# Study on pyroglutamyl peptides in Japanese rice wine, Sake

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## Contents

# Chapter 1

# General introduction

Glutamine is one of the most abundant amino acids in food and the human body. However, glutamine is converted to L-2-pyrrolidone-5-carboxylic acid (pyroglutamic acid; pyroGlu) with condensation between the amino groups at α position and side chain in water. This reaction, which is irreversible, proceeds even in low temperature and is accelerated by heating (Sato et al., 1998; Suzuki et al., 1999). Peptides with a glutaminyl residue at the amino-terminus can be converted to pyroglutamyl peptide (Sato et al., 1998) as shown in Figure 1. It has been demonstrated that pyroglutamyl peptide is widely distributed in enzymatic hydrolysates of food proteins; they account for nearly 10% (w/w) in some cases (Sato et al., 1998; Suzuki et al., 1999). Short chain pyroglutamyl peptides resist amino peptidase digestion (Sato et al., 1998) and can be absorbed as peptides into the portal blood of rats after ingestion (Higaki-Sato et al., 2006). 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 (Schlichtherle-Cerny et al., 2002). More recently, it has been demonstrated that pyroGlu-Leu attenuates D-galactosamine induced acute hepatitis in rat (Sato et al., 2013) and dextran sulfate sodium induced colitis in mice (Wada et al., 2013).

Japanese fermented foods such as soy sauce, *shoyu*, and Japanese rice wine, *sake*, are prepared using *Aspergillus oryzae*, which produces extracellular acid proteinases and acid carboxypeptidases (Kitano et al., 2002; Rao et al., 1998). Large amounts of amino acid and short-chain peptides are produced by these enzymes. In addition, these foods

contain relatively high contents of free pyroGlu (Ito et al., 2013; Tadenuma, M., 1966), which suggests the presence of short-chain pyroglutamyl peptides in these fermented foods. Recently, pyroGlu-Gln and pyroGlu-Gly were identified in shoyu and demonstrated to enhance the umami taste (Kaneko et al., 2011). However, there is still limited information on the structure of the pyroglutamyl peptides present in these fermented foods.

Japanese rice wine, sake, is a traditional fermented alcoholic beverage made from steamed rice, water, and lactic acid using fermentation with two microorganisms. 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 *A. 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 and acidified by addition of lactic acid to suppress the growth of various non-acid-resistant microorganisms, and then inoculated with sake brewer's 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. This liquid is pasteurized to inactivate the microorganisms, and then mixed with alcohol for brewing and water if necessary. Completed sake contains various oligosaccharides, amino acids and peptides derived rice starch and protein, respectively, because *A. oryzae* secretes extracellular amylases and proteases.

Some unique compounds with potential health-promoting activities have been

identified in sake. It has been demonstrated that ethyl α-D-glucoside is produced by *A. oryzae* transglutaminase during sake brewing (Imanari et al., 1971; Oka et al., 1976), which has potential to moderate UVB-induced epidermal barrier disruption (Hirotsune et al., 2005) and D-galactosamine-induced hepatitis (Izu et al., 2007) by oral administration in animal models. Short chain peptides in sake have been demonstrated to have inhibitory activities against angiotensin I converting enzymes (Saito et al., 1992 and 1994 a) and prolyl endpeptidase (Saito et al., 1997), and exert anti-hypertensive activity in a spontaneous hypertensive rat by oral administration (Saito et al., 1994 b).

To my best knowledge, there is, however, no information on short-chain pyroglutamyl peptides in sake. The objective of the present study was to identify the pyroglutamyl peptides in sake, which is one of the salt-free fermented alcohol beverages using *A. oryzae* with high amylases and proteases activity, to obtain basic knowledge of the pyroglutamyl peptides in sake and explore their potential health-promoting effects.

## Figures

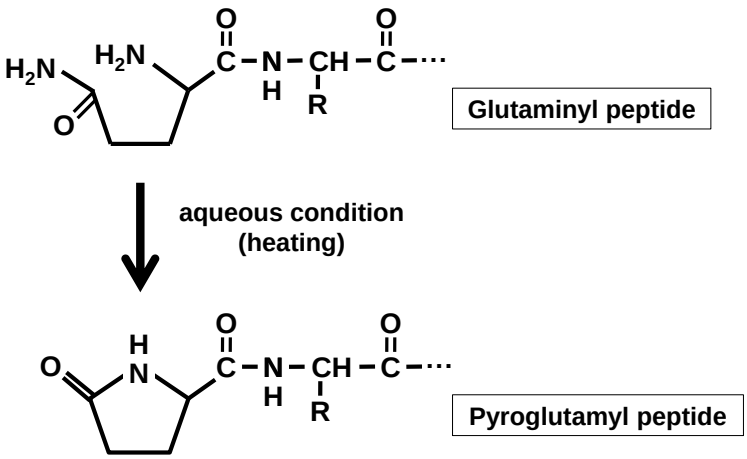


Figure 1. Production of pyroglutamyl peptide from peptide with a glutaminyl residue at the amino-terminal position.

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# Chapter 2

# Identification of pyroglutamyl peptides from sake: presence of hepatoprotective pyroGlu-Leu

## Introduction

As mentioned in chapter 1, pyroglutamyl peptide is widely distributed in enzymatic hydrolysate of food protein (Sato et al., 1998; Suzuki et al., 1999). Recent studies has reported the various beneficial effects of short-chain pyroglutamyl peptides in food protein hydrolysate, such as enhancing umami taste (Schlichtherle-Cerny et al., 2002), attenuating acute hepatitis (Sato et al., 2013) and colitis (Wada et al., 2013), which indicate that food-derived pyroglutamyl peptide may have the beneficial effects for human health.

Japanese rice wine, *sake* is a traditional fermented alcoholic beverage made from steamed rice, water, and lactic acid using multiple parallel fermentation (see chapter 1 for detail). In Japan, sake is not only consumed as alcohol beverage, but also used as one of major ingredients for Japanese cuisine, which is frequently used to add special flavor and taste to Japanese style dishes. As mentioned in chapter 1, sake may contain unique bioactive compounds including short-chain pyroglutamyl peptides. Therefore, daily intake of these compounds in sake might affect human health. However, there is very limited information on the pyroglutamyl peptides present in sake.

The objective of study in chapter 2 was to obtain basic knowledge of the pyroglutamyl peptides in sake.

## Materials and methods

### 1. 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 Shotoku Brewery (Kyoto, Japan). *Koji*, *shubo*, and *moromi* were also prepared from 60% polished rice on an industrial scale at the Shotoku Brewery. The steamed rice was cooled and inoculated with brewers' grade *Aspergillus 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 culture medium of sake brewers' yeast (*Saccharomyces 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.

**2. Reagents.**

*Pyrococcus furiousus* pyroglutamate aminopeptidase was purchased from Takara Bio (Otsu, Japan). Protease and Taka-diastase from *A. oryzae* were purchased from Sigma-Aldorich (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 (TEA) was purchased from Thermo Fisher Scientific (Waltham, MA). Other reagents were of analytical grade or better.

**3. Fractionation of peptides in sake.**

To separate pyroglutamyl peptides from peptides with an amino group, solid-phase extraction using a strong cation exchange resin was performed as described previously (Higaki-Sato et al., 2006). 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 × 7 mm i.d., 5.0 μm pore size, Ultrafree-MC, Millipore, Billerica, MA, Division of Merck, Darmstadt, Germany). 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 (Aito-Inoue et al., 2006). 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% (v/v) TFA containing 30% (v/v) 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.

**4. Identification of pyroglutamyl peptides.**

The sequence of the pyroglutamyl peptides was determined by the method as described previously (Higaki-Sato et al., 2003). 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 phenyl thiocarbamyl (PTC) derivatives were resolved by reversed phase-high performance liquid chromatography (RP-HPLC) as described previously (Higaki-Sato et al., 2003). Peaks that appeared only in the

pyroglutamate aminopeptidase digest were collected. The fractions were dried under vacuum. To residual PTC derivatives, was added 20 μL of a “redrying solution” consisting of methanol, water, and TEA (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 operated on 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 (Sato et al., 1999).

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 4 volumes of 0.1% formic acid containing 50% acetonitrile and directly injected to a LCQ (Thermo Fisher Scientific) or API 3200 (AB Sciex, Foster City, CA) using a syringe pump. The detection of parent and product ions were performed in positive mode and optimized using the auto tune program (Xcaliber, Thermo Fisher Scientific or Analyst, AB Sciex).

**5. 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 (Asano et al., 1999) 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 clarified by passing through a filter paper (No. 5C, Advantec, Tokyo, Japan), and 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 clarified by passing through the filter paper as described above and used in the subsequent liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis.

**6. Production of pyroGlu-Leu from $^{13}$C-Leu added in shubo.**

To investigate the production of pyroGlu-Leu from free Leu in shubo, 1-$^{13}$C-labeled Leu was added to the 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 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, 4 mm i.d., 0.45 μm pore size,

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), which was 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 (solvent A) and 0.1% formic acid containing 80% acetonitrile (solvent 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.

**7. 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 blendar. 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.

**8. Amino acid analysis.**

Vapor-phase 6 M HCl hydrolysis was performed according to the method of Bidlingmeyer et al. (1984). 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.

**9. 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; and then, aliquots of the samples were diluted with ultrapure water (1:10) and filtered using a 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 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), 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 (solvent A) and 0.1% formic acid containing 80% acetonitrile (solvent 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, B 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).

**10. Statistical analysis.**

The pyroGlu-Leu contents at the different brewing steps of sake were expressed as mean of triplicate ± standard deviations. Differences between these mean were evaluated by Tukey's test using Ekuseru-Toukei 2012 Version 1.11 (Social Survey Research Information, Tokyo, Japan).

## Results

### 1. 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 1, 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 (Fr.) 30–40 were considered to be short-chain pyroglutamyl peptide fractions and used for subsequent analyses.

The compounds in SEC Fr. 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 2, 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 thiohydantoin (PTH)-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 2), 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, -Gly, and -Ala, respectively, which supports the validity of the estimated structures as summarized in Table 1. SEC Fr. 38–40 did not yield significant amounts of

PTC derivatives that could yield PTH-amino acids even after pyroglutamate aminopeptidase digestion; however, they yielded Glu after HCl hydrolysis (Figure 1). It has been demonstrated that free pyroGlu is eluted in these fractions under the same condition. These facts indicate that Fr. 38–40 predominantly consist of free pyroGlu.

To further confirm the sequences of the pyroglutamyl peptides listed in Table 1, SEC Fr. 32–37 that had not been subjected to pyroglutamate aminopeptidase digestion were subjected to ESI-MS and ESI-MS/MS analyses. The MS spectrum of SEC Fr. 34 is shown in Figure 3A. Ion peaks with *m/z* values of 229.1 (c), 243.0 (i), 299.9 (e), 357.1 (b), and 390.9 (h) 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 3B, 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 Fr. 34. As summarized in Table 1, all protonated parent ions and their fragment ions were observed by MS and MS/MS analyses. Details for MS and MS/MS patterns are shown in the supplemented data attached in the end of this chapter. 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 2, 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 2, the pyroGlu-Leu and pyroGlu-Gln contents were estimated to be approximately 36.2% and 21.3% of the total pyroglutamyl peptides, respectively.

**2. PyroGlu-Leu content in sake.**

Figure 4I shows 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 4II 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/mL) pyroGlu-Leu, shubo at 2 days after mixing contained 23.2 μM (6 mg/L) 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.

**3. Production of pyroGlu-Leu.**

First, the production of pyroGlu-Leu by yeast (*Saccharomyces cerevisiae*) 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 (Figure 5).

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 (Figure 6C), even at 5 μmol/g of shubo, which was five times more than the Leu content originally present in shubo. 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.

