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Identification of Pyroglutamyl Peptides in Japanese Rice Wine (Sake): Presence of Hepatoprotective PyroGlu-Leu

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ABSTRACT: Japanese rice wine, *sake*, is made from steamed rice, water, and lactic acid by "multiple parallel fermentation" with mold (*Aspergillus oryzae*) and yeast (*Saccharomyces cerevisiae*). Nineteen pyroglutamyl peptides were identified in commercially available sake. Among them, pyroGlu-Leu and pyroGlu-Gln were the major constituents. PyroGlu-Leu has been demonstrated to attenuate hepatitis and colitis in animal models. Commercial products ($n = 5$) contained pyroGlu-Leu at concentrations ranging from 40 to 60 μM (10−15 mg/L). The pyroGlu-Leu content in sake mash increased during the fermentation processes. However, no pyroGlu-Leu was produced by yeast inoculated into preheated mash. Furthermore, addition of ^{13}C-Leu to the mash did not increase the ratio of pyroGlu-^{13}C-Leu to pyroGlu-^{12}C-Leu. On the other hand, digestion of steamed rice with *A. oryzae* proteases increased the pyroGlu-Leu content. These results indicate that pyroGlu-Leu in sake is produced from rice proteins by digestion with *A. oryzae* proteases.

KEYWORDS: *pyroglutamyl, pyroGlu-Leu, sake, rice wine, Aspergillus oryzae*

■ INTRODUCTION

Japanese rice wine, *sake*, is a traditional, fermented alcoholic beverage made from steamed rice, water, and lactic acid. The rice for sake brewing is polished to 45−70% of the starting material by dry milling before steaming. A part of the steamed rice is inoculated with *Aspergillus oryzae*, incubated at approximately 30 °C for a few days, and used as mold starter (*koji*). Koji, another part of the steamed rice, and water are mixed, acidified by addition of lactic acid to suppress the growth of various non-acid-resistant microorganisms, and then inoculated with sake brewers' yeast (*Saccharomyces cerevisiae*). The mixture is incubated at 15−20 °C for approximately 1 week to increase the amount of yeast, which is used as sake yeast starter (*shubo*). The other part of steamed rice, water, and koji are added to shubo (three times) and fermented for approximately 20 days after the final, third addition step; this mixture is referred to as sake mash (*moromi*). Moromi is pressed between cloths to obtain the liquid phase and then pasteurized to inactivate the microorganisms.

Glutamine is one of the most abundant amino acids in foods and the human body. However, glutamine is converted to L-2-pyrrolidone-5-carboxylic acid (pyroglutamic acid: pyroGlu) with condensation between the amino group at the α position and a side chain in water. This reaction, which is irreversible, proceeds even in low temperature and is accelerated by heating.[1,2] Peptides with a glutaminyl residue at the amino-terminal position can be converted to pyroglutamyl peptide[1] as shown in Figure 1. It has been demonstrated that pyroglutamyl peptides are widely distributed in enzymatic hydrolysates of food proteins; they account for nearly 10% (w/w) in some cases.[1,2] Short-chain pyroglutamyl peptides resist amino peptidase digestion[1] and can be absorbed as peptides into the portal blood of rats after ingestion.[3] In addition, it has been demonstrated that some pyroglutamyl peptides in wheat gluten hydrolysates exert biological activities. PyroGlu-Pro, pyroGlu-Pro-Ser, pyroGlu-Pro-Glu, and pyroGlu-Pro-Gln show umami taste.[4] More recently, it has been demonstrated that pyroGlu-Leu attenuates D-galactosamine induced acute hepatitis in rat[5] and dextran sulfate sodium induced colitis in mice.[6]

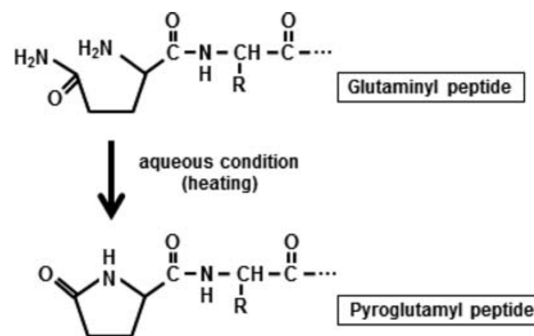

Figure 1. Production of pyroglutamyl peptide from peptide with a glutaminyl residue at the amino-terminal position.

Japanese fermented foods such as soy sauce, *shoyu*, and Japanese rice wine, sake, are prepared using *A. oryzae*, which produces extracellular acid proteinases and acid carboxypeptidases; large amounts of amino acid and short-chain peptides are produced by these enzymes. In addition, these products contain relatively high contents of free pyroGlu,[7,8] which suggests the

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presence of short chain pyroglutamyl peptides in these fermented foods. There is, however, limited information on the structure of the pyroglutamyl peptides present in these fermented foods. Recently, pyroGlu-Gln and pyroGlu-Gly were identified in shoyu and demonstrated to enhance the umami taste.[9] In the present study, we have focused on pyroglutamyl peptides in sake, in which the presence and structure of pyroglutamyl peptides have not yet been reported.

The objective of the present study was to identify pyroglutamyl peptides in sake to obtain basic knowledge of the pyroglutamyl peptides in sake.

■ MATERIALS AND METHODS

Samples. Five bottles of commercially available sake were obtained from different suppliers (A−E). In all cases, rice that had been polished to 60% was used.

