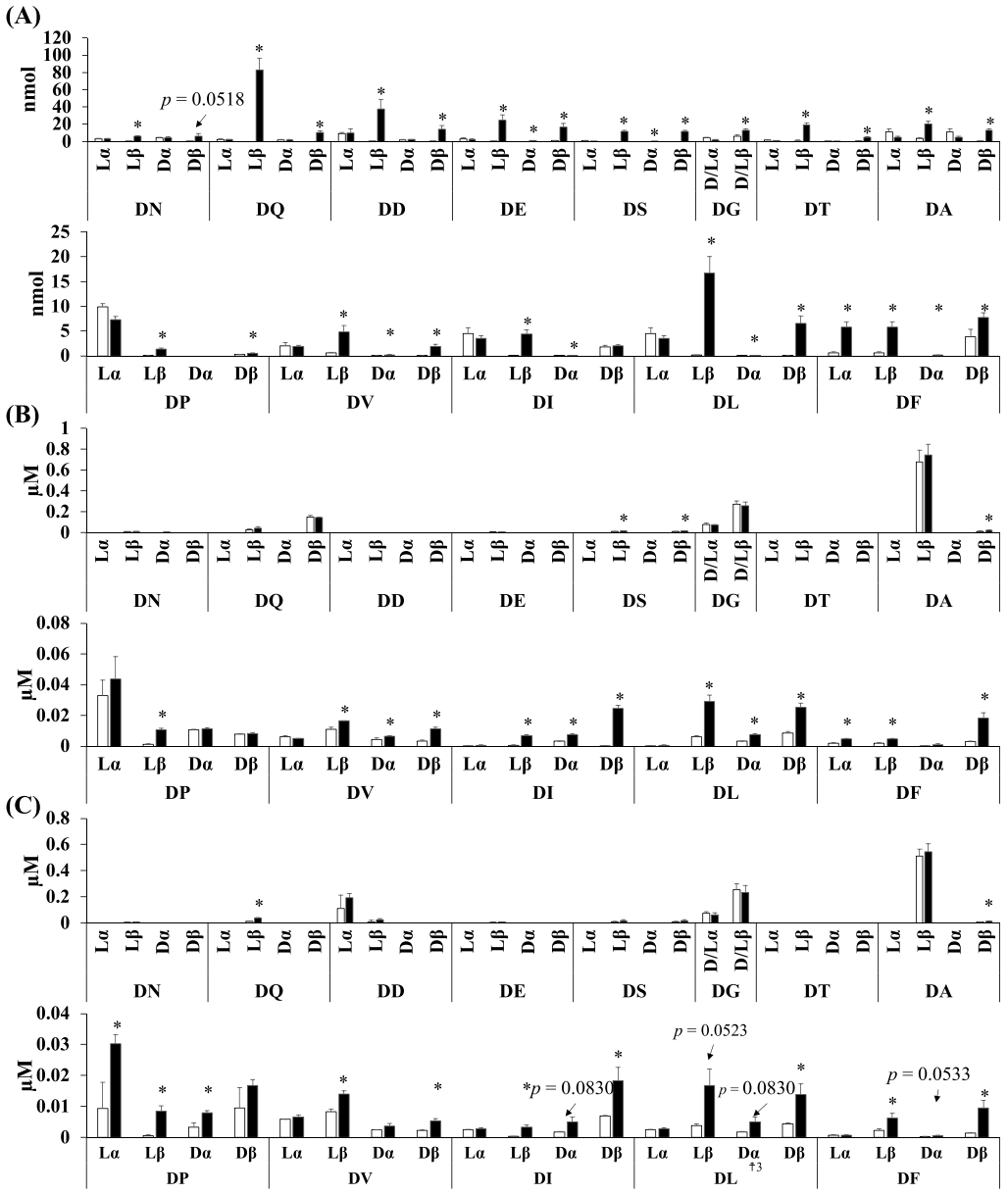

Figure 6. Four isomers of aspartyl dipeptides levels in inner content of small intestine (A), blood plasma from portal vein (B), and abdominal vena cava (C) of rats 1 h after administration of the vehicle (open bar) or aqueous extract of soybean-*koji miso* (close bar). Inner contents levels are presented by total amount (nM). Blood levels are represented as concentration (μM). Data are presented as mean ± SD (n = 3). * indicates a significant increase by Welch's t-test ($p < 0.05$). L-β-Asp-L-Ser and D-β-Asp-L-Ser, L-α-Asp-L-Ile and L-α-Asp-L-Leu, and D-α-Asp-L-Ile and D-α-Asp-L-Leu could not be separated using the present method. Contents of these unresolved peptides are expressed as the sum of two peptides.

Furthermore, D-Asp-Gly and L-Asp-Gly cannot theoretically be separated. All of the isomers were detected in the five commercially available *miso* samples (Figure 4). Soybean-*koji miso* had considerably higher ratios of L-aspartyl β-isopeptides (Lβ) to L-aspartyl α-peptides (Lα) than the other types of *miso*. The D-form of the Asp residue is also present in the α- and β-peptides (Dα and Dβ, respectively).