## Discussion

As summarized in Table 1, 19 pyroglutamyl peptides were identified in sake, which for the first time provides basic knowledge about the structure of pyroglutamyl peptides in sake. Among them, pyroGlu-Gln and pyroGlu-Leu were major constituents (Figure 2). The presence of pyroGlu-Gln has also been demonstrated in Japanese-style soy sauce, shoyu, at 1.6 mM (Kaneko et al., 2011). It has been demonstrated that pyroGlu-Gln enhances umami taste at approximately 0.3 mM. The sake (supplier A) contained pyroGlu-Gln at approximately 35 μM. Sake is frequently used in Japanese cuisine to enhance flavor and taste. Depending on the recipe, relatively large volumes of sake (compared shoyu) are used. Thus, pyroglutamyl peptides in sake, including pyroGlu-Gln, 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 (Sato et al., 2013) and colitis (Wada et al., 2013) 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 4I). In addition, pyroGlu-Leu was not produced from free Leu in sake yeast starter, shubo, when too much amount of free Leu was added in the shubo (Figure 6A–C). However, this peptide increased with digestion of rice protein by *A. oryzae* (Figure 6D). PyroGlu-Leu has been demonstrated to resist protease digestion (Sato et al., 2013). Therefore, pyroGlu-Leu may not be degraded in the sake brewing process. In addition, *A. oryzae* secretes proteases into the extracellular medium (Rao et

al., 1998 and Kitano et al., 2002), whereas yeast and lactic acid bacteria do not (Rao et al., 1998 and Savijoki et al., 2006). 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, non-alcoholic 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 human.

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

## Figures and tables

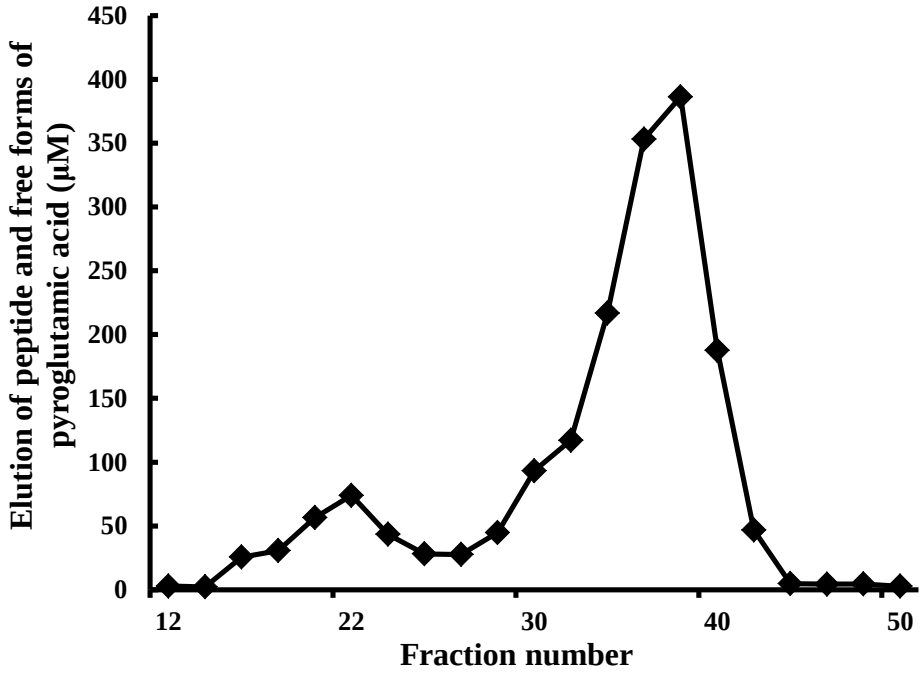
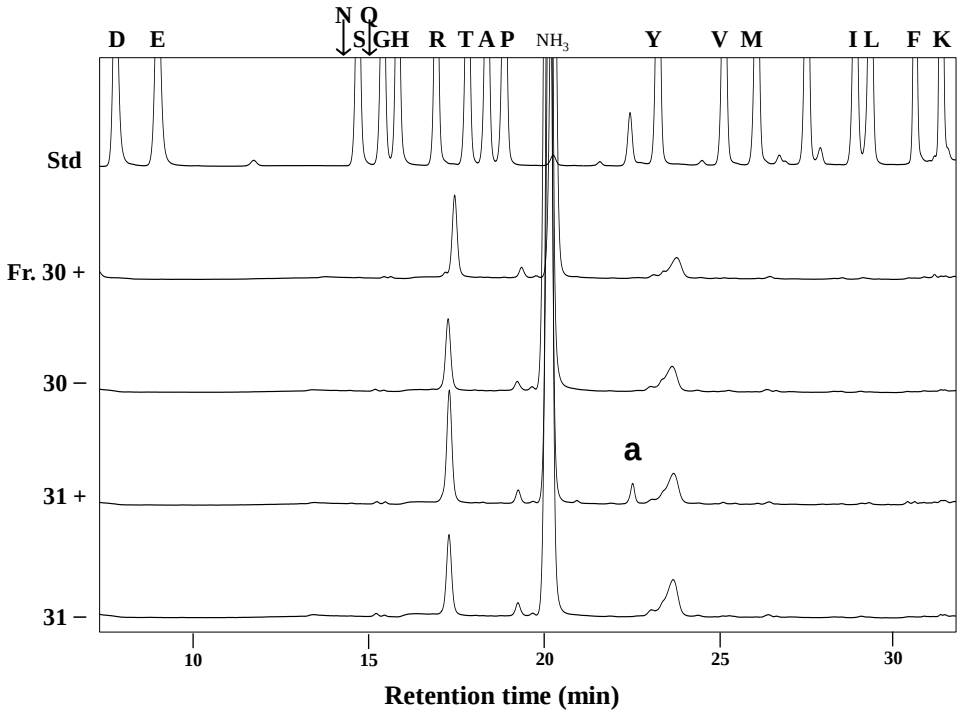



Figure 1. Elution profile of peptides in the unabsorbed fractions of solid-phase extraction of sake by size exclusion chromatography (SEC) using Superdex Peptide 10/30 GL (GE Healthcare). Peptide contents are expressed as the sum of the constituting amino acids in the SEC fractions after HCl hydrolysis. Free pyroglutamic acid and pyroglutamyl residues in peptides are converted to glutamic acid by the HCl hydrolysis.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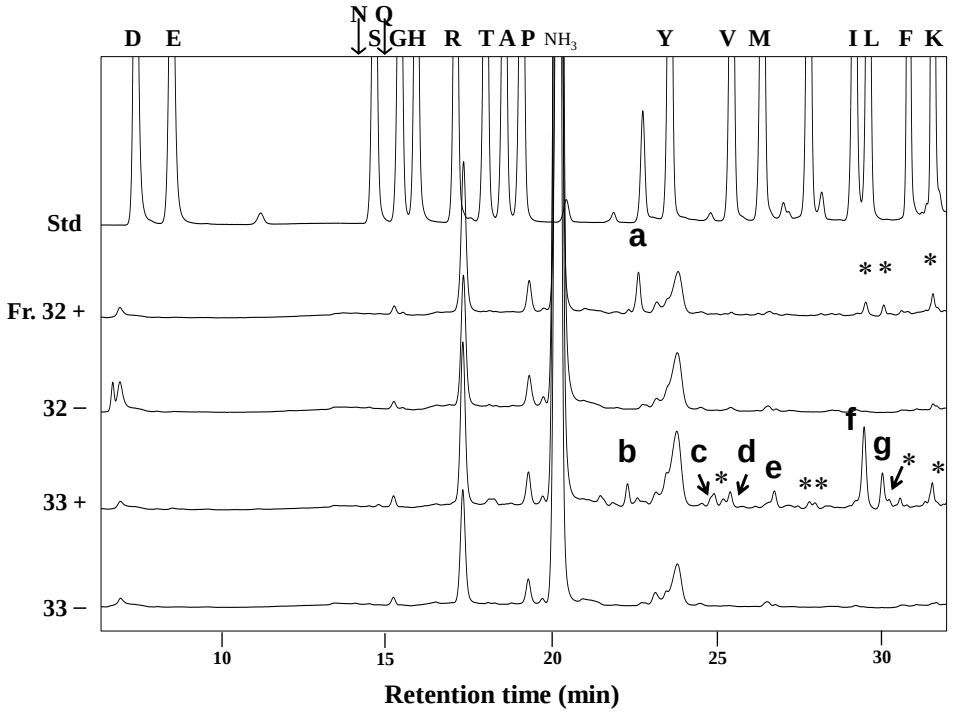
30 –
31 +
31 –
a
10
15
20
25
30
Retention time (min)


D E N Q S G H R T A P NH3 Y V M I L F K
Std
Fr. 32 +
32 –
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33 –
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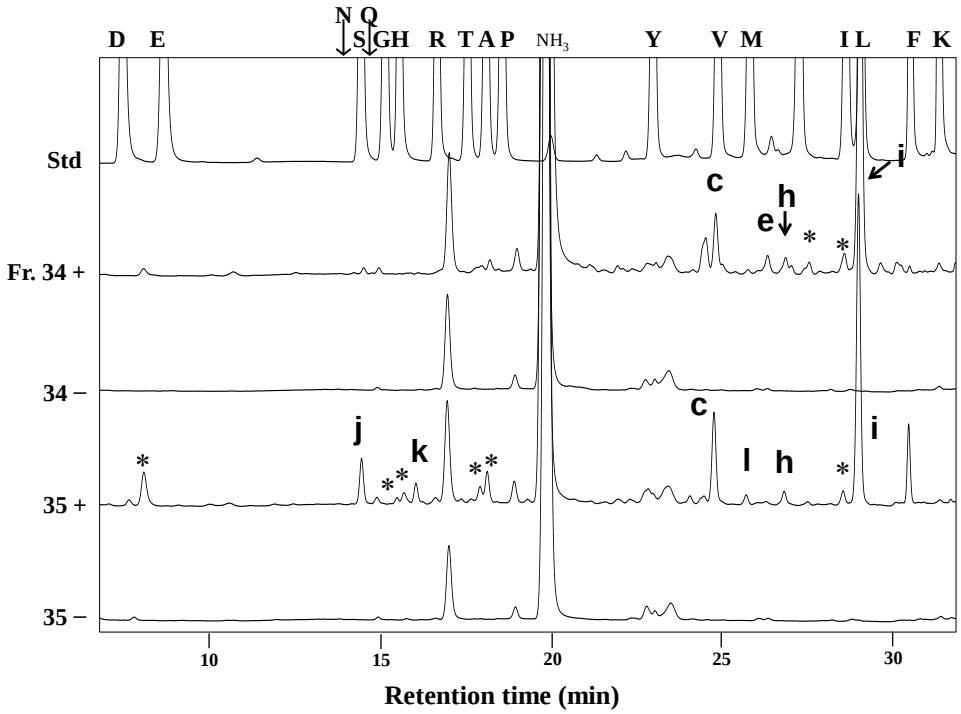
Retention time (min)


D E N Q S G H R T A P NH3 Y V M I L F K
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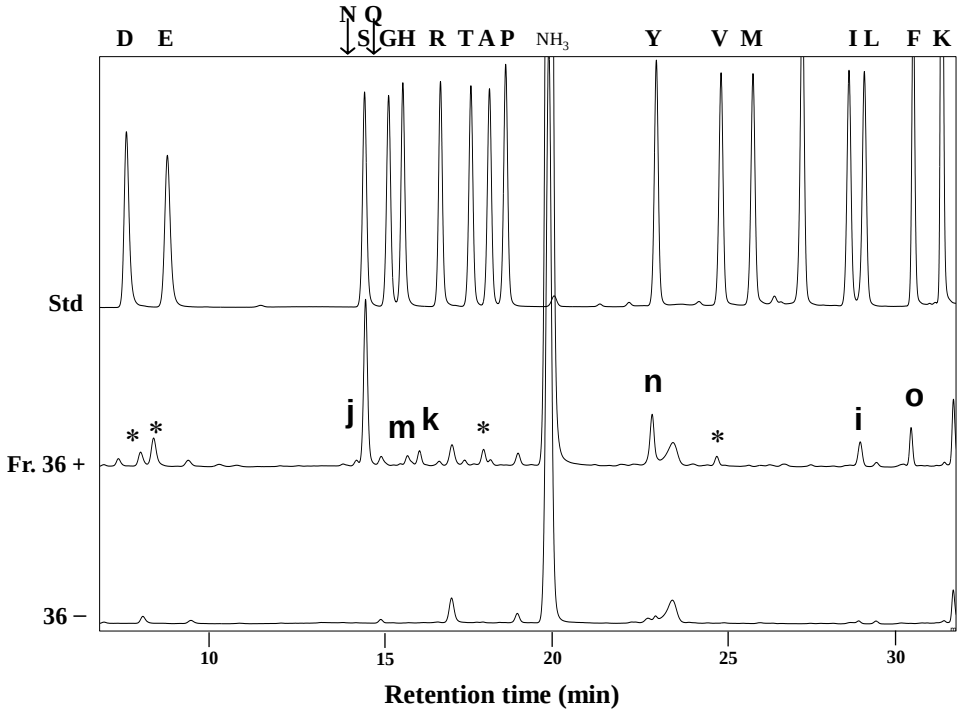
Retention time (min)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Retention time (min)