Steamed rice was prepared from three different cultivars for sake brewing (*Yamadanishiki*, *Nihonbare*, and *Gohyakumangoku*) that had been polished to 45%, 60%, and 60%, respectively. The polished rice was soaked in water for 1 h and then steamed at 120 °C for 45 min on an industrial scale at the Shoutoku Brewery (Kyoto, Japan). Koji, shubo, and moromi were also prepared from 60% polished rice on an industrial scale at the Shoutoku Brewery. The steamed rice was cooled and inoculated with brewers' grade *A. oryzae*, (Hishiroku, Kyoto, Japan), incubated at 30 °C for 2 days, and then used as koji. The koji from 40 kg of polished rice was mixed with water (160 L) containing 1 L of food-grade lactic acid (Musashino Chemical Laboratory, Tokyo, Japan). The mixture was stirred thoroughly and inoculated with 1.2 L of slant of sake brewers' yeast (*S. cerevisiae*; Kyokai No. 9, Brewing Society of Japan, Tokyo, Japan). Then, steamed rice from 90 kg of polished rice was added. The mixture was incubated at approximately 22 °C for 4 days and at 14 °C for additional 2 days with stirring. The final product was used as shubo. Another part of the steamed rice, water, and koji were added to shubo (three times). This mixture was incubated at approximately 10 °C for 20 days and used as moromi. These products were brought to our laboratory in an ice box and stored at −20 °C until use.

Reagents. *Pyrococcus furiosus* pyroglutamate aminopeptidase was purchased from Takara Bio (Otsu, Japan). Protease and Taka-diastase from *A. oryzae* were purchased from Sigma-Aldrich (St. Louis, MO). L-Leucine (1-^{13}C, 99%) was purchased from Cambridge Isotope Laboratories (Andover, MA). Phenyl isothiocyanate (PITC), acetonitrile (HPLC grade), and trifluoroacetic acid (TFA) were purchased from Wako Chemicals (Osaka, Japan). Triethylamine was purchased from Thermo Fisher Scientific (Waltham, MA). Other reagents were of analytical grade or better.

Fractionation of Peptides in Sake. To separate pyroglutamyl peptides from peptides with an amino group, solid-phase extraction using a strong cation exchanger was performed as described previously.[3] The strong cation exchange resin (AG50W-x8, hydrogen form, 100−200 mesh, Bio-Rad Laboratories, Hercules, CA) was washed with 50% methanol and packed into a spin column (15 mm × 7 mm i.d., 5.0 μm pore size, Ultrafree-MC, Millipore, Billerica, MA). Then, 200 μL of 10 mM HCl was added onto the column and eluted by centrifugation at 7,000 rpm for 1 min for equilibration (three times). The resin can capture amino acids and peptides with an amino group in the presence of 75% ethanol.[10] Next, 200 μL of sake (Supplier A) was loaded onto the column and eluted by centrifugation. This procedure was repeated 10 times. After elution of the sample, the resin was washed with 100 μL of 50% methanol (twice). Unabsorbed effluents, which contained pyroGlu and pyroglutamyl peptides, were combined, dried under vacuum, and then dissolved in 200 μL of 0.1% TFA containing 30% acetonitrile.

The sample was clarified by passing through a spin column packed with Sephadex G-25 fine grade (GE Healthcare, Buckinghamshire, U.K.), which was pre-equilibrated with 0.1% TFA containing 30% acetonitrile, as described earlier. After passing the sample, the spin column was washed with 50 μL of 0.1% TFA containing 30% acetonitrile. The effluents were combined. The clarified sample (200 μL) was loaded onto a size exclusion chromatography (SEC) column, Superdex Peptide 10/30 GL (GE Healthcare), which was equilibrated with 0.1% TFA containing 30% acetonitrile at flow rate of 0.5 mL/min. Fractions were collected every 1 min from 10 to 50 min corresponding to fractions 11−50.

Identification of Pyroglutamyl Peptides. The sequence of the pyroglutamyl peptides was determined by the method as described previously.[11] Two sets of aliquots (100 μL) of the SEC fractions were transferred to 1.5-mL centrifugal tubes and dried under vacuum. One set was used as blank, and the other set was used for pyroglutamate aminopeptidase digestion. To the blank tubes was added 100 μL of 50 mM sodium phosphate buffer, pH 7.0, containing 10 mM dithiothreitol and 1 mM ethylenediaminetetraacetic acid. To the tubes used for the digestion were added 80 μL of the same buffer and 20 μL of pyroglutamate aminopeptidase solution (0.4 mU/20 μL of the same buffer). The enzymatic reaction was carried out at 60 °C for 1 h. The reaction was terminated by drying under vacuum. The amino groups liberated by pyroglutamate aminopeptidase digestion were reacted with PITC. The resultant phenylthiocarbamyl (PTC) derivatives were resolved by reversed phase-high performance liquid chromatography (RP-HPLC) as described previously.[11] Peaks that appeared only in the pyroglutamate amino peptidase digest were collected and dried under vacuum. To residual PTC derivatives, was added 20 μL of a "redrying solution" consisting of methanol, water, and triethylamine (7:1:2), and the mixture was redried under vacuum to remove ammonia. Then, the residues were dissolved in 30% methanol and applied to an automatic peptide sequencer that operatedon the basis of the Edman degradation (PPSQ-21, Shimadzu, Kyoto, Japan). Programs of the peptide sequencer were changed to start from the cleavage reaction with TFA.[12]

SEC fractions that were not subjected to pyroglutamate aminopeptidase digestion or derivatization with PITC were also subjected to electrospray ionization-ion trap mass spectrometry (ESI-MS) and -tandem mass spectrometry (ESI-MS/MS) analyses. Aliquots of SEC fractions (100 μL) were mixed with four volumes of 0.1% formic acid containing 50% acetonitrile and directly injected to a LCQ (Thermo Fisher Scientific, Waltham, MA) or API 3200 (AB SCIEX, Foster City, CA) using syringe pump. The detection of parent and product ions was performed in positive mode and optimized using the auto tune program.

Production of PyroGlu-Leu by Yeast. To examine the production of pyroGlu-Leu by sake brewers' yeast (*S. cerevisiae*), three yeast preparations were inoculated into koji hot-water extracts. Koji hot-water extracts were prepared on laboratory scale as described previously[13] with a slight modification. Koji, steamed rice, and distilled water were mixed in the ratio 1:1:2 (volume) and then incubated overnight at 55 °C. The extracts were filtered through filter paper (No. 5C, Advantec, Tokyo, Japan), adjusted to 5° Baume with distilled water, and then sterilized in an autoclave at 120 °C for 20 min. Three strains of yeast (Koshi No. 2, 2NF, and 221) from the Kyoto Municipal Institute of Industrial Technology and Culture (Kyoto, Japan) were used. A loopful of these yeast slant cultures was transferred to 40 mL of sterilized koji hot-water extract and incubated at 30 °C for 3 days without shaking. The mediums were filtered through filter paper as described above and used in the subsequent liquid chromatography−tandem mass spectrometry (LC−MS/MS) analysis.