All of the pyroglutamyl peptides and diketopiperazines listed in Table S2 were present in five commercially available *miso* samples. The pyroglutamyl peptide contents of five commercially available *miso* samples are shown in Figure 5 A. PyroGlu-Gln, pyroGlu-Asp, pyroGlu-Glu, pyroGlu-Gly, and pyroGlu-Pro were abundant in all types of *miso*. Soybean-*koji miso* contained higher amounts of hydrophobic pyroglutamyl peptides, such as pyroGlu-Tyr, pyroGlu-Val, pyroGlu-Ile, pyroGlu-Leu, and pyroGlu-Phe than the other types of *miso* (rice-*koji* and barley-*koji miso*). Pyroglutamyl ethyl ester was detected in rice-*koji miso* B and C. As shown in Table S2, pyroglutamyl methyl ester was detected in the AG50 nonabsorbed fraction of the soybean-*koji miso* extracts. However, it was not detected in aqueous extracts of any types of *miso*. Thus, pyroglutamyl methyl ester was generated from pyroglutamate and methanol by the catalytic activity of the strong-acid sulfonated polystyrene (AG50W-×8).

Twelve prolyl diketopiperazines: cyclo-(Asn-Pro), cyclo-(Asp-Pro), cyclo-(Glu-Pro), cyclo-(Ser-Pro), cyclo-(Thr-Pro), cyclo-(Ala-Pro), cyclo-(Pro-Pro), cyclo-(Tyr-Pro), cyclo-(Val-Pro), cyclo-(Ile-Pro), cyclo-(Leu-Pro), and cyclo-(Phe-Pro) and ten nonprolyl diketopiperazines: cyclo-(Ser-Leu), cyclo-(Ser-Ile), cyclo-(Val-Ile), cyclo-(Val-Phe), cyclo-(Ile-Ile),

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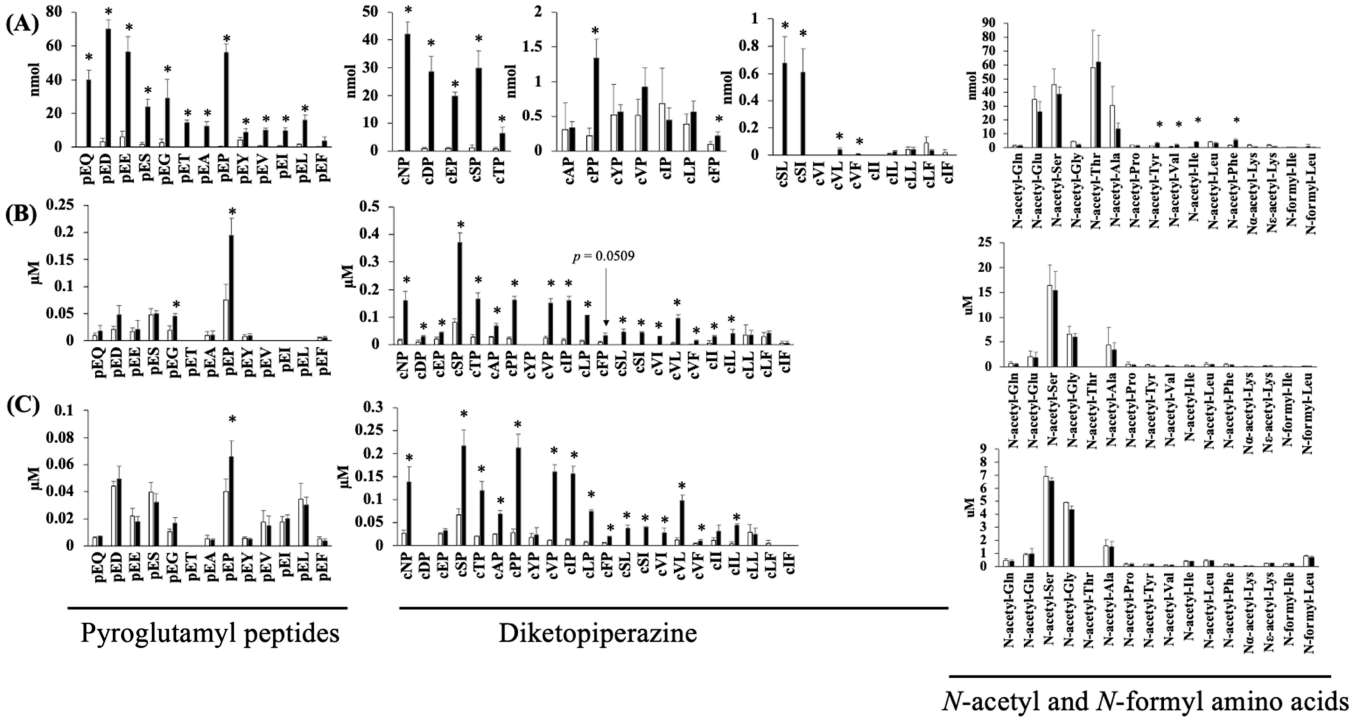

Figure 7. Levels of pyroglutamyl peptides, diketopiperazines, *N*-acetyl, and *N*-formyl amino acids in inner content of small intestine (A), blood plasma from portal vein (B), and abdominal vena cava (C) of rats 1 h after administration of the vehicle (open bar) or aqueous extract of soybean-*koji miso* (close bar). Data are presented as mean ± SD (n = 3). * Indicates a significant increase by Welch's t-test ($p < 0.05$).

cyclo-(Leu-Leu), cyclo-(Leu-Phe), and cyclo-(Ile-Phe) were detected in the five commercially available *miso* samples and quantified (Figure 5B). The prolyl diketopiperazine contents were higher than that of nonprolyl diketopiperazines in any types of *miso*. In all types of *miso*, cyclo-(Asn-Pro) was most abundantly present (approximately 0.05−0.4 μmol/g each *miso*). The contents of all diketopiperazines examined were higher in soybean-*koji miso* than in other types of *miso*.