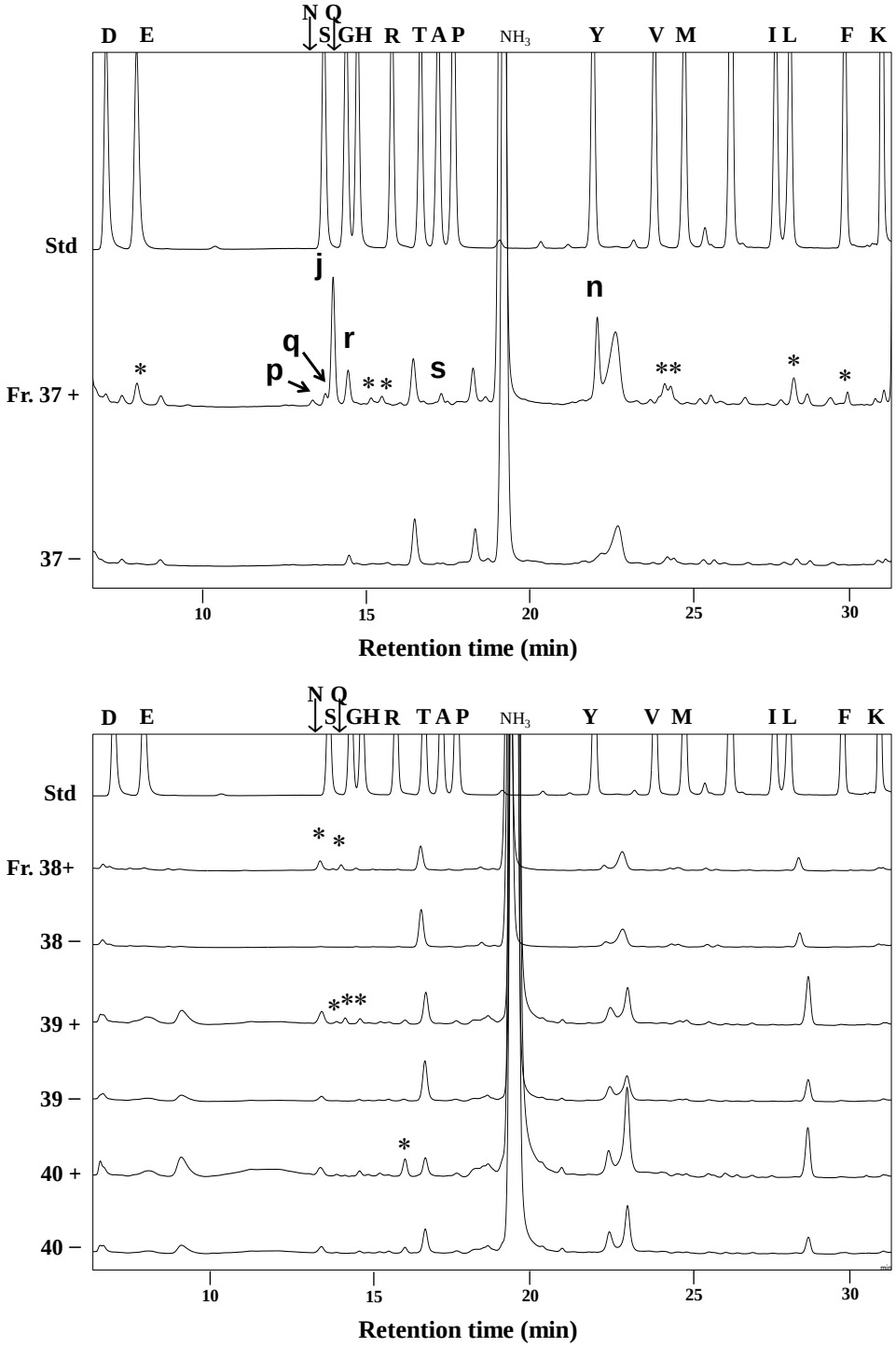


Figure 2. Isolation of phenyl thiocarbamyl-amino acids/-peptides in pyroglutamate aminopeptidase digests (+) and non-digests (−) of size exclusion chromatography fractions 30–40. Peaks marked with letters yielded phenyl thiohydantoin (PTH)-amino acids upon Edman degradation. Peaks marked with an asterisk did not yield PTH-amino acids.

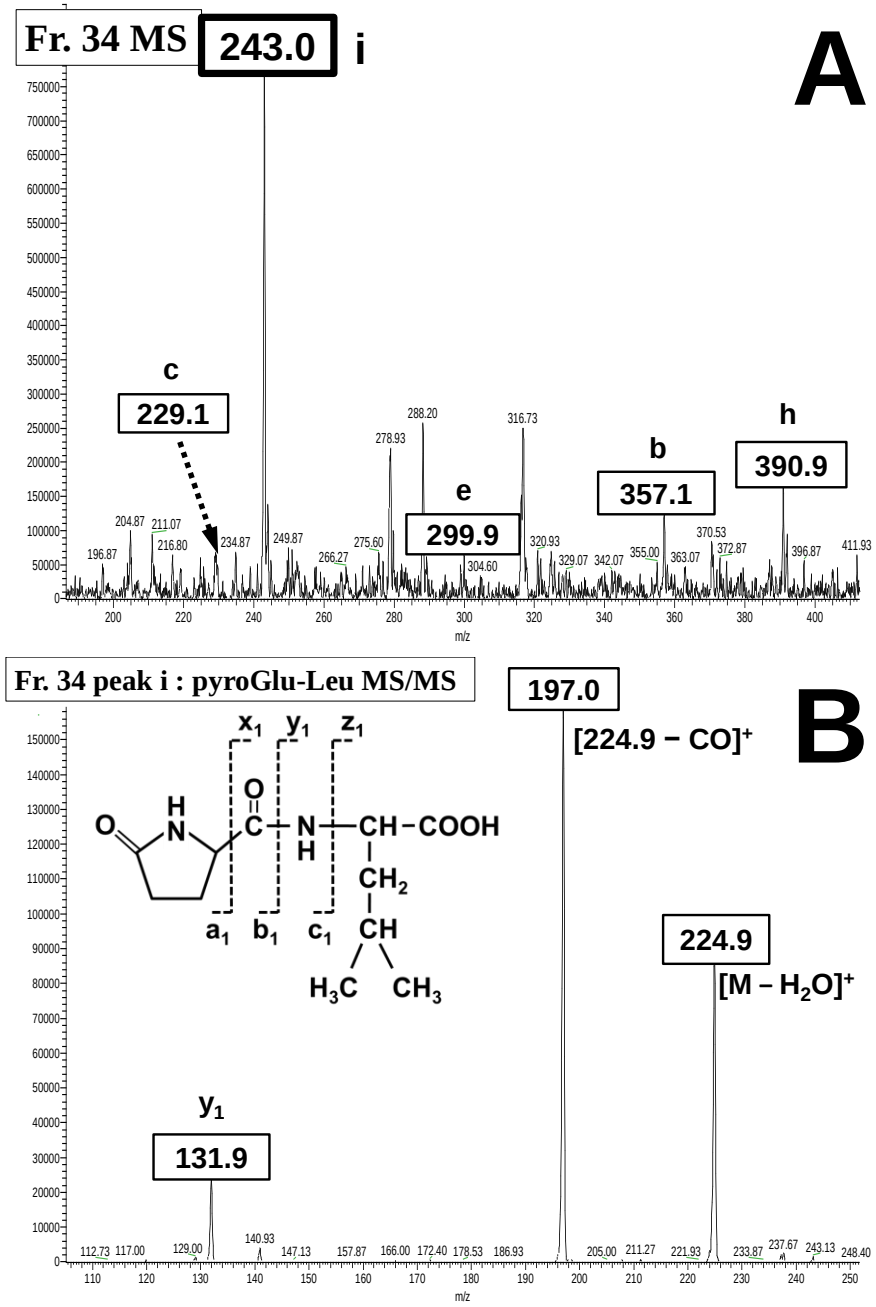


Figure 3. Electrospray ionization-mass spectrometry (ESI-MS) spectrum of the intact compounds in size exclusion chromatography fractions 34 (A) and ESI-tandem mass spectrometry (MS/MS) spectrum of the peak with an *m/z* value of 243.0 (B). Peaks marked with letters in panel A have *m/z* values of precursor ions corresponding to the estimated pyroglutamyl peptides indicated with the same letters in Table 1.

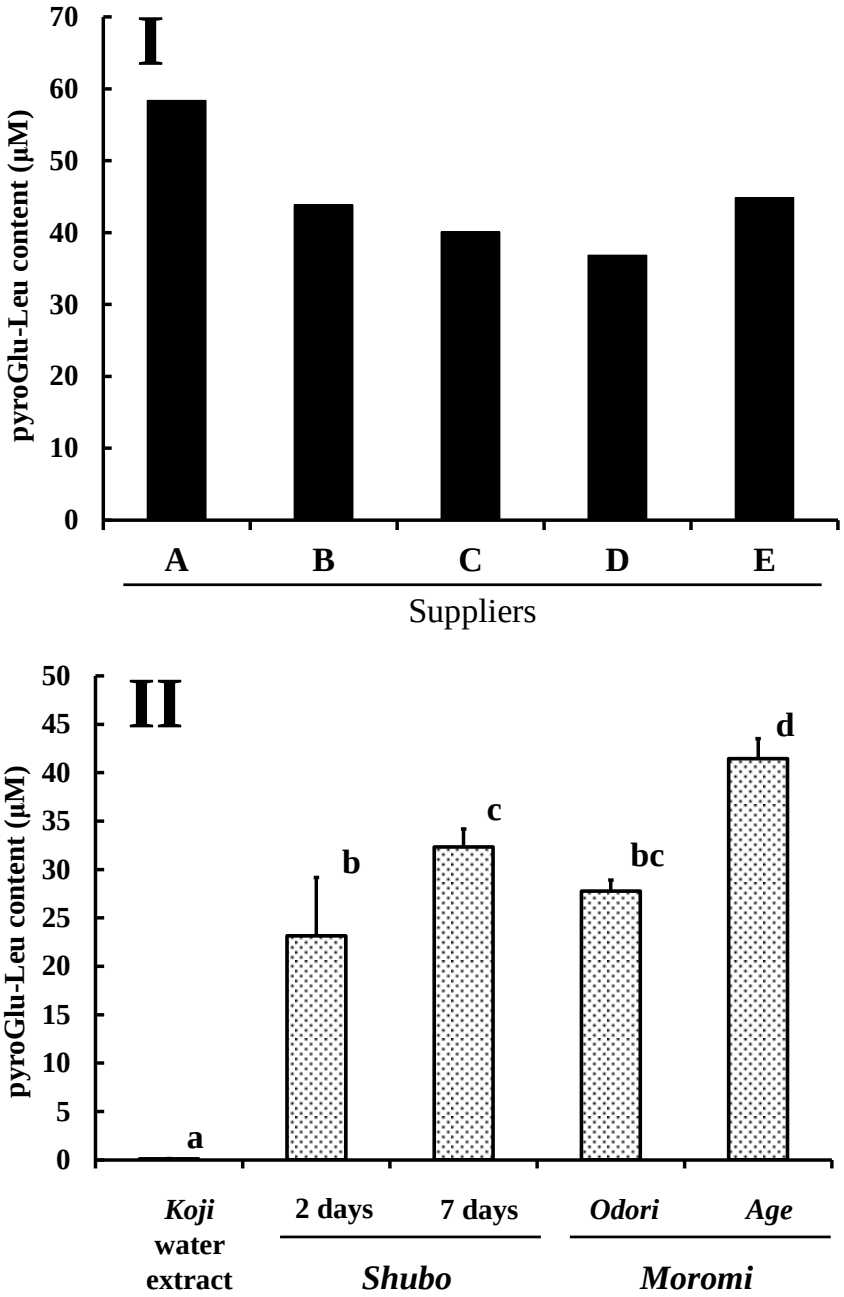


Figure 4. PyroGlu-Leu content in commercially available sake from five different suppliers (I) and change in the content during the brewing process (II). “Shubo 2 days” and “7 days” indicate 2 and 7 days after mixing the materials of shubo, respectively. “Moromi odori” refers to the first addition of 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 shown as mean ± standard deviations ($n$ = 3). Different letters indicate a significant difference by Tukey’s test ($p < 0.05$).