Production of PyroGlu-Leu from ^{13}C-Leu in Shubo. To investigate the production of pyroGlu-Leu from free Leu in shubo, 1-^{13}C-labeled Leu was added to shubo. The production of pyroGlu-^{13}C-Leu was monitored by LC−MS/MS as described below. Shubo, which was prepared in an industrial scale at the Shoutoku brewery, was homogenized using a blender (Ace Homogenizer, Nihonseiki, Tokyo, Japan). The homogenate (1.5 g) was placed into 2.0-mL centrifugal tubes. Various concentrations of aqueous ^{13}C-Leu solutions (100 μL) were added to the homogenates to give final concentrations of 0.5, 1.0, 2.5, and 5.0 μmol/g. The homogenate to which no ^{13}C-Leu was added was used as blank. These reaction mixtures were incubated at 22 °C for 4 days and at 14 °C

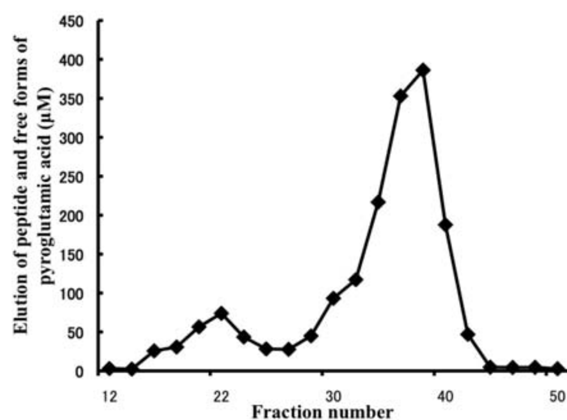

Figure 2. Elution profile of peptides in the unabsorbed fraction of solid-phase extraction of sake by size exclusion chromatography (SEC). Peptide contents are expressed as the sum of the constituting amino acids in the SEC fractions after HCl hydrolysis. Free pyroglutamyl acid and pyroglutamyl residues in peptides are converted to Glu by acid hydrolysis.

for an additional 2 days with shaking. Aliquots of these samples were collected every day and mixed with the same volume of distilled water. The samples were centrifuged at 13,000 rpm for 5 min. The supernatants were mixed with 3 volumes of ethanol. The resultant precipitates were removed by centrifugation. The ethanol-soluble fractions (200 μL) were subjected to solid-phase extraction as described above. Unabsorbed effluents were combined and then dried under vacuum. The dried fractions were dissolved with distilled water (200 μL) and clarified by passing through a filter (Cosmonice filter W, 0.45 μm pore size, 4 mm i.d., Nacalai Tesque, Kyoto, Japan). The clarified samples (180 μL) were injected to a RP-HPLC column, Inertsil ODS-3 (250 × 4.6 mm i.d., 5 μm, GL Science, Tokyo, Japan), equilibrated with 0.1% formic acid at a flow rate of 1.0 mL/min. Elution was performed using a binary linear gradient that consisted of 0.1% formic acid (A) and 0.1% formic acid containing 80% acetonitrile (B). The gradient profile was as follows: 0−15 min, B 0−50%; 15−15.1 min, B 50−100%; 15.1−20 min, B 100%; 20−20.1 min, B 100−0%; 20.1−30 min, B 0%. The column was maintained at 45 °C. The pyroGlu-Leu fraction, which was eluted between 15.5 and 16.5 min, was collected and used in the subsequent LC−MS/MS analysis.

Enzymatic Digestion of Steamed Rice. To investigate the production of pyroGlu-Leu from rice proteins by protease digestion, steamed rice prepared from three cultivars of rice grain was digested with *A. oryzae* enzymes. Steamed rice was homogenized with 2.2 volumes of distilled water using a blender. An aliquot of the homogenate (10 g) was mixed with 10 mL of distilled water and digested by 18.6 mg of Taka-diastase and 12.5 mg of a protease from *A. oryzae* at 50 °C for 5 h with shaking. Then, the reaction mixture was centrifuged at 3,000 rpm for 10 min. The supernatant was mixed with 3 volumes of ethanol. The pyroGlu-Leu fraction was prepared from the ethanol-soluble fraction by RP-HPLC as described above and used in the subsequent LC−MS/MS analysis.

Amino Acid Analysis. Vapor-phase HCl hydrolysis was performed according to the method of Bidlingmeyer et al.[14] Briefly, amino acids were derivatized with PITC, and the resulting PTC-amino acids were resolved by applying the same condition used for the PTC-peptides as described above.

Determination of PyroGlu-Leu using LC−MS/MS. PyroGlu-Leu in koji was extracted with water. Koji was mixed with water at a weight ratio of 15:85 and homogenized using a glass homogenizer. Shubo and moromi were homogenized without adding water. These suspensions were centrifuged at 2,000 rpm for 20 min. The supernatants were diluted (1:10) with distilled water. In case of the bottled sake, a sample was directly diluted with distilled water. The yeast-inoculated koji hot-water extracts were diluted with distilled water (1:10). These samples (200 μL) were subjected to solid-phase extraction using the strong cation exchange resin as described above. The unabsorbed fractions were collected and dried under vacuum. The dried samples were dissolved in 200 μL of ultrapure water; then, aliquots of the samples were diluted with ultrapure water (1:10) and filtered using Cosmonice filter W. The pyroGlu-Leu fractions from ^{13}C-Leu-added shubo (300 μL) and the enzyme digests of steamed rice (1 mL) were dried under vacuum and dissolved in 100 μL and

Table 1. Sequences, Precursor, and Product Ions of Pyroglutamyl Peptides in Size Exclusion Chromatography (SEC) Fractions of Sake