Quantification of *N*-Acetyl and *N*-Formyl Amino Acids in *Miso*. Fourteen *N*-acetyl amino acids, such as *N*-acetyl-Gln, *N*-acetyl-Glu, *N*-acetyl-Ser, *N*-acetyl-Gly, *N*-acetyl-Thr, *N*-acetyl-Ala, *N*-acetyl-Pro, *N*-acetyl-Tyr, *N*-acetyl-Val, *N*-acetyl-Ile, *N*-acetyl-Leu, *N*-acetyl-Phe, *Nα*-acetyl-Lys, *Nε*-acetyl-Lys, *N*-formyl-Ile, and *N*-formyl-Leu, were present in the five commercially available *miso* samples and quantified (Figure 5C). Contents of all *N*-acetyl or *N*-formyl amino acids were higher in soybean-*koji miso* (approximately 0.05−0.8 μmol/g *miso*) compared to other types of *miso*. Amounts of the acetyl and formyl amino acids were approximately a few % (0.1−5%) (Figure 5C) of each free amino acid (Figure S2).

Quantification of Biogenic Amines in *Miso*. Some monoamines such as tyramine, phenethylamine, isopentylamine, and 2-methylbuthylamine were detected in soybean-*koji miso* by LC-MS/MS in precursor ion scan mode. Furthermore, other amino-acid-derived monoamines (*β*-alanine amide, *β*-alanine, GABA, ethanolamine, histamine, pyrrolidine, L-DOPA, isobutylamine, tryptamine, and amylamine) and polyamines (cadaverine, putrescine, spermidine, spermine, *N*-acetyl putrescine, and *N*-acetyl spermine), which are not listed in Table S1, were also quantified (Figure 5D). Only negligible amounts of tryptamine, amylamine, spermine, *N*-acetyl spermidine, and *N*-acetyl spermine were detected, whereas the presence of *N*-acetyl spermidine was suggested by AccQ derivatization and LC-MS/MS analyses of the aqueous extract of soybean-*koji miso* (Table S1). The peak tentatively identified as AccQ-*N*-acetyl spermidine showed different retention times compared to those of the standard. The absence of *N*-acetyl spermidine was also confirmed using another derivatization technique with benzyl chloride. The most abundant monoamine was ethanolamine, which is a decarboxylated compound of serine (approximately 1.0−1.5 μmol/g of *miso*), followed by GABA and *β*-alanine (approximately 0.2−1.5 μmol/g of *miso*). Contents of isopentylamine and phenethylamine were higher in soybean-*koji miso* (approximately 0.1−0.2 μmol/g *miso*), compared to other types of *miso*. Contents of putrescine and *N*-acetyl putrescine were higher than those of other polyamines (approximately 0.1−0.5 μmol/g in *miso*).

***Miso*-Derived Compounds in the Rat Body upon Ingestion.** *Miso*-derived compounds as shown in Figures 3−5 were quantified in the inner contents of the small intestine and plasmas from the portal vein and abdominal vena cava 1 h after administration of the vehicle or soybean-*koji miso* extract. Four aspartyl peptide isomers were quantified separately (Figure 6). The levels of aspartyl peptides with *β* peptide bonds (L*β*, D*β*) in the lumen 1 h after administration of soybean-*koji miso* extract were significantly higher than those after administration of the vehicle (Figure 6A). However, no significant differences were observed in the levels of L-aspartyl peptides with *α*-peptide bonds (L*α*) between the two groups. There were significant differences in the levels of some D-aspartyl peptides with *α*-peptide bonds (D*α*) between the two groups; however, their contents were not as high as those of the other forms. In addition, some aspartyl peptides with *β* peptide bonds and D-aspartyl residues (L*β*, D*α*, D*β*) were significantly increased in the bloods from the portal vein and abdominal vena cava upon administration of the soybean-*koji miso* extract compared to the vehicle (Figure 6B,C). In addition, the levels of L-*α*-Asp-L-