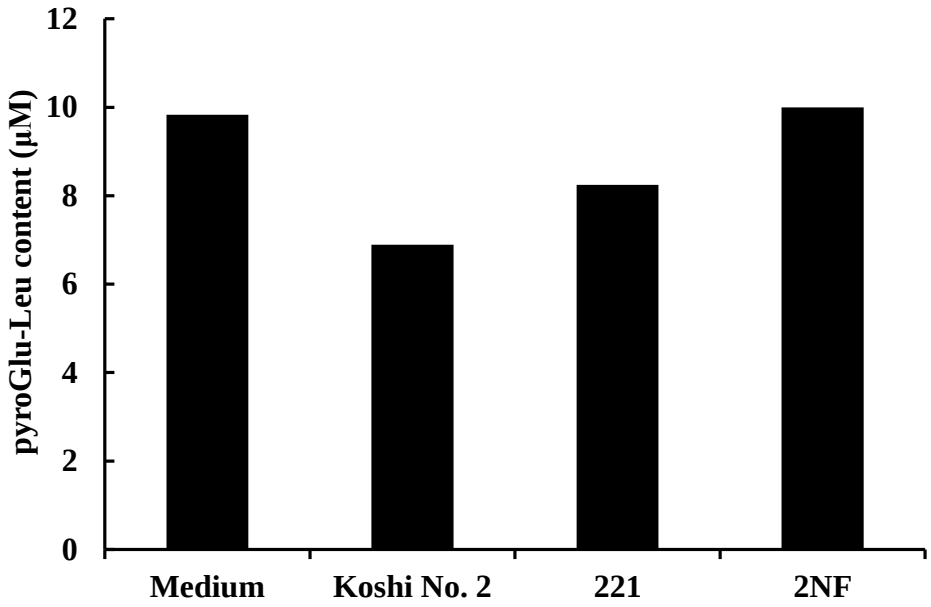


Figure 5. PyroGlu-Leu content in koji hot-water extracts three days after inoculation of three strains of brewers' yeast (Koshi No. 2, 221, and 2NF). The extracts were kept at 30°C for three days. Medium: the koji hot-water extract without inoculation of yeast.

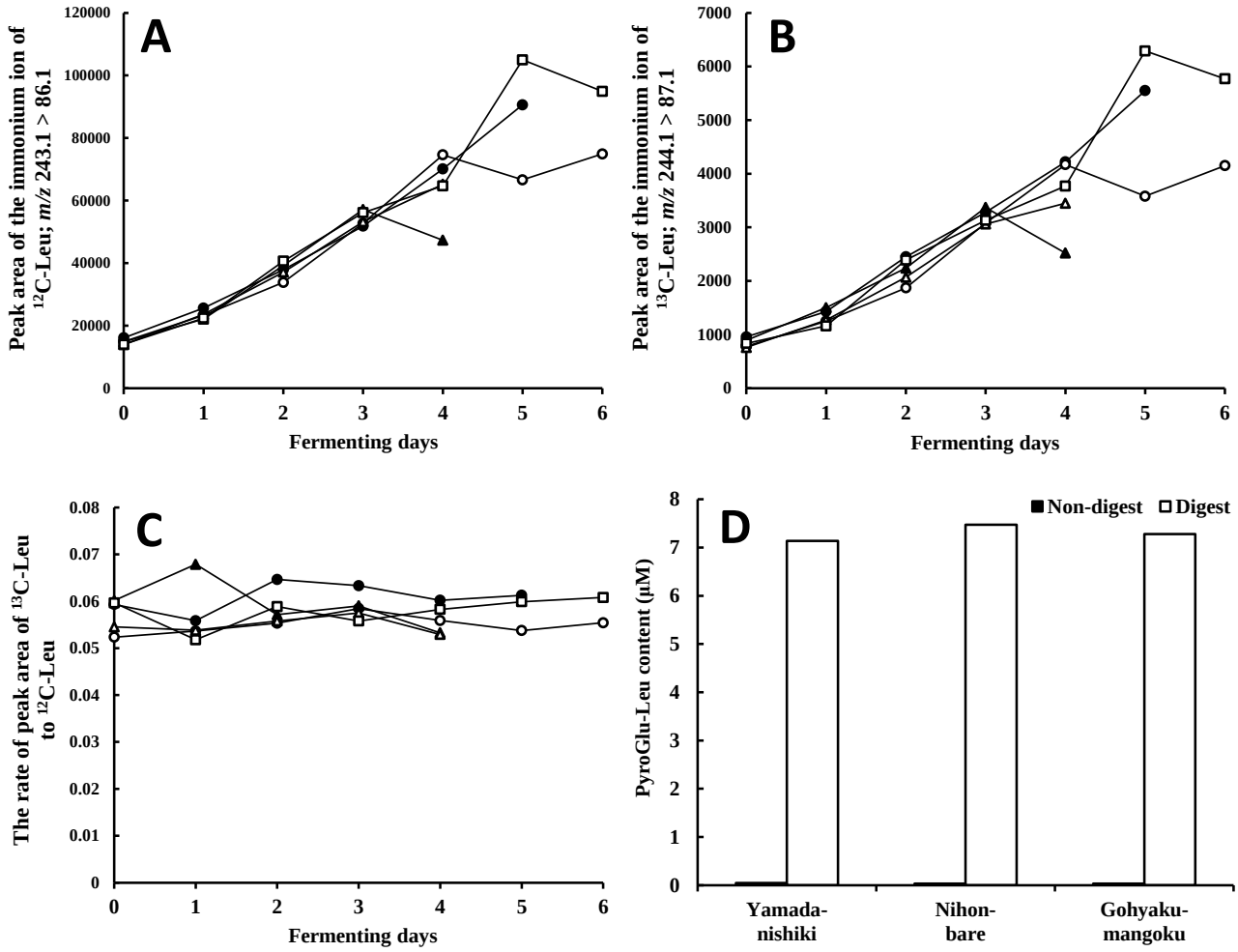


Figure 6. Production of pyroGlu-Leu by incubation of shubo (A–C) and digestion of steamed rice by *Aspergillus oryzae* enzymes (D). Different levels of $^{13}$C-Leu were added to shubo, and the production of pyroGlu-$^{12}$C-Leu (A), pyroGlu-$^{13}$C-Leu (B), and the ratio of pyroGlu-$^{13}$C-Leu/-$^{12}$C-Leu (C) are shown. Shymbols: (●) 0; (○) 0.5; (▲) 1.0; (△) 2.5; (□) 5.0 μmol/g of 1-$^{13}$C-Leu in shubo. 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 is shown in panel D.

Table 1. Sequences, precursor, and product ions of pyroglutamyl peptides in size exclusion chromatography (SEC) fractions of sake

| SEC Fr. | Peak | Peptide sequence* | Precursor ion (*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 ($[M\text{-}NH_3]^+$) |
| 33, 34 | b | pyropGlu-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. 7 (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 ($[M\text{-}COOH]^+$), 309.9 ($[M - H_2O]^+$) |
| 34, 35 | h | pyroGlu-Asn-Phe | 390.9 | 262. 7 (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 ($[M - H_2O]^+$) |

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

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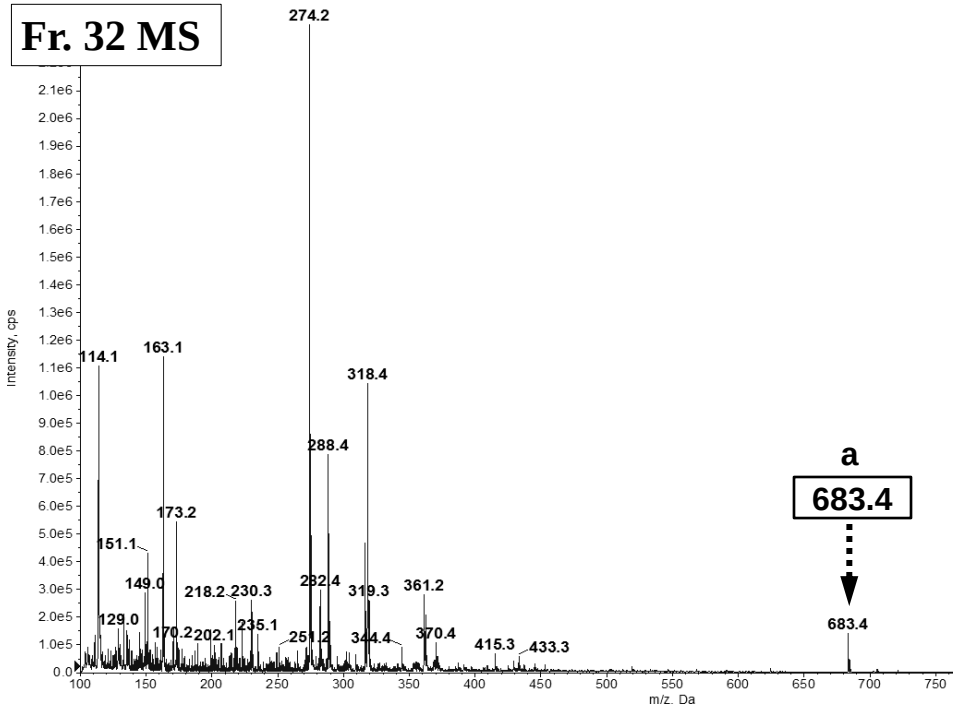
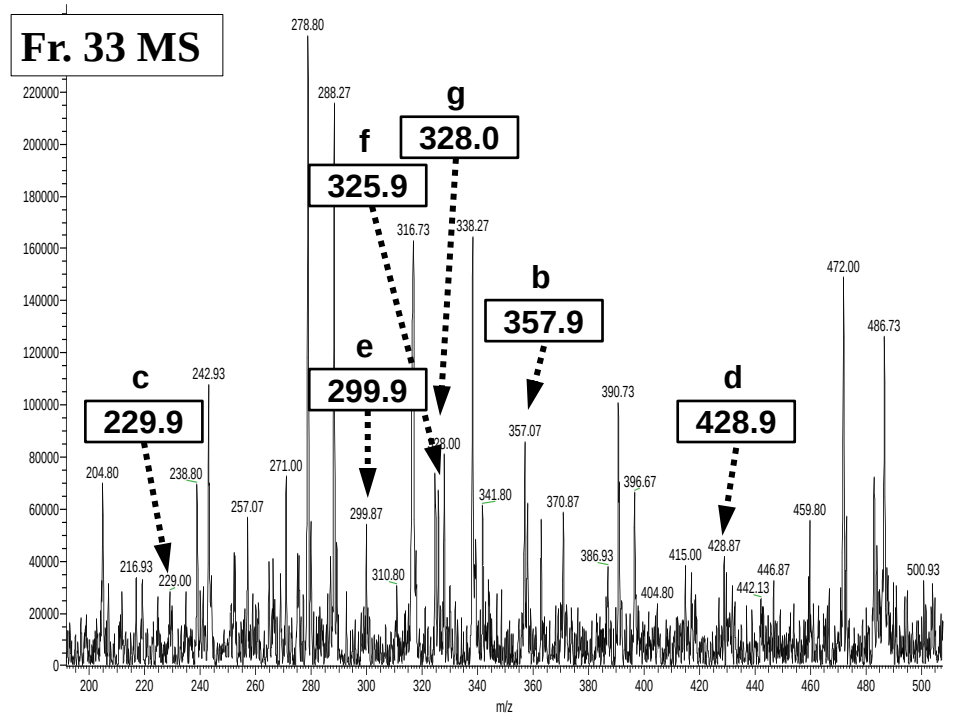
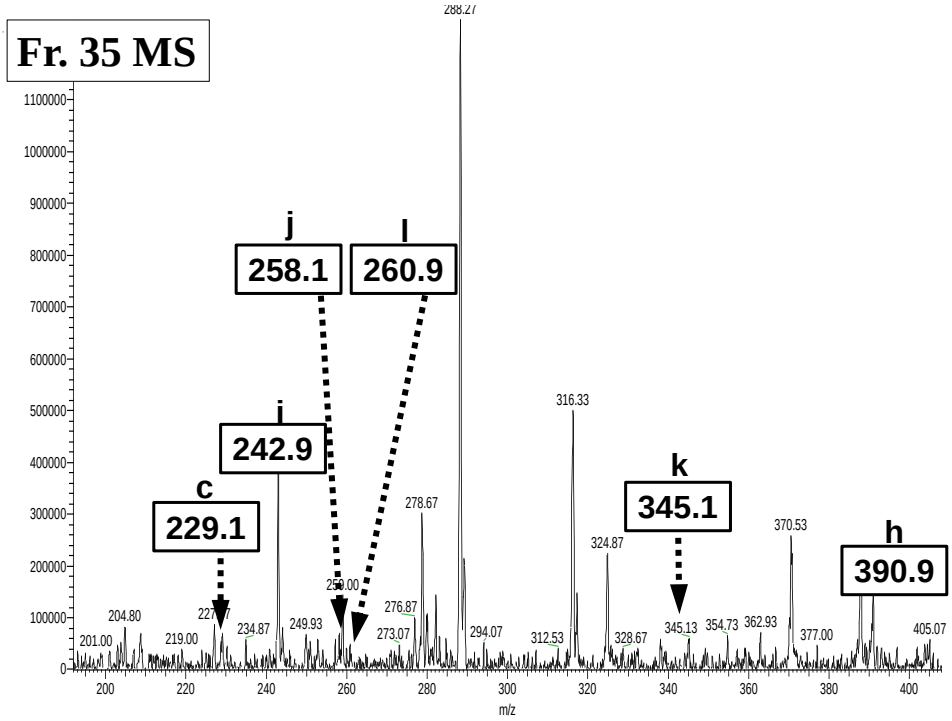
## Supplemental data