SEC fraction(s)	peak	peptide sequence[a]	precursor ions (m/z)	product ions (m/z)
32	a	pyroGlu-Asn-Ile-Asp-Asn-Pro	683.4	69.6 (immonium ion of Pro), 86.0 (immonium ion of Ile), 116.0 (y1), 311.2 (a3), 339.1 (b3), 454.2 (b4), 568.2 (b5), 666.2 ([M − NH_3]$^+$)
33, 34	b	pyroGlu-Asn-Ile	357.1	225.9 (b2), 243.7 (c2), 158.1 (x1), 131.9 (y1), 272.8 (x2), 229.4 (z2), 338.9 ([M − H_2O]$^+$)
33, 34, 35	c	pyroGlu-Val	229.1	118.5 (y1), 182.9 ([210.9 − CO]$^+$), 210.9 ([M − H_2O]$^+$)
33	d	pyroGlu-Leu-Trp	428.9	205.7 (y1), 188.1 (z1), 316. Seven (y2), 301.9 (z2), 411.1 ([M − H_2O]$^+$)
33, 34	e	pyroGlu-Val-Ala	299.9	183.0 (a2), 210.9 (b2), 115.7 (x1)
33	f	pyroGlu-Val-Pro	325.9	112.9 (b1), 128.8 (c1), 182.2 (a2), 210.9 (b2), 116.0 (y1), 214.9 (y2), 198.0 (z2)
33	g	pyroGlu-Val-Val	328.0	183.1 (a2), 210.9 (b2), 144.3 (x1), 118.0 (y1), 243.1 (x2), 283.3 ([M − COOH]$^+$), 309.9 ([M − H_2O]$^+$)
34, 35	h	pyroGlu-Asn-Phe	390.9	262. Seven (z2), 373.0 ([M − H_2O]$^+$)
34, 35, 36	i	pyroGlu-Leu	243.0	131.9 (y1), 197.0 ([224.9 − CO]$^+$), 224.9 ([M − H_2O]$^+$)
35, 36, 37	j	pyroGlu-Gln	258.1	129.1 (c1), 146.9 (y1), 212.9 ([M − COOH]$^+$), 240.0 ([M − H_2O]$^+$)
35, 36	k	pyroGlu-Ser-Gln	345.1	199.0 (b2), 215.9 (c2), 146.9 (y1), 129.9 (z1), 234.1 (y2), 327.0 ([M − H_2O]$^+$)
35	l	pyroGlu-Met	260.9	149.9 (y1), 214.9 ([242.9 − CO]$^+$), 242.9 ([M − H_2O]$^+$)
36	m	pyroGlu-Gly-Gln	314.9	141.1 (a2), 169.0 (b2), 146.9 (y1), 129.9 (z1), 187.2 (z2), 269.0 ([297.1 − CO]$^+$), 297.1 ([M − H_2O]$^+$)
36, 37	n	pyroGlu-Tyr	293.0	182.0 (y1), 247.0 ([274.9 − CO]$^+$), 274.9 ([M − H_2O]$^+$)
36	o	pyroGlu-Phe	276.9	166.0 (y1), 230.9 ([259.0 − CO]$^+$), 259.0 ([M − H_2O]$^+$)
37	p	pyroGlu-Asn	244.2	132.9 (y1), 197.8 ([225.9 − CO]$^+$), 225.9 ([M − H_2O]$^+$)
37	q	pyroGlu-Ser	216.9	106.0 (y1), 171.1 ([199.0 − CO]$^+$), 199.0 ([M − H_2O]$^+$)
37	r	pyroGlu-Gly	187.1	84.0 (a1), 75.9 (y1), 169.1 ([M − H_2O]$^+$)
37	s	pyroGly-Ala	200.9	89.9 (y1), 155.1 ([182.9 − CO]$^+$), 182.9 ([M − H_2O]$^+$)

[a]Estimated sequences by mass spectrometry and tandem mass spectrometry analyses of SEC fractions, together with Edman degradation based on sequences of phenyl thicarbamyl-amino acids of peptides in the pyroglutamate aminopeptidase digests (peaks a−s, Figure 3).