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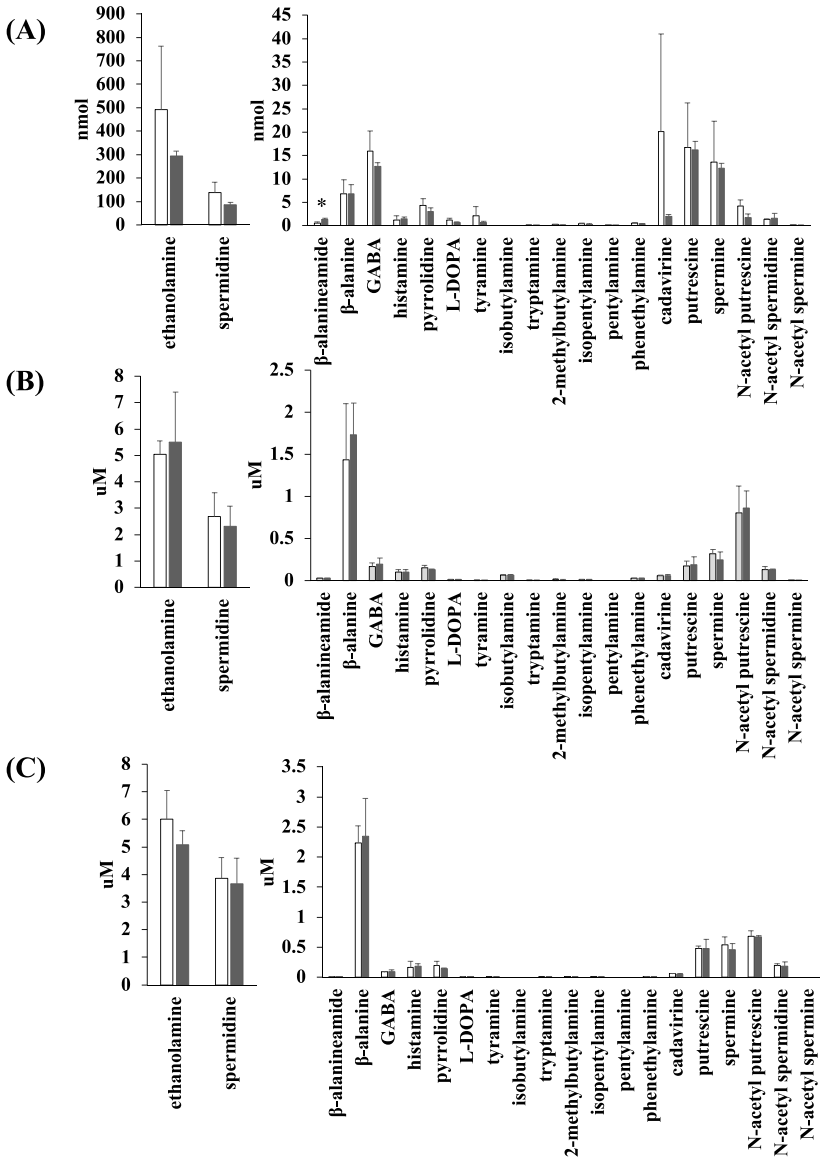

Figure 8. Contents of monoamines and polyamines in inner content of small intestine (A), blood plasma from portal vein (B), and abdominal vena cava (C) of rats 1 h after administration of the vehicle (open bar) or aqueous extract of soybean-*koji miso* (close bar). Data are presented as mean ± SD (n = 3). * indicates a significant increase by Welch's t-test ($p < 0.05$).

Pro in abdominal blood plasma were significantly higher after administration of the extract. Even after administration of the vehicle, some aspartyl peptides with β-peptide bonds and D-aspartyl residues ($_{L}\beta$, $_{D}\alpha$, $_{D}\beta$) were present in the lumen and blood, indicating the presence of endogenous aspartyl iso- and racemized peptides.

The levels of all pyroglutamyl peptides in the lumen 1 h after administration of soybean-*koji miso* extract were significantly higher than those of the vehicle, except for pyroGlu-Phe (Figure 7A). However, only pyroGlu-Gly and pyroGlu-Pro, and pyroGlu-Pro significantly increased in the portal and abdominal blood plasma upon administration of the extract, respectively. All of the examined pyroglutamyl peptides were detected in the lumen and blood, even after vehicle administration.

Levels of some diketopiperazines, cyclo-(Asn-Pro), cyclo-(Asp-Pro), cyclo-(Glu-Pro), cyclo-(Ser-Pro), cyclo-(Thr-Pro), cyclo-(Pro-Pro), cyclo-(Phe-Pro), cyclo-(Ser-Leu), cyclo-(Ser-Ile), cyclo-(Val-Leu), and cyclo-(Val-Phe), in the lumen 1 h after the administration of the soybean-*koji miso* extract were significantly higher than that of the vehicle (Figure 7B). The amount of hydrophilic diketopiperazines in the lumen was higher than that of the hydrophobic diketopiperazines. In the portal blood plasma, the levels of diketopiperazines, except for cyclo-(Tyr-Pro), cyclo-(Phe-Pro), cyclo-(Leu-Leu), cyclo-(Leu-Phe), and cyclo-(Ile-Phe), were significantly higher after administration of the extract. In the abdominal blood plasma, diketopiperazines, except for cyclo-(Asp-Pro), cyclo-(Glu-Pro), cyclo-(Tyr-Pro), cyclo-(Leu-Leu), cyclo-(Leu-Phe), and cyclo-(Ile-Phe), were significantly increased after administration of the extract. Some diketopiperazines were also observed in the lumen and blood, even after vehicle administration. The diketopiperazine composition in blood 1 h after the administration differed considerably from that in the lumen.