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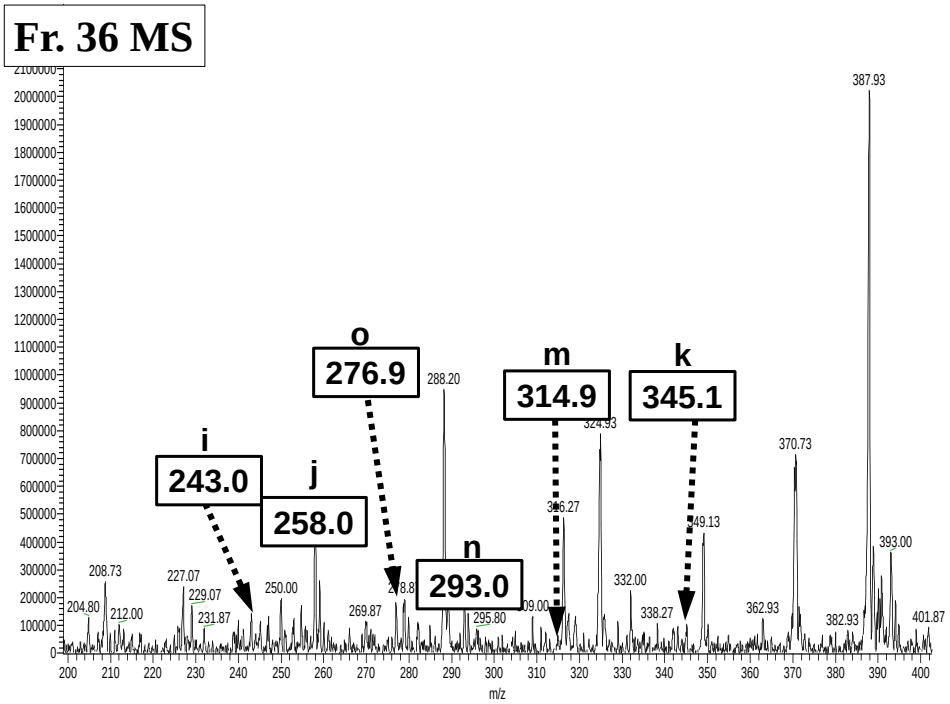
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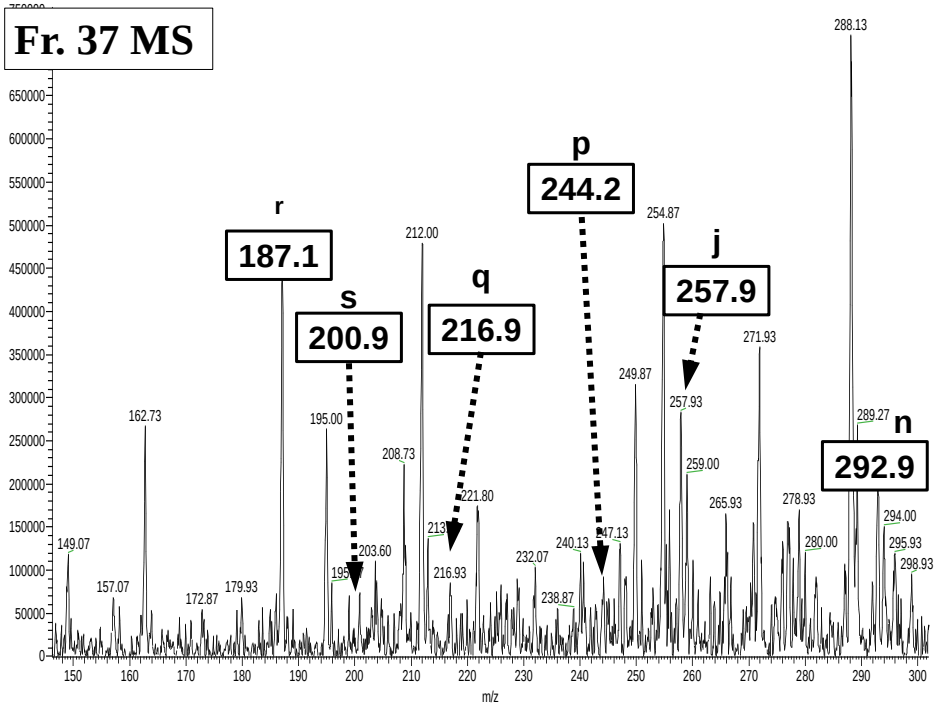
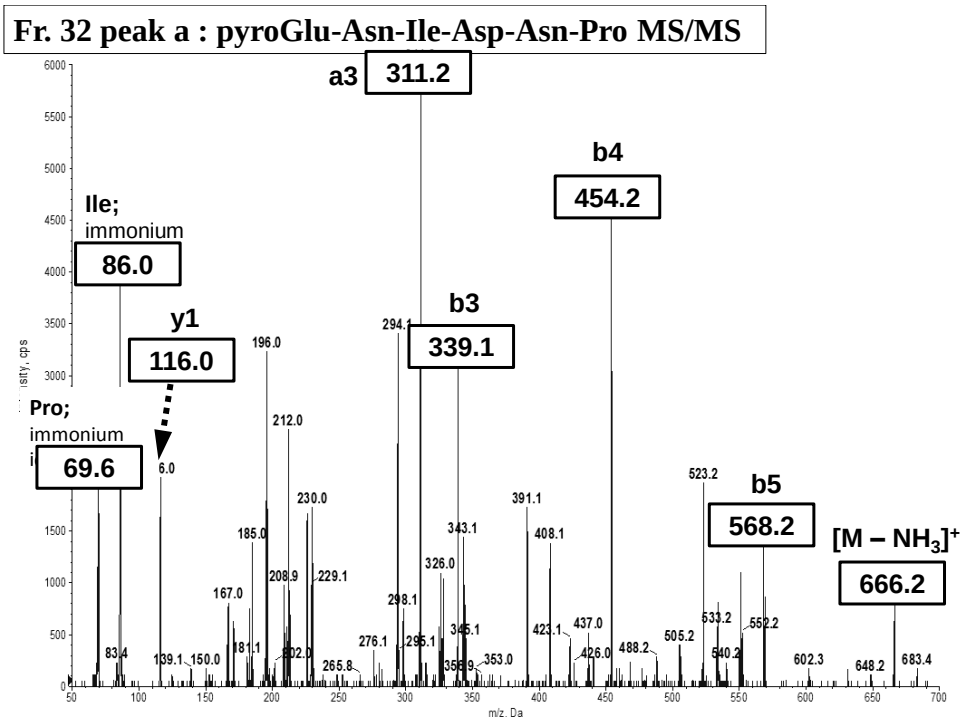
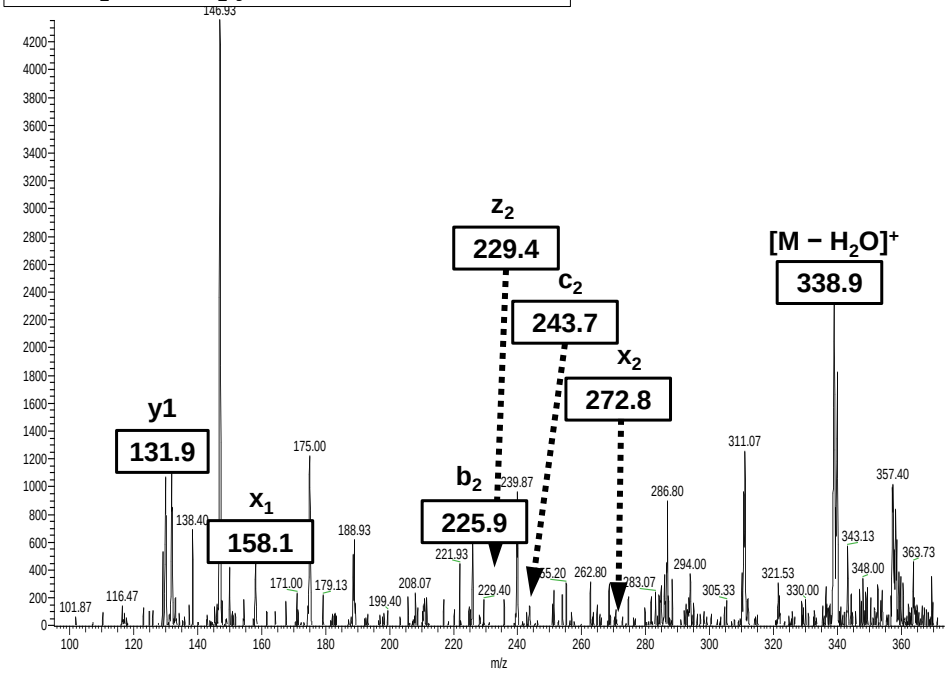



Supplemental figure 1. Electrospray ionization-mass spectrometry (ESI-MS) spectra of intact compounds in size exclusion chromatography (SEC) fractions 32, 33, 35, 36, and 37. Peaks marked with letters show *m/z* of precursor ions corresponding to the estimated pyroglutamyl peptides indicated with the same letters in Table 1.

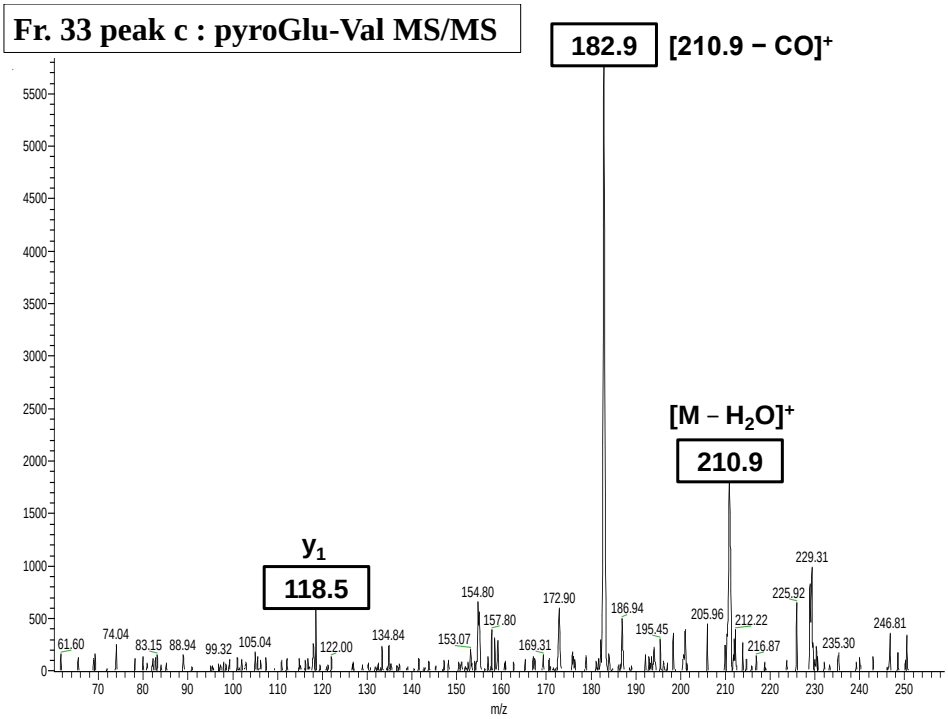



Supplement figure 2. ESI-tandem mass spectrometry spectrum (ESI-MS/MS) of the peak a in the ESI-MS spectrum of SEC Fr. 32. Fragment ions for pyroGlu-Asn-Ile-Asp-Asn-Pro.

