Identification of a Hepatoprotective Peptide in Wheat Gluten Hydrolysate against D-Galactosamine-Induced Acute Hepatitis in Rats

Kenji Sato,* Yukari Egashira,† Shin Ono,‡ Satoshi Mochizuki,§ Yuki Shimamura,¶ Yoshio Suzuki,∥ Megumi Nagata,† Kaori Hashimoto,† Tamami Kiyono,† Eun Young Park,† Yasushi Nakamura,† Mariko Itabashi,‡ Yuka Sakata,§ Seigo Furuta,∥ and Hiroo Sanada†

†Division of Applied Life Sciences, Kyoto Prefectural University, Shimogamo, Kyoto 606 8522, Japan
‡Graduate School of Horticulture, Chiba University, 648 Matsudo, Matsudo-Shi, Chiba 271 8510, Japan
§Graduate School of Science and Engineering, University of Toyama, 3190 Gofuku, Toyama 930 8555, Japan
¶Faculty of Education and Welfare Science, Oita University, 700 Dannohara, Oita 870 1192, Japan
∥Health Care Research Center, R&D Division, Nisshin Pharma Inc., 5-3-1 Tsurugaoka, Fujimino-city, Saitama 356 8511, Japan
△Graduate School of Health and Sports Science, Juntendo University, 1-1 Hiraga-Gakuenndai, Inzai, Chiba 276 1695, Japan

ABSTRACT: A hepatoprotective peptide, pyroglutamyl leucine (pyroGlu-Leu), was identified in wheat gluten hydrolysate through an in vivo activity-guided fractionation approach based on d-galactosamine-induced acute hepatitis in rats and fractionation of peptides with large-scale preparative ampholine-free isoelectric focusing. The active acidic fraction predominantly consisted of pyroglutamyl peptides and free pyroglutamic acid. Pyroglutamyl peptides were derivatized with phenyl isothiocyanate after removal of a pyroglutamyl residue by pyroglutamate aminopeptidase. The derivatives were purified by reversed-phase HPLC and subjected to sequence analysis. The active fraction contained pyroGlu-Ile, pyroGlu-Leu, pyroGlu-Gln, pyroGlu-Gln-Gln, and free pyroGlu. Ingestion of pyroGlu-Leu at 20 mg/kg body weight significantly decreased serum aspartate aminotransferase to approximately 30% and 20% of those values of the vehicle group, respectively, which were near the normal levels. Thirty minutes after ingestion of pyroGlu-Leu at 20 mg/kg, the concentration of pyroGlu-Leu in portal blood plasma increased to approximately 2 μM.

KEYWORDS: wheat gluten hydrolysate, pyroglutamyl, hepatitis, d-galactosamine, pyroGlu-Leu

INTRODUCTION

Hepatitis is inflammation of the liver that induces hepatocyte necrosis. It is primarily caused by viral infections, excessive alcohol consumption, toxins, and fatty liver.1−3 Acute hepatitis can induce life-threatening liver failure. Chronic hepatitis can develop into cirrhosis of the liver and consequently cirrhosis, which causes life-threatening liver damage.3

Crude extracts of some plants have been shown to exert a hepatoprotective effect in animal models.4−7 In addition, some amino acids8−11 and enzymatic hydrolysates of food proteins12,13 have also been shown to alleviate hepatitis in animal models. Therapeutic approaches based on these naturally occurring components are attractive. However, ingestion of relatively high doses of these food compounds is required to achieve a beneficial effect in animal models.4−13

Recently, it has been demonstrated that supplementation with 4−15 g/day of an enzymatic hydrolysate of wheat gluten (WGH) decreased serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), and γ-glutamyl transpeptidase levels in patients with hepatitis from different backgrounds without adverse effects.14 More recently, Suzuki et al. (2012) demonstrated that supplementation with the same WGH preparation (4% of total protein in diet) suppressed rat liver cirrhosis induced by chronic injection of carbon tetrachloride.15 These results suggest that oral administration of WGH might have a therapeutic effect against acute and chronic hepatitis at practical doses.