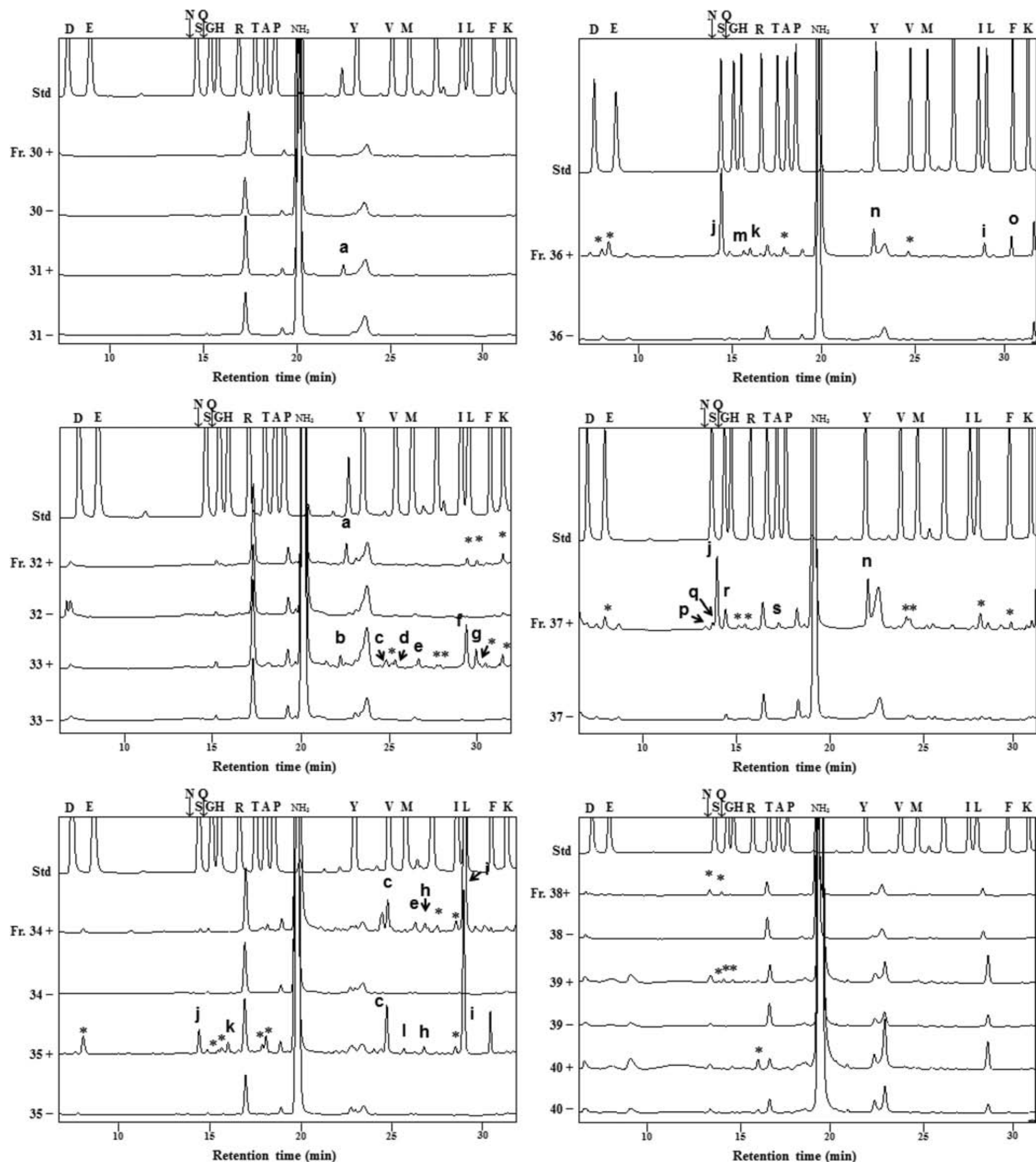

Figure 3. Isolation of phenyl thiocarbamyl-amino acids/peptides in pyroglutamate aminopeptidase digests (+) and nondigests (−) of size exclusion chromatography fractions 30−40. Peaks marked with letters yielded phenyl thiohydantoin-amino acids upon Edman degradation. Peaks marked with an asterisk did not yield phenyl thiohydantoin-amino acids.

1 mL of ultrapure water, respectively. The amounts of pyroGlu-Leu in these samples were determined using an LC−MS/MS that consisted of the Prominence 20A HPLC system (Shimadzu, Kyoto, Japan), the API 3200, and an Inertsil ODS-3 column (250 × 2.1 mm i.d., 5 μm). The column was equilibrated with 0.1% formic acid containing 5% acetonitrile at a flow rate of 0.2 mL/min. Elution was performed using a binary linear gradient consisting of 0.1% formic acid containing 5% acetonitrile (A) and 0.1% formic acid containing 80% acetonitrile (B). The gradient profile was as follows: 0−15 min, B 0−80%; 15−15.1 min, B 80−100%; 15.1−20 min, B 100%; 20−20.1 min, 100−0%; 20.1−30 min, B 0%. The column was maintained at 40 °C. Detection was performed by multi reaction monitoring in positive mode. Multi reaction monitoring conditions were optimized using Analyst Version.1.4.2 (AB SCIEX).

Statistical Analysis. The pyroGlu-Leu contents at the different brewing steps of sake were expressed as means of triplicate ± standard deviations. Differences between these means were evaluated by