**Fr. 33 peak b : pyroGlu-Asn-Ile MS/MS**




**Fr. 33 peak c : pyroGlu-Val MS/MS**



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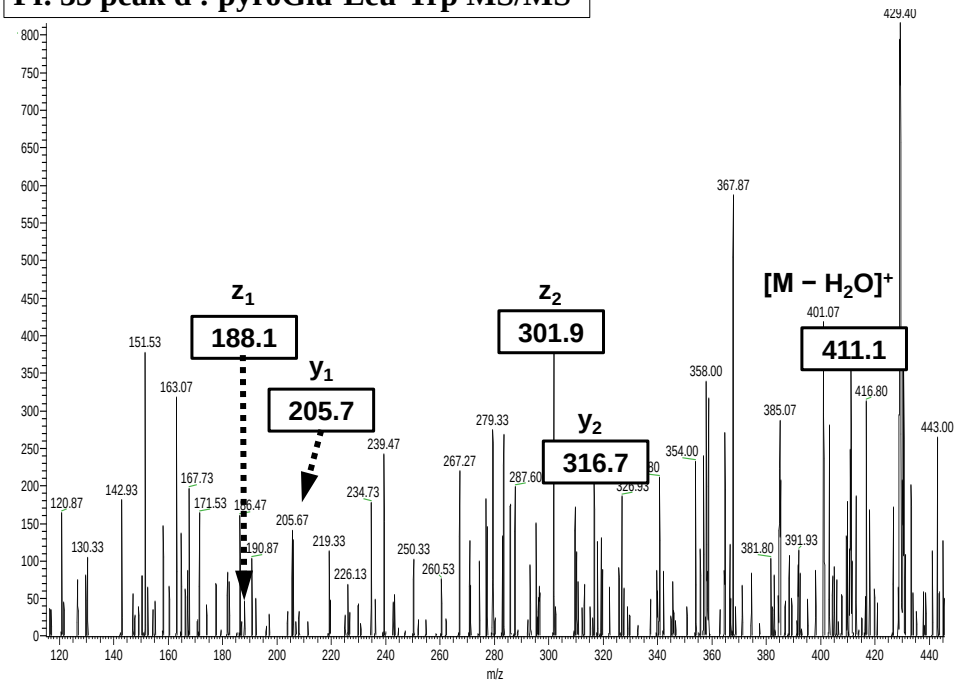


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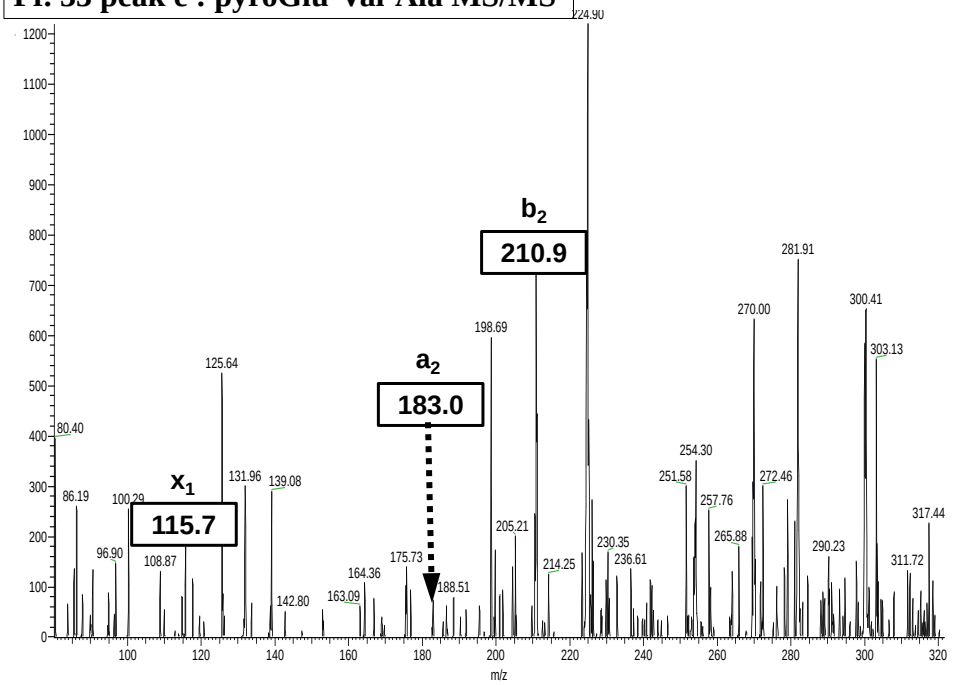
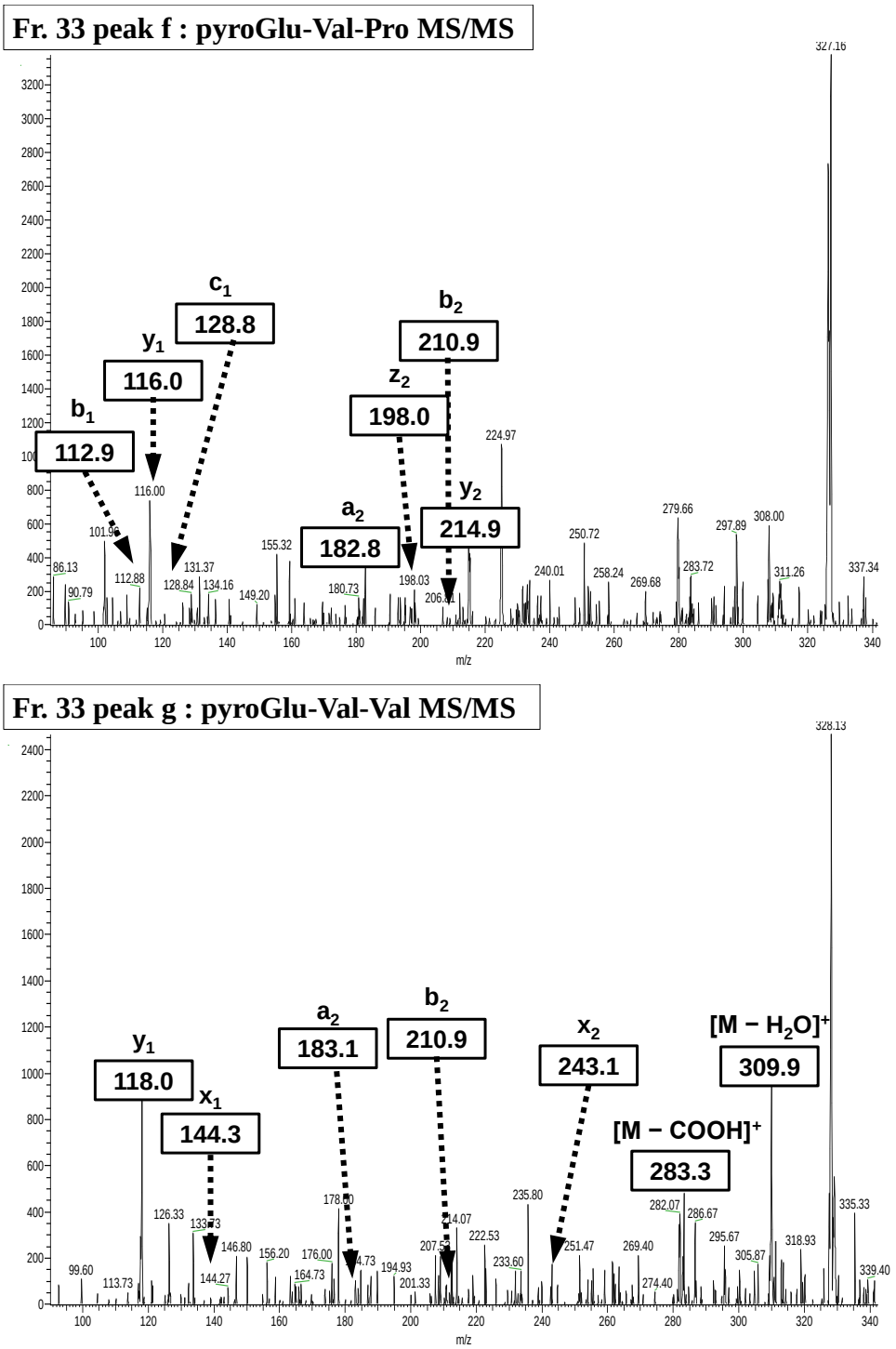
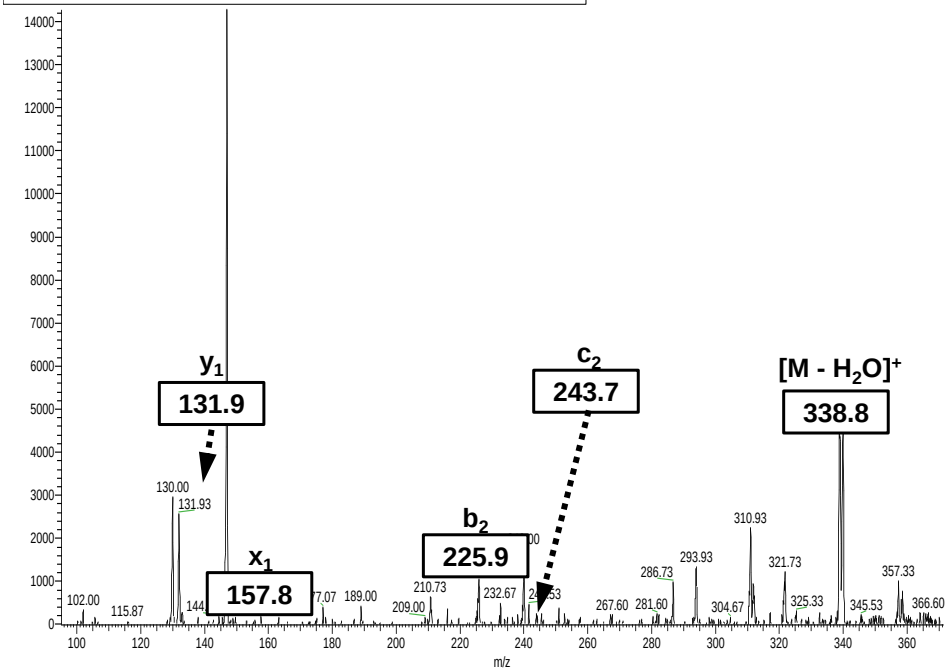