As wheat gluten is rich in glutaminyl residues, enzymatic hydrolysate of wheat gluten may contain non-negligible amounts of peptides with glutaminyl residues at their amino termini, and these amino-terminal glutaminyl residues can be converted to pyroglutamyl residues during processing.16,17 In some cases, pyroglutamyl peptide content in such preparations reached nearly 10% (w/w).16

The peptide responsible for the hepatoprotective activity in WGH has not yet been identified. To elucidate the mechanism underlying the hepatoprotective activity of WGH, it is crucial to isolate and identify the active peptides. The objective of the present study was to identify the peptide(s) in WGH that confer hepatoprotective activity through oral administration.

MATERIALS AND METHODS

Starting Material. An enzymatic digest of wheat gluten hydrolysate was obtained from a commercial source (WGH; Nisshin Pharma, Tokyo, Japan). Wheat gluten was digested with Aspergillus

Received: October 27, 2012
Revised: May 17, 2013
Accepted: June 7, 2013
Published: June 7, 2013

© 2013 American Chemical Society
oryzae protease. The reaction was terminated by heating at 90 °C. The digest was filtered and then spray-dried. Number average molecular weight and weight average molecular weight are 400 and 18,000, respectively (data from the supplier). This preparation is the same product used in the previous studies.

**Chemicals.** Pentobarbital sodium was obtained from Sigma (St. Louis, MO, USA). *Pyrococcus furiosus* pyroglutamate aminopeptidase was obtained from Takara Bio (Ohtsu, Japan). N-galactosamine hydrochloride, a transaminase assay kit (Transaminase C-II test), phenyl isothiocyanate (PITC), acetonitrile (HPLC grade), N,N-dimethylformamide (DMF), trithioacetic acid (TFA), dithiothreitol (DTT), disodium dihydrogen ethylenediaminetetraacetate (EDTA), triethylamine (TEA), and an amino acid mixture (Type H) were obtained from Wako Pure Chemicals (Osaka, Japan). Pyroglutamic acid was obtained from Nacalai Tesque (Kyoto, Japan). Pyroglutamyl glutamine (pyroGlu-Gln) was purchased from Bachem (Bubendorf, Switzerland). All amino acid derivatives and the 2-chlorotriyl chloride resin (Barlos resin) for peptide synthesis were purchased from Watanabe Chemical (Hiroshima, Japan). Reagents for peptide resin (Barlos resin) for peptide synthesis were purchased from GL Sciences Inc. (Osaka, Japan). Other reagents used were of analytical grade or better.

**Animal Experiment.** Four-week-old male Wistar rats were purchased from CLEA Japan (Shizuoka, Japan). All rats were fed the CE-2 commercial diet (CLEA Japan) for 4 days and then the AIN-93 diet for 9 days. All animals were housed in the same building (temperature: 22–24 °C, humidity: 40–60%) under a 12 h light–dark cycle and free access to diet pellets and filtered water. This study was conducted in accordance with the standards established by the Guide for the Care and Use of Laboratory Animals of Chiba University. The care and treatment of all animals were performed according to the ethical guidelines set by the Faculty of Horticulture of Chiba University.

Acute hepatitis was induced in an injection of n-galactosamine as previously described. Briefly, rats were fasted for 5 h before n-galactosamine injection. Then, 1 h before injection of n-galactosamine, the test component was administered. The samples were dissolved to suitable concentrations in distilled water and administered via a stomach sonde at a constant volume of 2 mL/kg body weight. n-Galactosamine hydrochloride was dissolved in distilled water to 300 mg/mL, neutralized to pH 7.0, and sterilized by membrane filtration (0.22 μm). One hour after ingestion of the test sample, n-galactosamine (800 mg/kg body weight) was intraperitoneally injected. Four hours after the injection, the rats were allowed access to food and water. Whole blood samples were collected from the abdominal aorta under pentobarbital anesthesia 24 h after n-galactosamine injection. Serum AST and ALT levels were measured by using a commercial kit (Wako Pure Chemicals).