The examined *N*-acetyl and *N*-formyl amino acids were present in the lumen and blood even after vehicle administration (Figure 7C). The levels of *N*-acetyl-Tyr, *N*-

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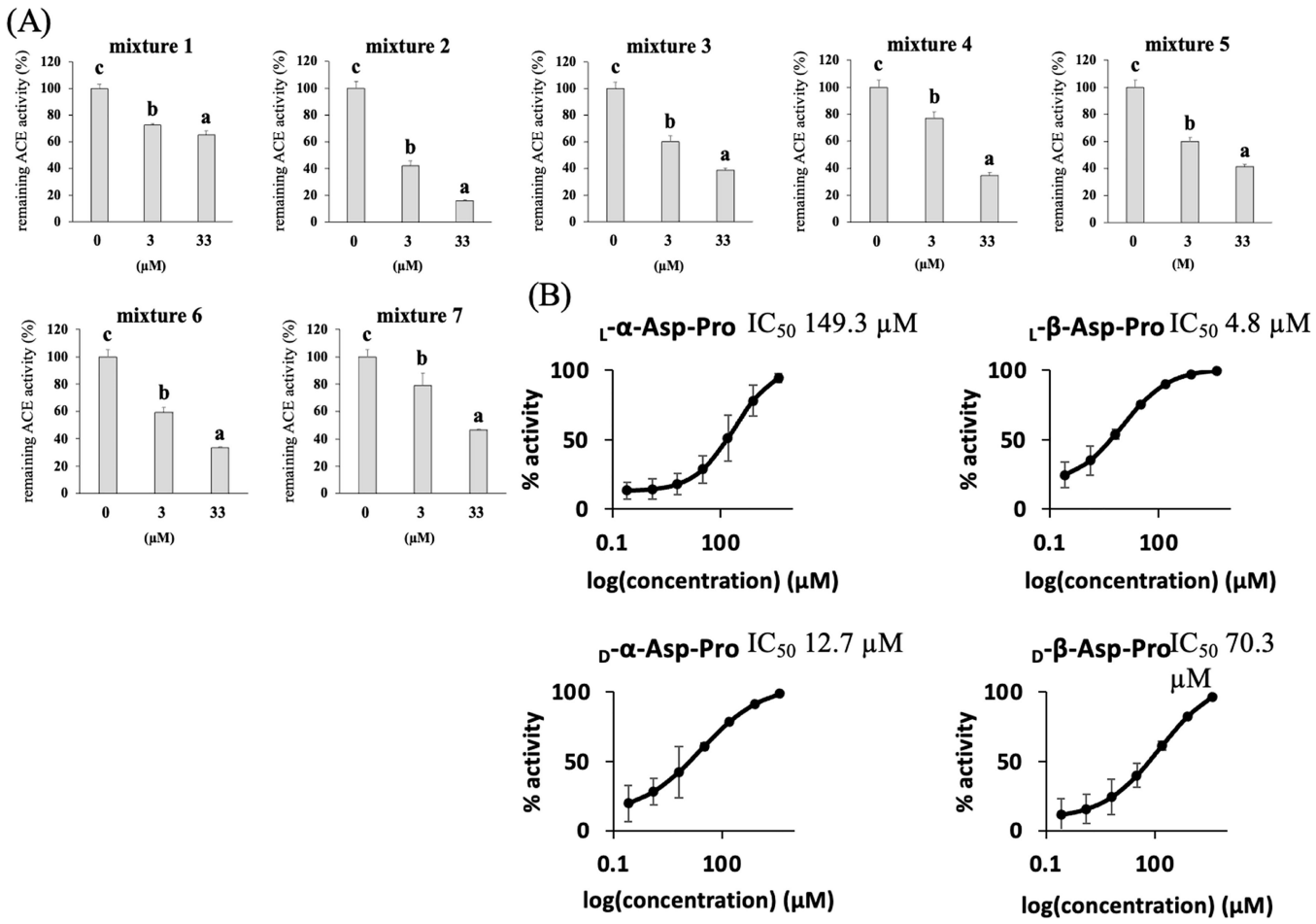

Figure 9. ACE inhibitory activity of peptide mixture (A) and each isomer of Asp-Pro (B). (A) Peptide mixture 1 included cyclo-(Asn-Pro), cyclo-(Asp-Pro), cyclo-(Glu-Pro), cyclo-(Ser-Pro), cyclo-(Thr-Pro), cyclo-(Ala-Pro), cyclo-(Pro-Pro), cyclo-(Val-Pro), cyclo-(Ile-Pro), cyclo-(Leu-Pro), cyclo-(Phe-Pro), mixture 2; L-α-Asp-Pro, L-β-Asp-Pro, D-α-Asp-Pro, mixture 3; D-β-Asp-Ala, L-β-Asp-Val, D-β-Asp-Val, mixture 4; L-β-Asp-Ile, D-α-Asp-Ile, D-β-Asp-Ile, mixture 5; L-β-Asp-Leu, D-α-Asp-Leu, D-β-Asp-Leu, mixture 6; L-β-Asp-Phe, D-α-Asp-Phe, D-β-Asp-Phe, and mixture 7; L-β-Asp-Gln, pyroGlu-Gly, pyroGlu-Pro. Each mixture contained the same concentration of peptides listed on the *X*-axis of the graph. The remaining ACE activities were calculated and expressed as mean ± SD ($n = 3$). Different letters indicate significant differences ($p < 0.05$) by Steel−Dwass test. (B) IC_{50} values of four isomers of Asp-Pro were calculated.

acetyl-Val, *N*-acetyl-Ile, and *N*-acetyl-Phe in the lumen 1 h after administration of the soybean-*koji miso* extract were significantly higher than those of the vehicle. By contrast, the two groups had no remarkable changes in the levels of all examined *N*-acetyl- and *N*-formyl amino acids in the blood. *β*-Alanine amide significantly increased in the lumen following the administration of soybean-*koji miso* extract (Figure 8A). By contrast, there were no remarkable changes in the blood levels of all monoamines and polyamines between the two groups (Figure 8B,C). All the examined mono- and polyamines were also observed in the lumen and blood, even after vehicle administration.