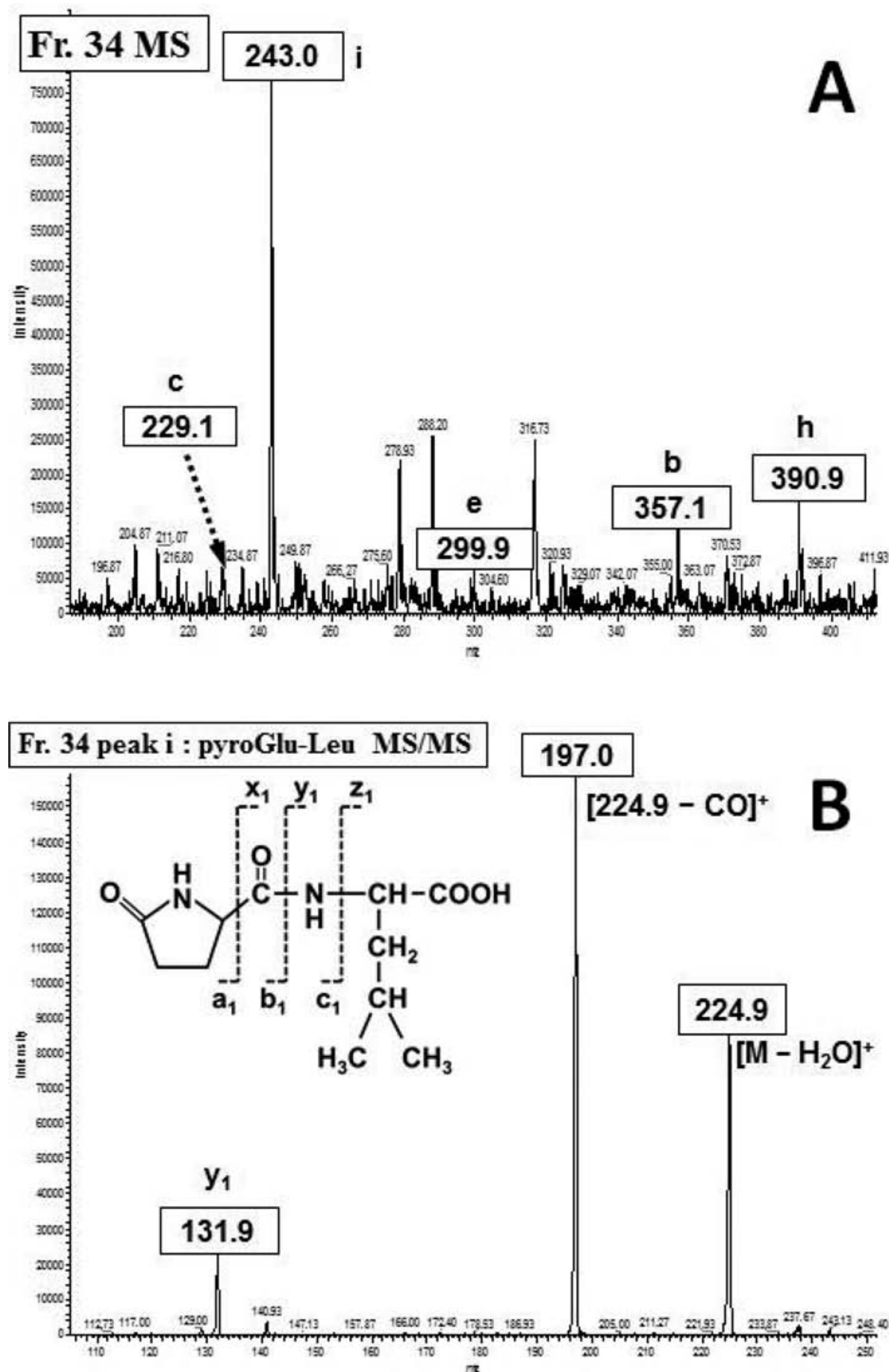

Figure 4. Electrospray ionization-mass spectrometry (ESI-MS) spectrum of the intact compounds in size exclusion chromatography fraction 34 (A) and electrospray ionization-tandem mass spectrometry (ESI-MS/MS) spectrum of the peak with an m/z value of 243.00 (B). Peaks marked with letters in panel A have m/z values of precursor ions corresponding to the estimated pyroglutamyl peptides indicated with the same letters in Table 1.

Tukey's test using Ekuseru-Toukei 2012 Version 1.10 (Social Survey Research Information, Tokyo, Japan).

■ RESULTS

Identification of Short-Chain Pyroglutamyl Peptides in Sake. Pyroglutamyl peptides and pyroGlu in the unabsorbed fraction obtained by solid-phase extraction were first fractionated by SEC based on the molecular mass. The elution of these compounds was monitored by amino acid analysis following HCl hydrolysis. As shown in Figure 2, nearly 80% of these compounds were eluted after 30 min. On the basis of the elution volume, these fractions might contain peptides with a molecular mass of less than approximately 1,000 Da. Fractions 30−40 were considered to be short-chain pyroglutamyl peptide fractions and used for subsequent analyses.

The compounds in SEC fractions 30−40 of samples that had or had not been subjected to pyroglutamate aminopeptidase digestion were reacted with PITC. The resultant PTC-amino acids and -peptides were resolved by RP-HPLC and detected at 254 nm. As shown in Figure 3, some peaks appeared only after pyroglutamate aminopeptidase digestion, indicating that they

could be potentially derived from pyroglutamyl peptides. These peaks were collected and subjected to Edman degradation analysis. Peaks marked with letters (a−s) yielded phenyl thiohydantion-amino acids, while peaks marked with an asterisk did not. On the basis of the Edman degradation analysis of the PTC-derivatives (peaks a−s in Figure 3), the estimated sequences of pyroglutamyl peptides in sake (Supplier A) are listed in Table 1. PTC-derivatives (peaks c, i, j, l, n, o, p, q, r, and s in Figure 3) showed the same retention time PTC-Val, -Leu, -Gln, -Met, -Tyr, -Phe, -Asn, -Ser, -Glu, and -Ala, respectively, which supports the validity of the estimated sequences of these peptides listed in Table 1. SEC fractions 38−40 did not yield significant amounts of PTC-derivatives that could yield phenyl thiohydantoin-amino acids even after pyroglutamate aminopeptidase digestion; however, they yielded Glu after HCl hydrolysis (Figure 2). It has been demonstrated that free pyroGlu is eluted in these fractions under the same condition.[5] These facts indicate that fractions 38−40 predominantly consist of free pyroGlu.

To further confirm the sequences of the pyroglutamyl peptides listed in Table 1, SEC fractions of samples that had not been subjected to pyroglutamate aminopeptidase digestion were subjected to ESI-MS and ESI-MS/MS analyses. The MS spectrum of SEC fraction 34 is shown in Figure 4A. Ion peaks with m/z values of 229.1, 243.0, 299.9, 357.1, and 390.9 were observed, which correspond to protonated ions of pyroGlu-Val, pyroGlu-Leu, pyroGlu-Val-Ala, pyroGlu-Asn-Ile, and pyroGlu-Asn-Phe, respectively. The major ion with an m/z value of 243.0 corresponding to pyroGlu-Leu was subjected to MS/MS analysis. As shown in Figure 4B, product ions from pyroGlu-Leu were observed that correspond to the y1 ion, the loss of a water group, and the loss of a carboxyl group. These data also confirm the presence of pyroGlu-Leu in SEC fraction 34. As summarized in Table 1, all protonated parent ions and their fragment ions were observed by MS and MS/MS analyses. Together with the results obtained using Edman degradation analysis, the presence of pyroglutamyl peptides as listed in Table 1 in sake is strongly indicated.

As shown in Figure 3, pyroGlu-Leu and pyroGlu-Gln were the major constituents of the pyroglutamyl peptides in sake (Supplier A). On the basis of the peak areas shown in Figure 3, the pyroGlu-Leu and pyroGlu-Gln contents were estimated to be approximately 36.2% and 21.3% of the total pyroglutamyl peptides, respectively.

PyroGlu-Leu Content in Sake. Figure 5A shows the pyroGlu-Leu content in commercially available sake from different suppliers. In all cases, pyroGlu-Leu was detected at concentrations ranging from 40 to 60 μM (10−15 mg/L). Figure 5B shows the pyroGlu-Leu content at different steps of sake brewing. Whereas the water extract of koji contained less than 0.1 μM (0.02 mg/L) pyroGlu-Leu, shubo at 2 days after mixing contained 23.2 μM pyroGlu-Leu. The pyroGlu-Leu content tentatively decreased in moromi on the first day (odori) because new ingredients were added to shubo but also increased significantly during the shubo and moromi processes.

Production of PyroGlu-Leu. First, the production of pyroGlu-Leu by yeast was examined. Three types of sake brewers' yeast preparation were inoculated into koji hot-water extracts. After incubation at 30 °C for 3 days, no increase in the pyroGlu-Leu content was observed (data not shown).