To estimate the absorption of pyroGlu-Leu into the blood, synthesized pyroGlu-Leu was dissolved in distilled water and administered orally at 20 mg/kg body weight via a stomach sonde. Rats were fasted 24 h before administration. As a control, the vehicle (distilled water) was administered. Thirty and sixty minutes after ingestion, blood was collected from the portal vein under ether anesthesia (n = 3 for each group). Plasma was prepared in the presence of heparin by centrifugation at 3000g and then mixed with 3 volumes of ethanol. The precipitate was then removed by centrifugation at 16300g for 5 min. The ethanol-soluble fraction was used to determine pyroGlu-Leu levels.

**Fractionation of Peptides in the WGH.** For the animal experiment, the peptides in WGH were fractionated based on the anaphoretic nature of the sample peptides using the method of Hashimoto et al., which has been referred to as autofocusing, using an apparatus with 12 compartments (66.5 mm in length × 80 mm in width × 80 mm in height). The compartments at either ends of the tank were used as the anode and cathode compartments. The other compartments were used as sample compartments and were numbered from the anode side (No. 1) to the cathode side (No. 10). Sample compartments 5 and 6 were filled with 500 mL of a 5% WGH solution in water. The other compartments were filled with deionized water. Autofocusing was performed in constant voltage mode at 500 V for 26 h. All fractions from the autofocusing were collected and adjusted to pH 6.0 and subsequently freeze-dried.

**High-Performance Liquid Chromatography (HPLC).** For fractionation of the peptides in WGH and amino acid analysis, either a Shimadzu low-pressure gradient system consisting of a LC-10Avp pump, an SPD-10Avi UV–vis detector, and an FCV-10AL gradient valve unit or a high-pressure gradient system consisting of two LC-10Advp pumps and an SPD-10Avp UV–vis detector was used. For purification of the chemically synthesized peptide, a Jasco HPLC system consisting of two PU 980 pumps and a Shimazu SPD-6A UV detector was used.

**Identification of Peptides in the Active Autofocusing Fraction.** Peptides in the active autofocusing fraction were further fractionated by size exclusion chromatography (SEC) using Superdex peptide 10/30 HR (GE Healthcare, Buckinghamshire, UK) equilibrated with 30% (v/v) acetonitrile containing 0.1% TFA. A 136 mg amount of dried sample was dissolved in 1 mL of 30% (v/v) acetonitrile containing 0.1% TFA. The sample solution was filtered through a Column Guard LCR4 (Millipore, Billerica, MA, USA). A 200 μL amount of sample was injected into the column. Elution was performed at a rate of 0.5 mL/min, and a fraction was collected every minute.

Two sets of 200 μL aliquots of the SEC fractions were put into centrifuge tubes (1.5 mL) and dried under vacuum. One set was used for pyroglutamate aminopeptidase digestion to liberate the amino group coupled to the pyroglutamyl residue. To both sets of tubes, 100 μL of 50 mM sodium phosphate buffer, pH 7.4, containing 10% acetonitrile and resolved by reversed-phase HPLC using a Lichrocart Supersphere RP-18 (c) column (250 mm × 4 mm i.d.; Merck, Darmstadt, Germany) equilibrated with 150 mM ammonium acetate buffer (pH 6.0, containing 5% (v/v) acetonitrile (solvent A) at 0.8 mL/min. Elution was performed using a binary gradient system with 60% (v/v) acetonitrile (solvent B). The gradient profile was as follows: 0–5 min, 0% B; 5.1–25 min, 10–47.5% B; 25–30 min, 47.5–100% B; 30–37 min, 100% B; 37.1–40 min, 0% B. The column was maintained at 45 °C, and absorbance at 254 nm was monitored. Peaks were collected and dried under vacuum. The dried sample was mixed with 10 μL of redrying solution (methanol/triethylamine/water, 7:2:1 (v/v)) and then dried under vacuum to remove ammonia. The dried sample was dissolved in 30% acetonitrile and loaded on a protein sequencer (PPSQ-21; Shimadzu, Kyoto, Japan), and the program was changed to start the cleavage reaction.