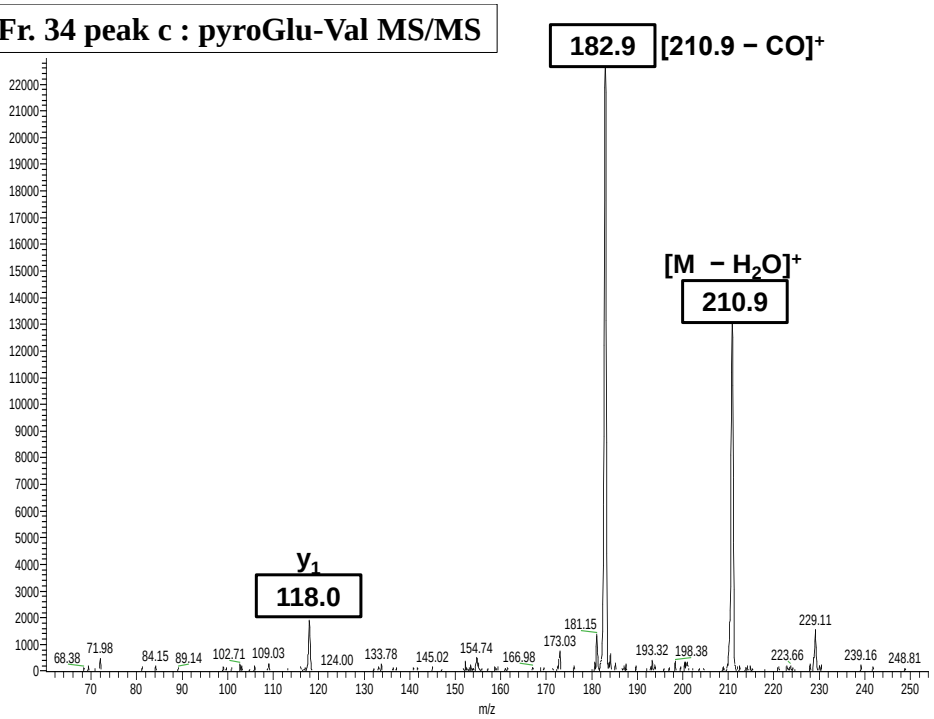


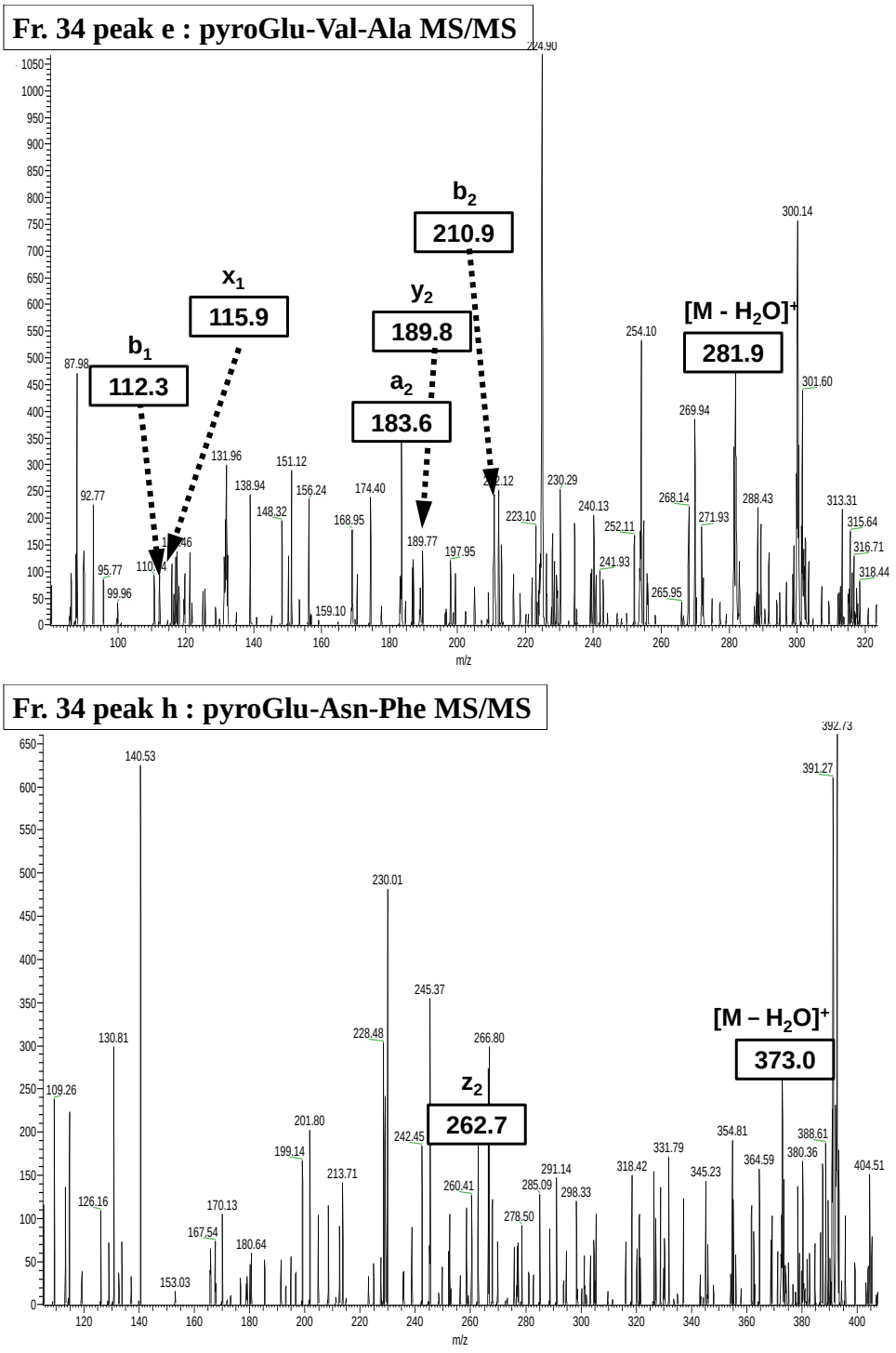

Supplement figure 3. ESI-MS/MS spectra of the peaks b, c, d, e, f, and g in the MS spectrum of SEC Fr. 33. Fragment ions of the sequences indicated on the spectra were observed.

**Fr. 34 peak b : pyroGlu-Asn-Ile MS/MS**



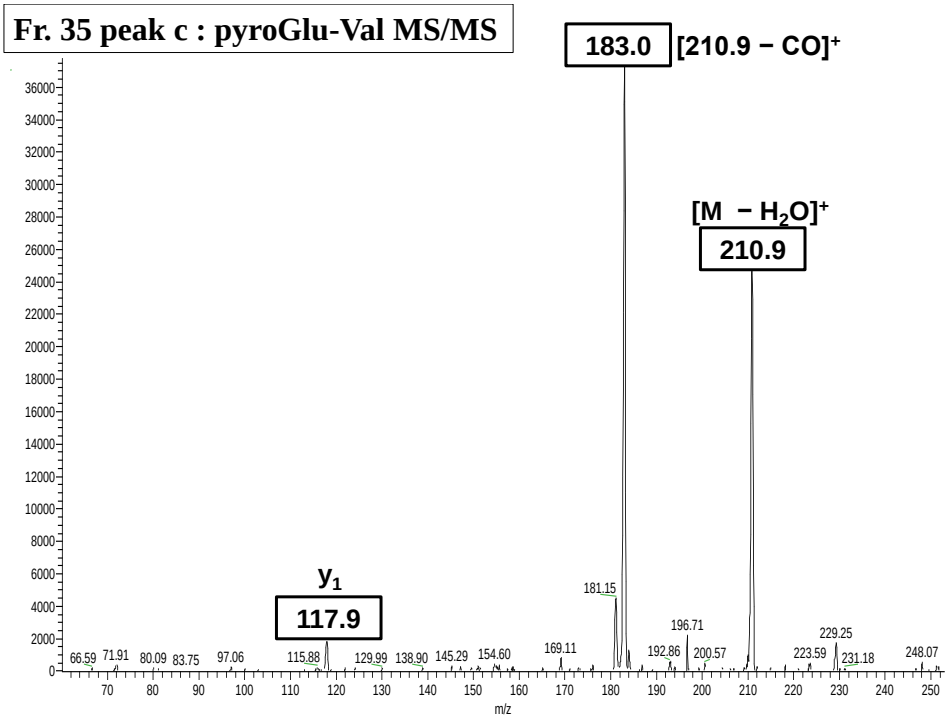

**Fr. 34 peak c : pyroGlu-Val MS/MS**






Supplement figure 4. ESI-MS/MS spectra of the peaks b, c, e, and h in the MS spectrum of SEC Fr. 34. Fragment ions of the sequences indicated on the spectra were observed.