ACE Inhibitory Activity of *Miso* Peptides. Stock solutions of synthetic diketopiperazines, which increased significantly in the blood upon ingestion, were mixed. Stock solutions of synthetic aspartyl dipeptides were divided into six groups and mixed, as shown in the legend of Figure 9. All peptide mixtures showed significant dose-dependent ACE inhibitory activity (Figure 9). Mixture 2, consisting of isomers of Asp-Pro, showed the highest ACE inhibitory activity. Therefore, the IC_{50} values of the four Asp-Pro isomers were evaluated. As shown in Figure 10, L-*β*-Asp-Pro showed the highest ACE inhibitory activity ($IC_{50} = 4.8$ *μ*M) among them, whereas normal peptide L-*α*-Asp-Pro showed the lowest inhibitory activity (IC_{50},149.3 *μ*M).

DISCUSSION

Early studies demonstrated the presence of dipeptides rich in aspartyl, glutamyl, and lysyl residues in *miso*, while these studies did not provide the sequences of these peptides.[10] Shirako et al. recently reported the presence of 12 pyroglutamyl dipeptides in *miso*.[11] In this study, comprehensive analyses of short-chain peptides, amino acids, and their modified forms were performed. The current study identified 86 normal short-chain peptides with amino group and *α* peptide bonds and 13 pyroglutamyl dipeptides in *miso*. In addition, the current study demonstrated the presence of other modified peptides and amino acids such as diketopiperazines, isomerized aspartyl dipeptides, and *N*-acetyl, and *N*-formyl amino acids in *miso*. These peptides can resist exopeptidases from fungi, yeasts, and bacteria involved in the production of *miso*.

The current study revealed that oral administration of the aqueous extract of soybean-*koji miso* to rats did not affect the

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levels of normal peptides in the lumen and blood, except for L-α-Asp-Pro (Figure 6 and Figure S3). These facts suggest that most normal short-chain peptides in soybean-*koji miso* are degraded to amino acids by the intestinal mucosa and blood exopeptidases or that the dose of these peptides is not enough to impact the peptide content in the rat body. On the other hand, some peptides were also present in the vehicle group. These peptides are considered to be degradation products of endogenous peptides. It is possible that these endogenous peptides also exert some physiological functions.

Ejima et al. demonstrated the presence of D/L-aspartyl dipeptides with α and β peptide bonds (Asp-Val, Asp-Ile, Asp-Leu, and Asp-Phe) in an exopeptidase digest of a porcine liver protein hydrolysate.[23] The current study also demonstrated the presence of these isomers in 13 aspartyl dipeptides in *miso,* as shown in Figure 4. It has been reported that the β-carboxyl group on the side chain of L-aspartyl residues in proteins can react with the peptide bonds on the carboxyl side of L-aspartyl residues to form succinimide.[30,31] D-Aspartyl residues and β-peptide bonds can be generated by the oxidation and reduction of succinimide and the cleavage of succinimide, respectively. This isomerization of aspartyl residue in protein is believed to be caused by nonenzymatic reactions in human tissues during aging.[30,31] The soybean-*koji miso* used in this study was aged for 3 years, which is considerably longer than that of other types of *miso* (a few weeks to a few months). The longer aging period of soybean-*koji miso* accounts for the higher content of isomerized and racemized aspartyl dipeptides in soybean-*koji miso*. Ejima et al. demonstrated that the levels of Lβ, Dα, and Dβ types of four aspartyl dipeptides (Asp-Val, Asp-Ile, Asp-Leu, and Asp-Phe) were significantly increased in the abdominal vein blood 1 h after oral administration of porcine liver protein hydrolysate.[23] The current study also demonstrated that Lβ, Dα, and Dβ types of aspartyl dipeptides with hydrophobic amino acid residues were significantly increased in the portal blood and abdominal vein blood plasma of rats upon ingestion of the aqueous extract of *miso* (Figure 6). It has been reported that dipeptides with a D-amino acid residue at the amino terminus and β-peptide bond show lower affinity for oligopeptide transporters than those with an L-amino acid residue and α-peptide bond,[32,33] while the D- and β-peptides are resistant to exopeptidases.[34] These facts indicate that the susceptibility to exopeptidases is more important for peptide bioavailability than the affinity for peptide transporters. By contrast, dipeptides with hydrophobic amino acid residues at the carboxyl terminus have higher affinities for peptide transporter 1 (PEPT1),[35] which is consistent with the higher blood levels of hydrophobic isomerized and racemized aspartyl peptides than hydrophilic ones. Therefore, the affinity for transporters also significantly affects the blood levels of exopeptidase-resistant peptides.