**Synthesis of Pyroglutamyl Peptides.** Pyroglutamyl leucine (pyroGlu-Leu) and pyroglutamyl isoleucine (pyroGlu-Ile) were synthesized using the conventional liquid-phase method. Pyroglutamyl glutaminyl glutamine (pyroGlu-Gln-Gln) was synthesized using the manual solid-phase method. The final products were characterized by reversed-phase HPLC using a TSKgel ODS-100S (3.0 × 150 mm; Tosoh, Tokyo, Japan) and electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS) with a Hitachi NanoFrontier LC-MS system (Chiba, Japan).

**pyroGlu-Leu.** Triethylamine (0.31 mL) and Boc-pyroGlu-OH (510 mg) were added to a solution of L-leucine tert-butyl ester hydrochloride (500 mg) in DMF (15 mL) at 0 °C. Then, HOBt (600 mg) and EDC-HCl (640 mg) were added, and the reaction...
mixture was stirred for 12 h at room temperature. After removal of the solvent under reduced pressure, the residue was dissolved in ethyl acetate, washed with 5% NaHCO₃ and 10% citric acid, and then dried over anhydrous sodium sulfate. After filtration, the filtrate was concentrated under reduced pressure, and the white product was precipitated by the addition of petroleum ether. As a result, 850 mg of protected peptide was obtained in 96% yield. The protected dipeptide was treated with 4 M HCl/dioxane at room temperature for 5 h to remove the protected groups. After removal of the excess HCl/dioxane, pyroGlu-Leu was precipitated with diethyl ether, yielding 430 mg of the desired product in 85% yield.

**pyroGlu-Ile.** Triethylamine (0.15 mL) and Boc-pyroGlu-OH (229 mg) were added to a solution of t-isoleucine tert-butyl ester hydrochloride (225 mg) in DMF (10 mL) at 0 °C. HOBT (271 mg) and EDC·HCl (287 mg) were added, and the reaction mixture was stirred for 12 h at room temperature. The protected peptide was recovered and deprotected as described above. As a result, 40 mg of pyroGlu-Ile was recovered in 64% yield.

**pyroGlu-Gln.** pyroGlu-Gln was obtained by solid-phase synthesis on 2-chlorotrityl chloride resin (Barlos resin) utilizing Fmoc/tBu chemistry. The coupling reaction was performed with the HBTU·HOBT using a 3-fold molar excess of Fmoc-amino acid in the presence of diisopropylethylamine in DMF. The Fmoc group was deprotected by treatment with 20% piperidine in DMF for 15 min. Cleavage of the peptide from the resin was achieved with TFA-containing water (95:5, v/v) for 30 min. After removing the resin by filtration, the filtrate was concentrated by flushing with nitrogen gas, and then the crude product was precipitated with diethyl ether. The obtained crude product was purified by preparative reversed-phase HPLC on a Shiseido C₁₈ column (Capcell Pak C18 UG120A, 20 × 250 mm) using an acetonitrile/TFA solvent system. The corresponding fraction was lyophilized, giving 44 mg of the desired product in 43% yield.

**Amino Acid Analysis.** Peptides were hydrolyzed with HCl vapor at 150 °C using the method of Bidlingmeyer et al. (1984). The resultant amino acids were derivatized with PITC, and the PTC-amino acids were resolved using the same conditions as described above.

**Determination of Free PyroGlu.** Free pyroGlu was determined by precolumn derivatization with 2-nitrophenyl hydrazine as described previously.