Second, the production of pyroGlu-Leu from free Leu in shubo was examined. The ratio of pyroGlu-^{13}C-Leu to pyroGlu-^{12}C-Leu in shubo, to which different amounts of ^{13}C-Leu had been added, was determined by monitoring the immonium ions of ^{12}C-Leu and ^{13}C-Leu from pyroGlu-Leu (m/z 243.1 > 86.1 and 244.1 > 87.1), respectively. As shown in Figure 6A and B, the amount of both forms of pyroGlu-Leu consisting of ^{12}C-Leu and ^{13}C-Leu increased during the incubation of shubo. However, the ratio of ^{13}C-Leu to ^{12}C-Leu in pyroGlu-Leu did not increase after addition of ^{13}C-Leu, even at 5 μmol/g of shubo, which was five times more than the Leu content originally present in shubo (Figure 6C). These results indicate that only a negligible amount of pyroGlu-Leu was produced from free Leu in shubo.

Third, the production of pyroGlu-Leu by proteolysis of rice proteins by *A. oryzae* enzymes was examined. As shown in Figure 6D, pyroGlu-Leu was produced from steamed rice and reached a concentration of 7 μM (2 mg/L) in the reaction mixtures from three different rice cultivars by enzyme treatment, whereas steamed rice contained only a negligible amount of pyroGlu-Leu before digestion.

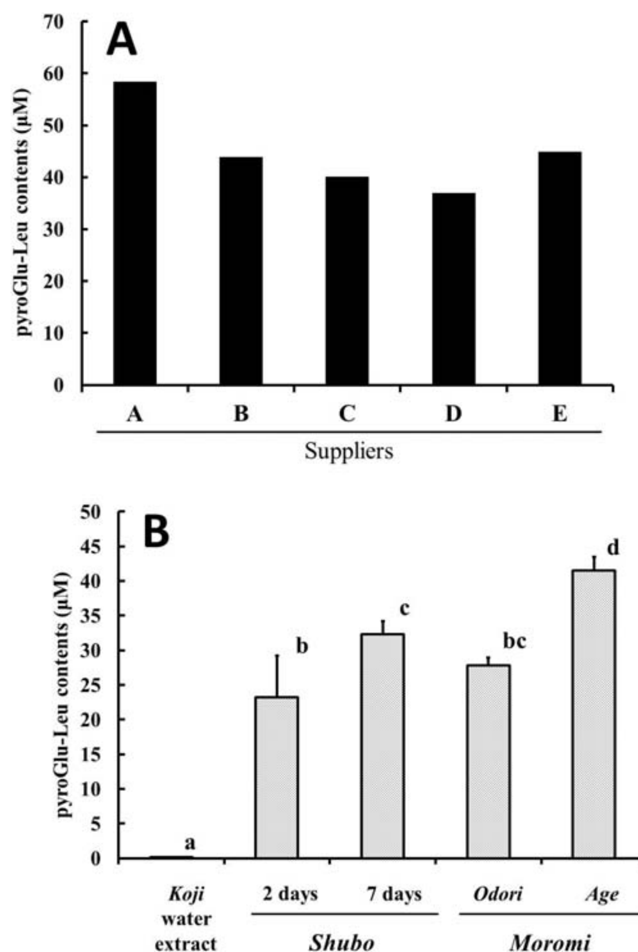

Figure 5. PyroGlu-Leu contents in commercially available sake from five different suppliers (A) and change in the contents during the brewing process (B). "Shubo 2 days" and "7 days" indicate 2 and 7 days after mixing the materials of shubo, respectively. Moromi odori refers to the first addition of steamed rice, water, and koji to completed shubo (7 days), and moromi age means completed moromi at approximately 20 days after the final, third addition step. Data are presented as mean ± standard deviations (n = 3). Different letters indicate a significant difference by Tukey's test (p < 0.05).

DISCUSSION

As summarized in Table 1, 19 pyroglutamyl peptides were identified in a Japanese rice wine (sake), which for the first time provides basic knowledge about the structure of pyroglutamyl peptides in sake. Among them, pyroGlu-Gln and pyroGlu-Leu