Pyroglutamyl peptides are generated from peptides with a glutaminyl residue at the amino terminus by a nonenzymatic reaction in a time- and temperature-dependent manner.[12] *Miso* also contains short-chain pyroglutamyl peptides.[11] The present study confirmed the presence of 13 pyroglutamyl dipeptides in all types of *miso* (Figure 5A) and demonstrated that nearly all pyroglutamyl peptides increased in the lumen after administration, while only a few pyroglutamyl peptides were increased in blood. These results are consistent with the previous study.[36]

Diketopiperazines feature a six-membered ring containing two amide groups at opposite positions and are cyclic dipeptides. The presence of diketopiperazines in some heated and fermented foods, such as chicken soup broth,[37] cocoa,[38] dried bonito,[39] and Japanese rice wine (*sake*),[40] has been reported. Diketopiperazines frequently contain prolyl residue(s). However, the presence of nonprolyl diketopiperazines has also been reported in some foods, such as dried bonito[39] and roasted cocoa nib.[41] The prolyl diketopiperazines have been reported to be formed from linear peptides by enzymatic reactions and nonenzymatic reactions such as the heating process,[24,39] whereas nonprolyl diketopiperazines cannot be formed under the same conditions. The current study demonstrates the presence of 12 prolyl diketopiperazines and 10 nonprolyl diketopiperazines in all types of *miso* and the contents of the prolyl diketopiperazines (approximately 0.002−0.4 μmol/g) were approximately 10 times higher than those of the nonprolyl diketopiperazines (0.0001−0.04 μmol/g), which is consistent with the previous studies.[39,41] The contents of all examined diketopiperazines, especially nonprolyl ones, were remarkably higher in soybean-*koji miso* than in the other types of *miso* (Figure 5B). The soybean-*koji miso* is aged for a long period (3 years) without a heating process, suggesting that diketopiperazines are generated by nonenzymatic cyclization during the long-aging period. Moreover, smaller but detectable amounts of diketopiperazines were present in the short-aging (a few weeks)-type *miso*, white *miso* (Figure 5B). There is a possibility that diketopiperazines can be generated by enzymes from *A. sojae* or *A. oryzae* and airborne lactic acid bacteria. Diketopiperazines are much more stable against exopeptidase digestion than linear dipeptides and can pass through PEPT1 as well as linear oligopeptides,[42] suggesting that diketopiperazines in foods might be absorbed into the body upon ingestion. Indeed, 1 h after administration of *miso* extract, most diketopiperazines were significantly increased in the abdominal blood except for cyclo-(Tyr-Pro), cyclo-(Leu-Leu), cyclo-(Ile-Phe), and cyclo-(Leu-Phe). The increase in hydrophilic diketopiperazines, such as cyclo-(Asp-Pro) and cyclo-(Glu-Pro), in portal and abdominal blood was relatively low, whereas these diketopiperazines were significantly increased in the lumen. By the contrast, hydrophobic cyclo-(Pro-Pro), cyclo-(Val-Pro), and cyclo-(Ile-Pro) were significantly increased in the blood, whereas increases in cyclo-(Val-Pro) and cyclo-(Ile-Pro) in the lumen were not significant. Furthermore, some nonprolyl diketopiperazines, cyclo-(Ser-Leu), cyclo-(Ile-Ser), cyclo-(Val-Leu), and cyclo-(Val-Phe), were significantly increased in the blood and showed similar levels to some prolyl diketopiperazines (Figure 7B,C), whereas the contents of nonprolyl diketopiperazines in the *miso* extract were considerably lower than those in the prolyl diketopiperazines (Figure 5B). These facts indicate that the speed of absorption of diketopiperazines into the blood and their clearance from the blood differ considerably, depending on their structure. Shigemura et al. reported that some diketopiperazines are generated from longer collagen peptides during digestion and absorption processes in humans.[43] Therefore, it is possible that some diketopiperazines, especially nonprolyl diketopiperazines, may be generated by the digestion of *miso*-derived longer peptides (>3 amino acid residues) in rat digestive organs.

Few studies have reported the presence of *N*-acetyl amino acids in foods.[44,45] The current study revealed that 10 *N*-acetyl amino acids and 2 *N*-formyl amino acids were present in all types of *miso*. The levels of *N*-acetyl and formyl amino acids were remarkably higher in soybean-*koji miso* than in the other

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miso amino acids (Figure 5C). It has been reported that the content of *N*-acetyl amino acids in Pu-erh tea increases during pile fermentation,[45] which is consistent with the higher content of *N*-acetyl amino acids in soybean-*koji miso* (aged for 3 years) than in the other types of *miso* (aged for a few weeks to a few months). The current study demonstrated that the levels of four *N*-acetyl amino acids slightly but significantly increased in the lumen after administration of *miso* extract, while all examined *N*-acetyl and *N*-formyl amino acids did not significantly increase in the portal and abdominal blood (Figure 7B,C). All examined *N*-acetyl and *N*-formyl amino acids were observed in rat lumen and plasma even after the administration of the vehicle, indicating that these *N*-acyl amino acids are also endogenously generated in the rat body.

The presence of biogenic amines in *miso* and other fermented foods has been demonstrated previously.[46] The current study also observed 14 monoamines and 7 polyamines in all *miso* types. The putrescine content in all types of *miso* examined was higher than that of the other polyamines (Figure 5D), which is consistent with a previous study.[46] The ratio of putrescine to spermidine in *miso* including the short-aging type is higher than that in the raw material (soybean),[46] whereas the fermentation process of *natto*, another fermented soybean product, does not affect the polyamine composition.[47] These facts indicate that some polyamines, such as putrescine, are generated during the fermentation process of *miso*. The levels of hydrophobic monoamines, such as isopentylamine and phenethylamine, were higher in soybean-*koji miso* than in the other types of *miso*, whereas there was no remarkable difference in the levels of hydrophilic monoamines, such as β-alanine, GABA, and ethanolamine, between all types of *miso* (Figure 5D). Therefore, hydrophobic monoamines were generated during the long-aging process.