**Determination of PyroGlu-Leu in Portal Blood.** The concentration of pyroGlu-Leu in rat portal blood plasma was estimated by LC-MS/MS using a Q-TRAP 3200 (AB SCIEX, Foster City, CA, USA). Ethanol-soluble fractions (1 mL) of plasma obtained from the rats that received pyroGlu-Leu or vehicle were subjected to solid-phase extraction using a spin column packed with a strong cation exchanger (AG 50W × 8; Bio-Rad Laboratories, Hercules, CA, USA), as described previously. After elution of the sample, the resin was washed with 200 μL of 10 mM HCl containing 50% ethanol. The eluents were combined, dried under vacuum, and dissolved in 250 μL of 0.1% formic acid. The samples (10 μL) were injected onto an Inertsil ODS-3 column (2 mm i.d. × 250 mm; GL Science, Tokyo, Japan). Binary gradient elution was performed with 0.1% formic acid (solvent A) and 80% acetonitrile containing 0.1% formic acid (solvent B) at a flow rate of 0.2 mL/min. The column was equilibrated with 100% solvent A, and the gradient profile was as follows: 0–12 min, 0–50% B; 12–20 min, 50–100% B; 20–24 min, 100% B; 24–24.1 min, 100–0% B; and 24.1–30 min, 0% B. The column was maintained at 40 °C throughout. MS/MS conditions were optimized in positive mode by using Analyst Version 4.2 (AB SCIEX) in auto select mode.

**Statistics.** The differences in serum transaminases and plasma pyroGlu-Leu levels were analyzed by one-way analysis of variance (ANOVA) using StatView 4.11 (Abacus Concepts Inc., Berkeley, CA, USA). Significant differences between the groups were evaluated by Scheffe’s post hoc test.

### RESULTS

**Hepatoprotective Autofocusing Fractions.** As shown in Figure 1a, a pH gradient from approximately 2 to 12 was developed by autofocusing of WGH. More than 98% of the peptides was recovered in Fr. 4–8, and only low amounts of peptides were recovered in Fr. 1–3 and Fr. 9 and 10 (Figure 1b). As shown in Figure 1c, the acidic and basic fractions of the hydrolysate were characterized by higher levels of glutamic acid and arginine, respectively, which indicates that the peptides in WGH were fractionated based on their isoelectric points.

Autofocusing fractions 1–10 were orally administered to rats 1 h prior to the injection of D-galactosamine. The doses of the autofocusing fractions are shown in Figure 2. Lower doses were used for Fr. 1–3 than for the other fractions due to low peptide recovery. Injection of D-galactosamine induced acute hepatitis in rats and increased serum AST activity approximately 800 IU/L. As shown in Figure 2, Fr. 2 and 4 significantly decreased serum AST activity, while Fr. 9 significantly increased serum AST activity. Since Fr. 2 exhibited hepatoprotective activity at the lowest dose, it was used to identify the hepatoprotective peptide.

Figure 1. Properties of autofocusing fractions of wheat gluten hydrolysate (WGH). pH's of Autofocusing fractions are shown in a. The distribution of peptides (b) is expressed as the sum of the constituent amino acids. Amino acid composition (c) expressed as molar ratios of the acidic (Asp and Glu), basic (Lys, Arg, and His), and neutral amino acids (other amino acids) in the HCl hydrolysates.