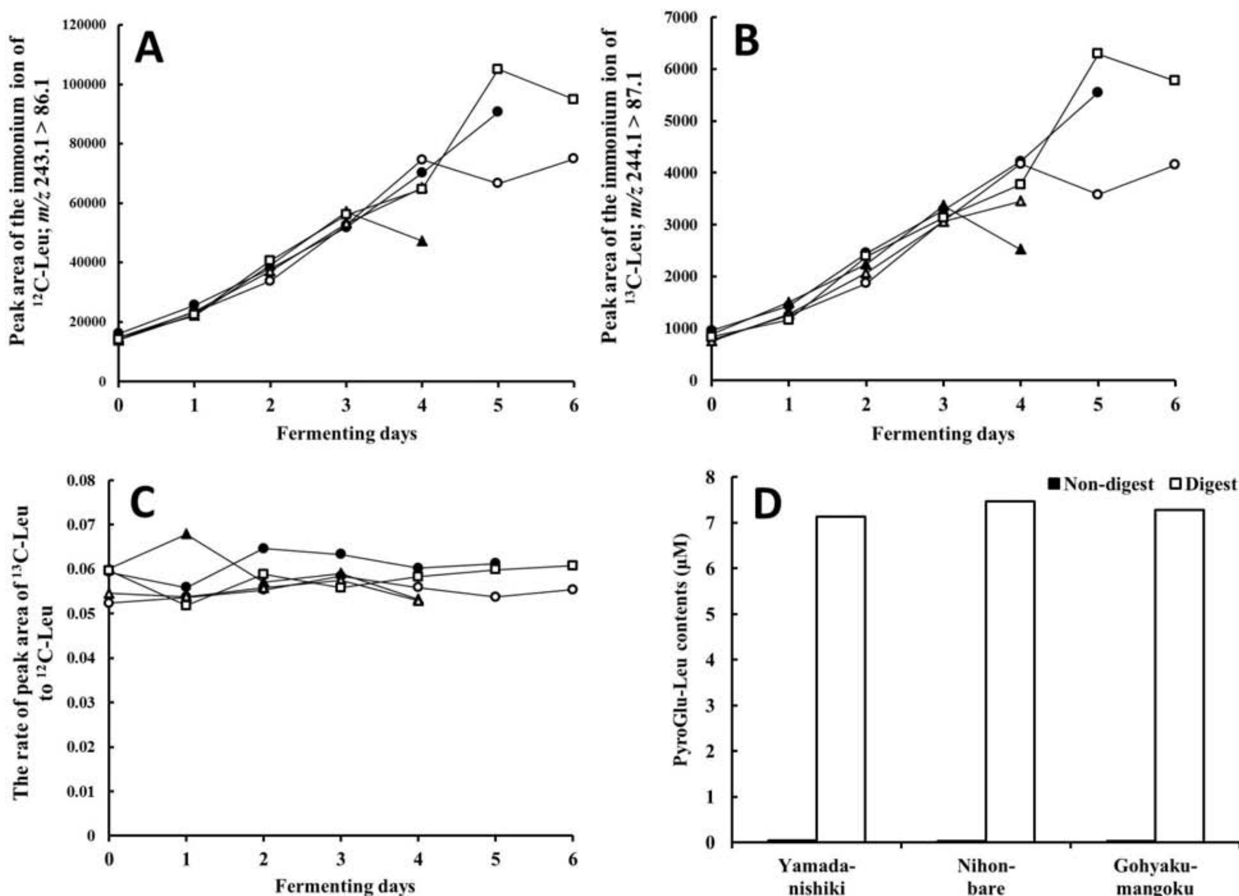

Figure 6. Production of pyroGlu-Leu by incubation of shubo (A−C) and digestion of steamed rice by *Aspergillus oryzae* enzymes (D). Different levels of ^{13}C-Leu were added to shubo, and the production of pyroGlu-^{12}C-Leu (A) and pyroGlu-^{13}C-Leu (B) and the ratio of pyroGlu-^{13}C-Leu/-^{12}C-Leu (C) are presented. Symbols: (●) 0; (○) 0.5; (▲) 1.0; (△) 2.5; (□) 2.0 μmol/g of 1-^{13}C-Leu. Contents of pyroGlu-^{12}C-Leu and pyroGlu-^{13}C-Leu are expressed as peak areas of the immonium ion of ^{12}C-Leu, *m/z* 243.1 > 86.1, and ^{13}C-Leu, *m/z* 244.1 > 87.1, as arbitrary unit. PyroGlu-Leu content in samples of steamed rice from three different cultivars that had or had not been subjected to digestion by *A. oryzae* enzymes (D).

were major constituents (Figure 3). The presence of pyroGlu-Gln has also been demonstrated in Japanese-style soy sauce (shoyu) at 1.6 mM. It has been demonstrated that pyroGlu-Gln enhances umami taste at approximately 0.3 mM.[9] Sake is frequently used in Japanese cuisine to enhance flavor and taste. Depending on the recipe, relatively large volumes of sake (compared to shoyu) are used. Thus, pyroglutamyl peptides, including pyroGlu-Gln, in sake can contribute to improving the flavor and taste of traditional Japanese dishes.

PyroGlu-Leu, the major constituent of pyroglutamyl peptides in sake, was first identified in a wheat gluten hydrolysate, which improved hepatitis[5] and colitis[6] at low doses (20 and 0.1 mg/kg body weight, respectively) in animal models. The present study demonstrated that pyroGlu-Leu is present in commercially available sake at concentrations ranging from more than 40 μM (10 mg/L) (Figure 5A). However, consuming sake is not directly linked to improvement of hepatitis and colitis, as it contains other compounds, such as high concentration (approximately 15% v/v) of ethanol.

As shown in Figure 5B, the water extract of *A. oryzae*-inoculated steamed rice (koji, a solid phase of fermented materials) contained a low level of pyroGlu-Leu. The peptide concentration increased in the subsequent fermentation processes (shubo and moromi), in which yeast (*S. cerevisiae*) and airborne lactic acid bacteria were involved, in addition to *A. oryzae*. However, the yeast in the koji hot-water extract, in which both *A. oryzae* and its enzymes were denatured by autoclave sterilization, did not produce pyroGlu-Leu, which indicates that yeast does not play a significant role in the production of pyroGlu-Leu. In addition, no significant amounts of pyroGlu-Leu were produced from free Leu by microorganisms in shubo (Figure 6A−C). On the other hand, pyroGlu-Leu was produced by digestion of steamed rice with an *A. oryzae* protease (Figure 6D). PyroGlu-Leu has been demonstrated to resist protease digestion.[5] Therefore, pyroGlu-Leu is not degraded in the sake brewing process. In addition, *A. oryzae* secretes proteases into the extracellular medium,[15,16] whereas yeast and lactic acid bacteria do not.[15,17] With these facts, it can be concluded that *A. oryzae* proteases play a significant role in the production of pyroGlu-Leu in sake brewing. Therefore, nonalcoholic food ingredients rich in pyroGlu-Leu can be produced from rice and other protein sources by fermentation with *A. oryzae* or digestion by its proteases, which have the potential for improving hepatitis and colitis in humans.

In addition to pyroGlu-Leu, nearly 20 pyroglutamyl peptides, whose biological activities remain to be determined, were identified in sake. Further studies on the production of pyroGlu-Leu by *A. oryzae* fermentation and on the biological activities of the other pyroglutamyl peptides in sake are in progress.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

A. oryzae, *Aspergillus oryzae*; ESI-MS, electrospray ionization-mass spectrometry; ESI-MS/MS, electrospray ionization-tandem mass spectrometry; LC–MS/MS, liquid chromatography-tandem mass spectrometry; PITC, phenyl isothiocyanate; PTC, phenyl thiocarbamyl; pyroGlu, pyroglutamic acid; RP-HPLC, reversed phase-high performance liquid chromatography; *S. cerevisiae*, *Saccharomyces cerevisiae*; SEC, size exclusion chromatography; TFA, trifluoroacetic acid

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