As mentioned in the Introduction, the consumption of *miso* exerts some health-promoting effects. Hydrophobic pyroglutamyl peptides in *miso,* such as pyroGlu-Leu, alleviate high-fat diet (HFD)-induced obesity in a rat model, possibly owing to the enhanced secretion of rat gut antimicrobial peptides,[11] which can explain the antiobesity effect of *miso*. Despite the high salt concentration in *miso*, it has been reported that the consumption of *miso* does not cause hypertension but rather suppresses hypertension.[4−6] The ACE inhibitory activity of peptides in *miso*[19,20] may be involved in the blood pressure-lowering effects of *miso*. However, ACE inhibitory peptides in the *miso* region have not been identified. Many ACE inhibitory peptides have been identified in foods.[14,17,18] However, only a few of these peptides have been examined for their bioavailability.[15,16] When doses of enzymatic hydrolysates of proteins or fermented foods that lower blood pressure were administered, the concentrations of the reported ACE inhibitory peptides in the blood were at several nanomolar levels, which were less than 1/1000 of the concentration used for the ACE inhibitory assay.[14−18] For example, Ile-Lys-Pro, Val-Tyr, Val-Pro-Pro, and Ile-Pro-Pro showed IC_{50} of 1.7,[14] 11,[17] 9,[18] and 5 μM,[18] respectively. Therefore, mechanisms other than ACE inhibition have been proposed; however, there are still large differences in the concentrations of food-derived peptides in target tissues compared with the amounts required by the proposed mechanisms.[48−50] Therefore, it is not fully understood why peptides in food lower blood pressure. In this study, we found that more than 30 modified peptides were significantly increased in the blood to 5−250 nM after consuming 1 g/kg of *miso* containing 20−800 nmol of these modified peptides, and the increased peptides showed ACE inhibitory activity in a dose-dependent manner, although there were differences in degree. The bioavailability (blood concentration relative to intake amount) of these modified peptides in *miso* is extremely high compared to the ACE inhibitory peptides reported so far.[15,16] It is possible that *miso*-derived peptides that enter the bloodstream act on the renin-angiotensin system in cooperation with other components of *miso* or endogenous peptides, thereby exerting an effect on blood pressure. Further studies on the effects of individual peptides on the renin-angiotensin system, blood pressure, and bioavailability of modified peptides in humans using realistic doses of *miso* are in progress.

Diketopiperazines and aspartyl isopeptides, which are significantly increased in the blood after oral administration, may exhibit activities other than ACE inhibition. We are currently investigating the inhibition of other enzymes and their effects on innate immune cells at various concentrations in the blood. It has been reported that monoamines and polyamines, which are decarboxylated metabolites of amino acids, have several biological functions. The decarboxylated amino acids in *miso* may be involved in health-promoting functions when consumed daily. Furthermore, the physiological functions of *N*-acetyl and *N*-formyl amino acids in *miso*, which were revealed in this study, remain largely unknown. These amino acid derivatives do not significantly increase in the blood; however, similar to the pyroglutamyl peptide, they may act on cells in the gastrointestinal tract, such as Paneth cells, and exhibit significant physiological functions.

In conclusion, we revealed that modified peptides exist in *miso* in addition to normal peptides. These modified peptides exist in the lumen, and some also exist in the blood upon ingestion and exert biological functions, such as ACE inhibition. These modified peptide and amino acids with high bioavailability are thought to be involved in the beneficial effects of *miso* consumption. *Miso* can be consumed nearly every day in Japan and has a long consumption history. Therefore, the modified peptides obtained by ingestion of *miso* are believed to exhibit no adverse effects.

Food generally contains peptides and their metabolites. However, most of these are broken down during digestion. The components that pass into the lumen and blood are limited to peptides with specific characteristics, and their numbers are smaller than those present in food. It is, therefore, easier to investigate the functions of a small number of the food-derived peptides in the body than to investigate the functions of so many peptides in food that should be checked their bioavailability.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.4c02603.

Precursor and product ions for estimation of amino compounds in soybean-*koji miso*; precursor and product ions for estimation of nonamino compounds in soybean-*koji miso*; mass chromatograms of four isomers of aspartyl peptides in soybean-*koji miso*; amino acid contents in five commercial *miso*; and contents of normal peptides in lumen, portal blood, and abdominal vein blood after administration of vehicle and aqueous extract of soybean-*koji miso* (PDF)

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

LC-MS/MS; liquid chromatography tandem mass spectrometer; *m*/*z*; mass-to-charge ratio; pyroGlu; pyroglutamyl; ACE; angiotensin-converting enzyme; PBS; phosphate buffered saline; Fmoc; 9-fluorenylmethyloxycarboxynyl; AccQ; 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate; RP-HPLC; reversed-phase high-performance liquid chromatography; MRM; multireaction monitoring; IC_{50}; half-maximal inhibitory concentration; GABA; γ-aminobutyric acid; DOPA; dihydroxyphenylalanine; HFD; high-fat diet; PEPT; peptide transporter

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