Figure 1a, a pH gradient from approximately 2 to 12 was developed by autofocusing of WGH. More than 98% of the peptides in Fr. 1–10 were orally administered to rats 1 h prior to the injection of D-galactosamine. The doses of the peptides in Fr. 1–10 are shown in Figure 2. Lower doses were used for Fr. 1–3 than for the other fractions due to low peptide recovery. Injection of D-galactosamine induced acute hepatitis in rats and increased serum AST activity approximately 800 IU/L. As shown in Figure 2, Fr. 2 and 4 significantly decreased serum AST activity, while Fr. 9 significantly increased serum AST activity. Since Fr. 2 exhibited hepatoprotective activity at the lowest dose, it was used to identify the hepatoprotective peptide.
Identification of the Hepatoprotective Peptide in Active Autofocusing Fraction. Peptides in autofocusing Fr. 2 were eluted in Fr. 34–48 by size exclusion chromatography (Figure 3, upper). These compounds were derivatized with phenyl isothiocyanate, and the resultant phenyl thiocarbamyl derivatives were resolved by reversed-phase HPLC (Figure 3, lower). Fr. 34–38 had only reagent peaks without pyroglutamate aminopeptidase digestion (as indicated by the “−” in Figure 3), which indicated that these fractions contained negligible amounts of compounds with amino and imino groups. The pyroglutamate amino peptidase digests of SEC Fr. 34–36 (indicated by the “+” in Figure 3) yielded peaks that were not present in the undigested samples. Peaks 1–4 were collected and subjected to sequence analysis. The retention times of peaks 1 and 2 in SEC Fr. 34 and 35 were identical to those of PTC-Ile and PTC-Leu. Sequence analysis of the compounds in peaks 1 and 2 yielded phenyl thiohydantoin-Ile and -Leu, respectively. Sequence analysis of peaks 3 (SEC Fr. 35 and 36) and 4 (SEC Fr. 35) yielded Gln and Gln-Gln, respectively. These results indicate that SEC Fr. 34–36 contain pyroGlu-Ile (peak 1), pyroGlu-Leu (peak 2), pyroGlu-Gln (peak 3), and pyroGlu-Gln-Gln (peak 4). In contrast, no new peak appeared in SEC Fr. 37 and 38 after pyroglutamate aminopeptidase digestion; however, a large amount of glutamic acid was liberated by HCl hydrolysis. Precolumn derivatization with 2-nitrophenylhydrazine revealed the occurrence of free pyroglutamic acid (pyroGlu) in SEC Fr. 37 and 38.
Based on the amino acid composition of the SEC fraction and the peak areas of the PTC-peptides (Figure 3), the molar ratios of pyroGlu-Ile, pyroGlu-Leu, pyroGlu-Gln, pyroGlu-Gln-Gln, and free pyroGlu in autofocusing Fr. 2 were approximately 1.0:1.3:3.2:3.6:30.0.

**Hepatoprotective Activity of Pyroglutamyl Peptides.**

The hepatoprotective effects of the pyroglutamyl peptides in autofocusing Fr. 2 (20 mg/kg of body weight) and WGH (1 g/kg of body weight) were evaluated using d-galactosamine-induced hepatitis in rats. Serum AST and ALT activities are presented as a percentage of those of the vehicle group that received vehicle in each experiment. As shown in Figure 4, ingestion of pyroGlu-Leu (pEL) decreased serum AST and ALT to approximately 30% and 20% of the values of the vehicle group, which were near the normal levels. Ingestion of WGH (1 g/kg of body weight) and pyroGlu-Ile (pEI) also weakly but significantly decreased serum ALT activity. In contrast, ingestion of pyroGlu-Gln (pEQ),
pyroGlu-Gln-Gln (pEQQ), and free pyroGlu (pE) did not significantly affect serum AST and ALT activities. The hepatoprotective activity of pyroGlu-Leu was also confirmed by an additional two animal experiments using the same protocol (data not shown).

Absorption of PyroGlu-Leu in Portal Blood. Figure 5 shows typical MRM chromatograms of pyroGlu-Leu in portal blood plasma 30 min after ingestion of vehicle or pyroGlu-Leu (20 mg/kg of body weight). After the ingestion of pyroGlu-Leu, major peaks of m/z 243.1 > 86.1 and m/z 243.1 > 84.0 with same retention time as the pyrGlu-Leu standard were observed. The ratios of the ion intensity of m/z 243.1 > 86.1 to m/z 243.1 > 84.0 for the sample and standard were identical, which indicates that the major peaks can be assigned to pyroGlu-Leu. In contrast, only a negligible amount of pyroGlu-Leu was detected after ingestion of vehicle. As shown in the inset, the levels of pyroGlu-Leu increased significantly 30 min after ingestion of pyroGlu-Leu compared to the levels following ingestion of vehicle (p < 0.05). These results indicate that at least a portion of the orally administered pyroGlu-Leu is absorbed into portal blood and delivered to the liver.

**DISCUSSION**

To identify the active compounds in foods, activity-guided fractionation based on in vitro assays using cell culture and enzyme reaction systems along with separation by HPLC has been generally employed. However, peptides are frequently degraded into smaller peptides and amino acids in the digestive tract and in blood. Therefore, the biological activities of the peptides detected by such in vitro assays cannot be directly linked to the response by oral ingestion. Therefore, in the present study, we attempted to identify the hepatoproteective peptide in WGH using an in vivo activity-guided fractionation approach. This approach was based on large-scale peptide fractionation by autofocusing and a D-galactosamine-induced hepatitis model in rats, which exhibit acute hepatic injury that is histologically and biochemically comparable to that in humans. In fact, the present animal model can detect hepatoprotective activity of WGH in a shorter period compared to a previous animal model. This approach enables identification of the active peptide in food protein hydrolysates.

As pyroGlu-Leu exerts a stronger hepatoprotective activity in a smaller dose (20 mg/kg body weight) compared to WGH (1 g/kg body weight), pyroGlu-Leu can be considered to be one of the main hepatoprotective peptides in WGH. However, autofocusing fraction 2 exhibited hepatoprotective activity at 8 mg/kg body weight, which corresponds to only 0.27 mg/kg of pyroGlu-Leu; yet, pyroGlu-Leu alone did not show a hepatoprotective effect at less than 2 mg/kg using the present animal model (data not shown). Therefore, it is possible that the other pyroGlu peptides and free pyroGlu in Fr. 2 might enhance the hepatoprotective effect of pyroGlu-Leu. In addition, hepatoprotective activity was also detected in autofocusing Fr. 4, which may have other hepatoprotective peptides.

LC-MS/MS analysis demonstrated that pyroGlu-Leu is absorbed into portal blood. Therefore, it could reach the target organ, the liver. This indicates that pyroGlu-Leu is resistant to peptidase digestion in the gastrointestinal tract and blood. The indigestibility of pyroGlu-Leu is one reason it exerts biological activity after oral administration.

To our best knowledge, no single peptide that exerts hepatoprotective activity by oral ingestion in a similar dose to pyroGlu-Leu has been reported before the present study. PyroGlu-Leu is a dipeptide that can be easily synthesized in large-scale by liquid-phase chemical methods, as shown in the present study. Using chemically synthesized pyroGlu-Leu, further studies on the mechanisms underlying its beneficial activities are currently in progress. As a follow-up to these animal studies, the efficacy of pyroGlu-Leu and foods containing pyroGlu-Leu should be evaluated in a well-designed human trial.

**AUTHOR INFORMATION**

Corresponding Author
*Tel/Fax: 81 75 723 3503. E-mail: k_sato@kpu.ac.jp.

Author Contributions
*Y. Egashira and S. Ono contributed equally to this work.

Notes
The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

We express our appreciation to Kyoto Integrated Science & Technology Bio-Analysis Center for the use of their LC-MS/MS.

**ABBREVIATIONS**

WGH, wheat gluten hydrolysate; AST, aspartate aminotransferase; ALT, alanine aminotransferase; TFA, trifluoroacetic acid; DTT, dithiothreitol; PITC, phenyl isothiocyanate; PTC, phenyl thiocarbamyl; PTH, phenyl thiohydantoin; Boc, tert-butyloxycarbonyl; Fmoc, 9-fluorenylmethyloxycarbonyl; Bz, tert-butyll; HBTU, 1-[bis(dimethylamino)methylene]-1H-benzotriazolium 3-oxide hexafluorophosphate; HOBt, N-hydroxybenzotriazole; DMF, N,N-dimethylformamide; EDC-HCl, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide monohydrochloride; SEC, size exclusion chromatography; ESI-TOF-MS, electrospray ionization time-of-flight mass spectrometry

**REFERENCES**