**Fr. 35 peak c : pyroGlu-Val MS/MS**

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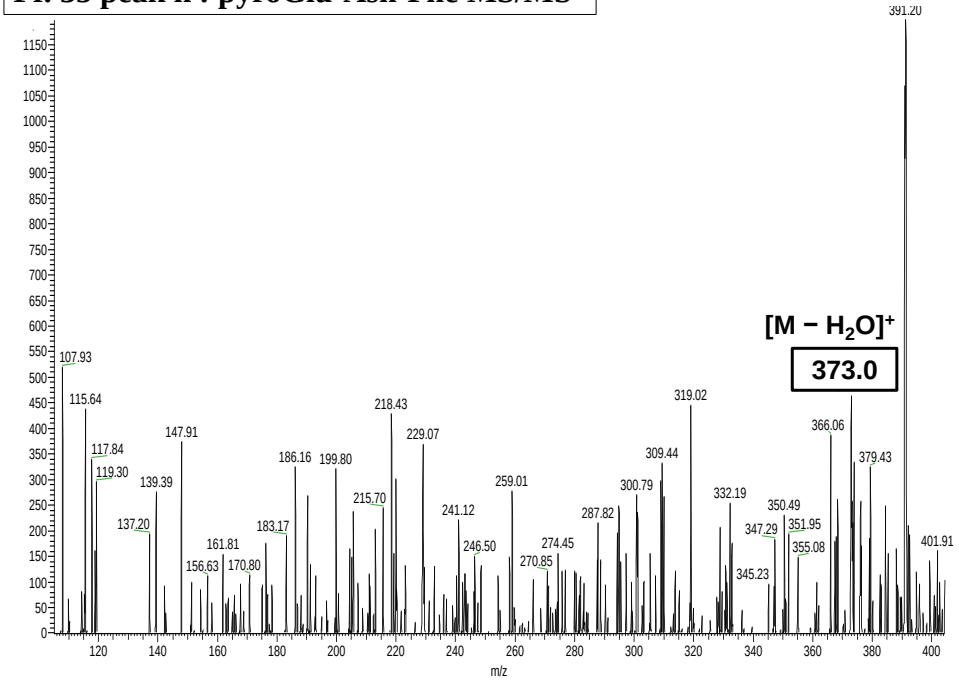
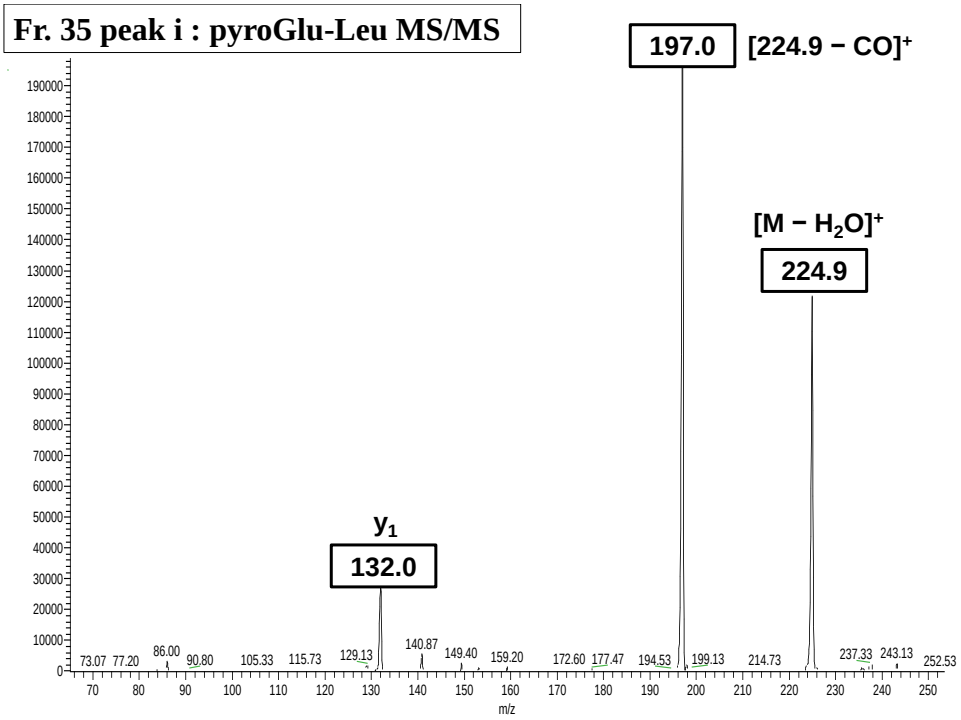
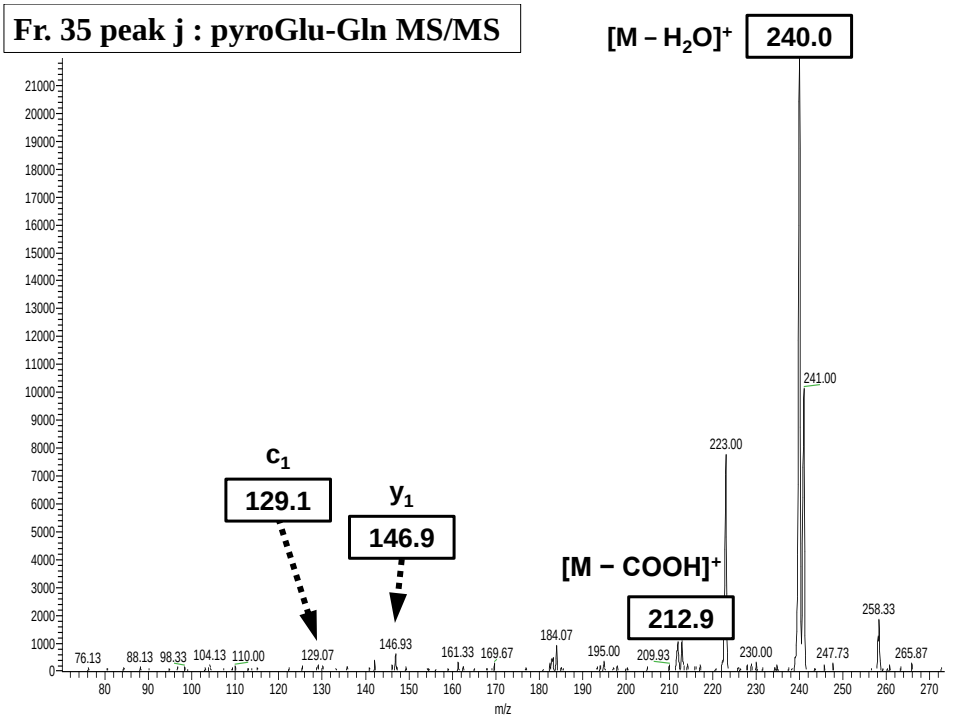
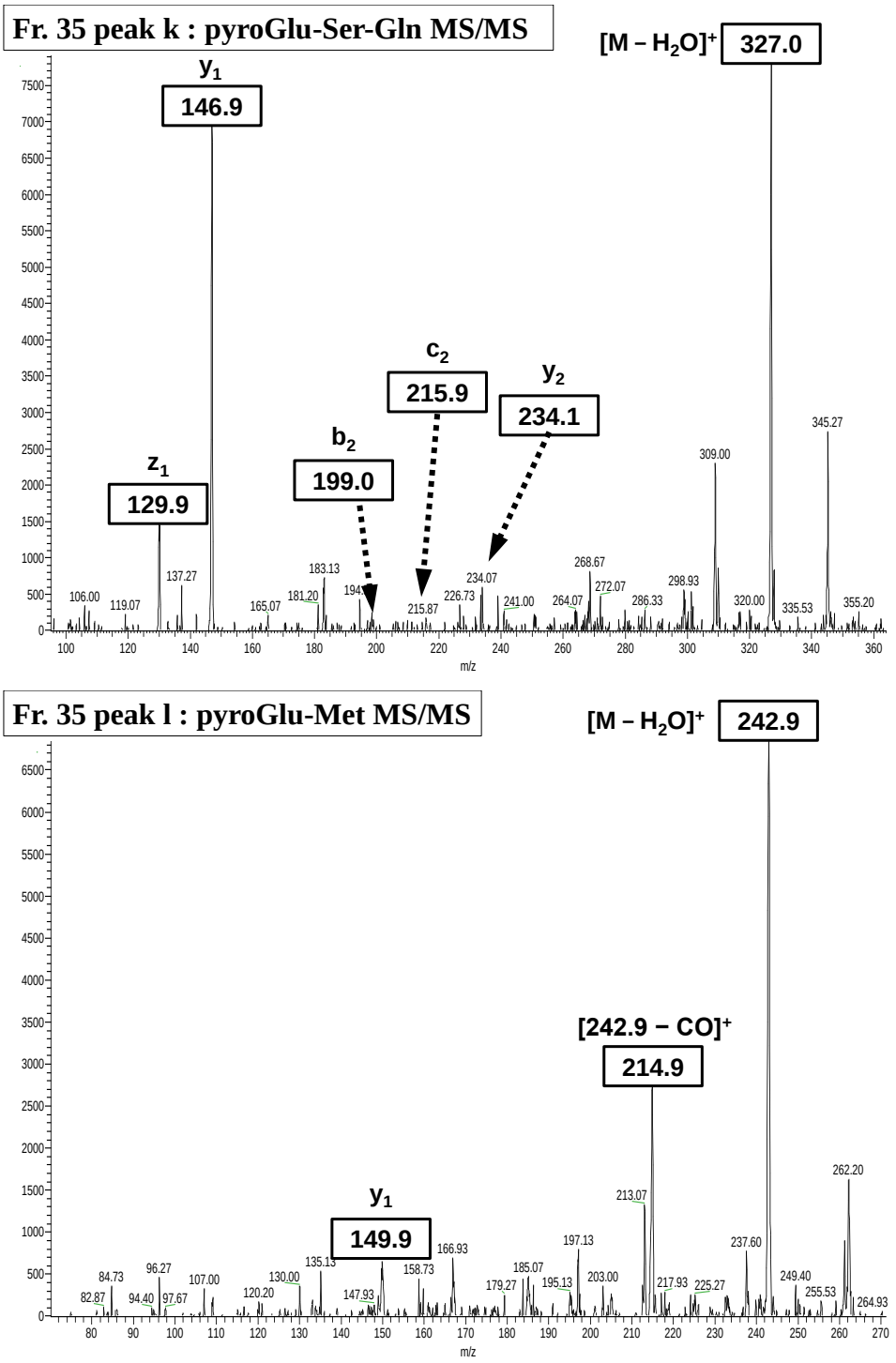


**Fr. 35 peak i : pyroGlu-Leu MS/MS**



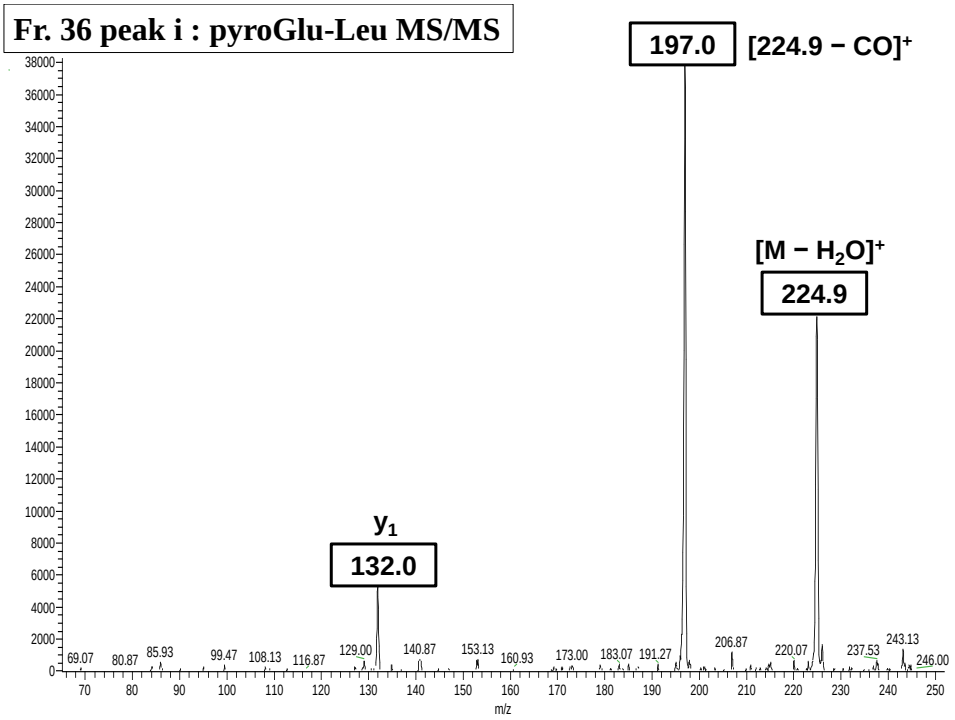

**Fr. 35 peak j : pyroGlu-Gln MS/MS**



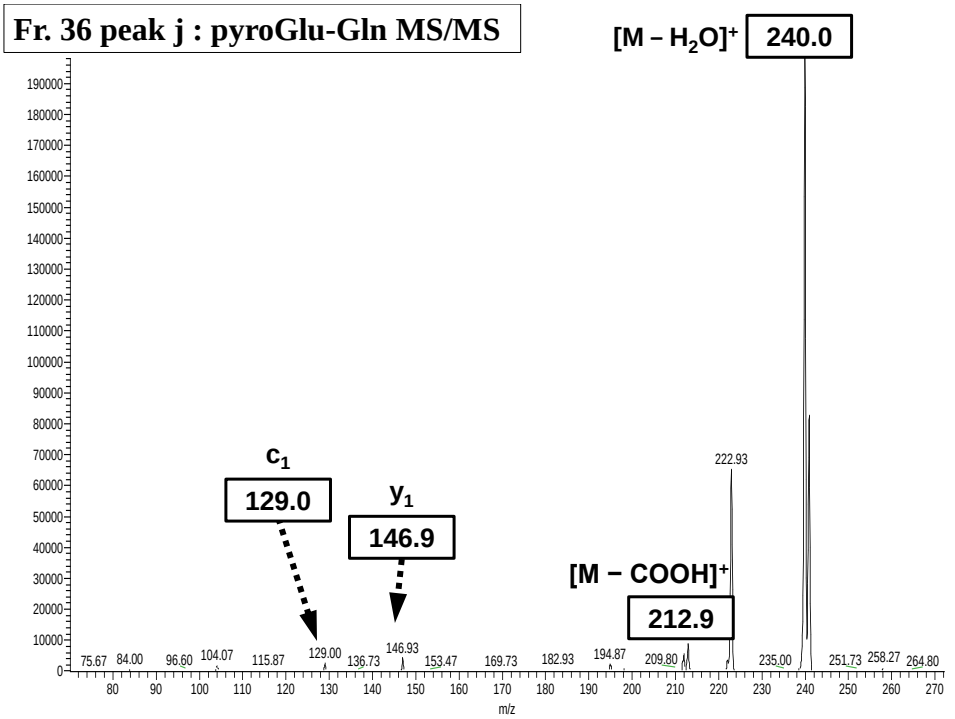



Supplement figure 5. ESI-MS/MS spectra of the peaks c, h, i, j, k, and l in the MS spectrum of SEC Fr. 35. Fragment ions of the sequences indicated on the spectra were observed.

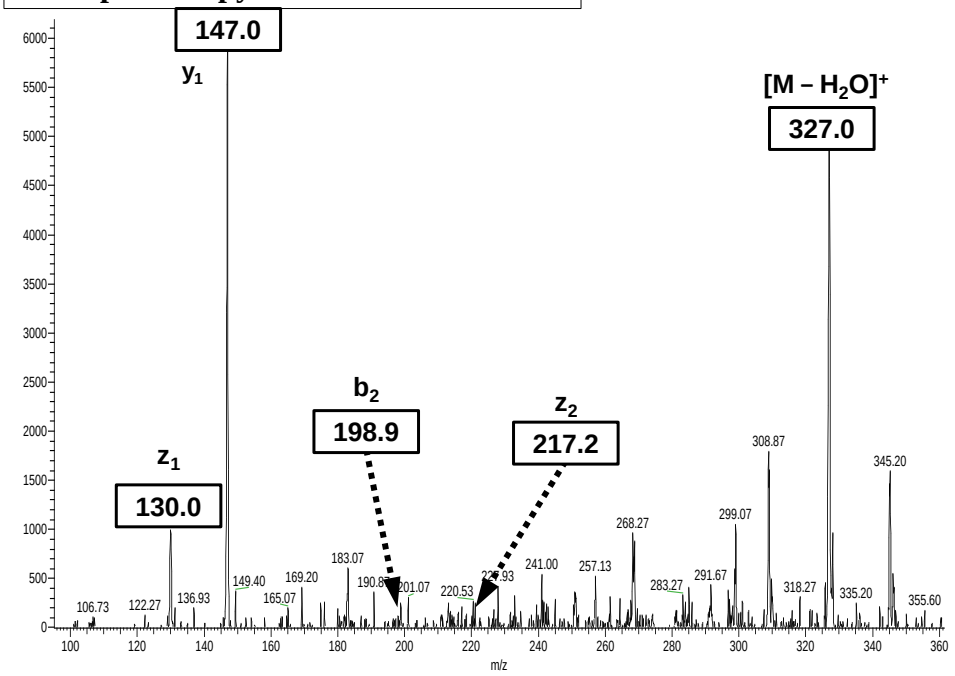
**Fr. 36 peak i : pyroGlu-Leu MS/MS**



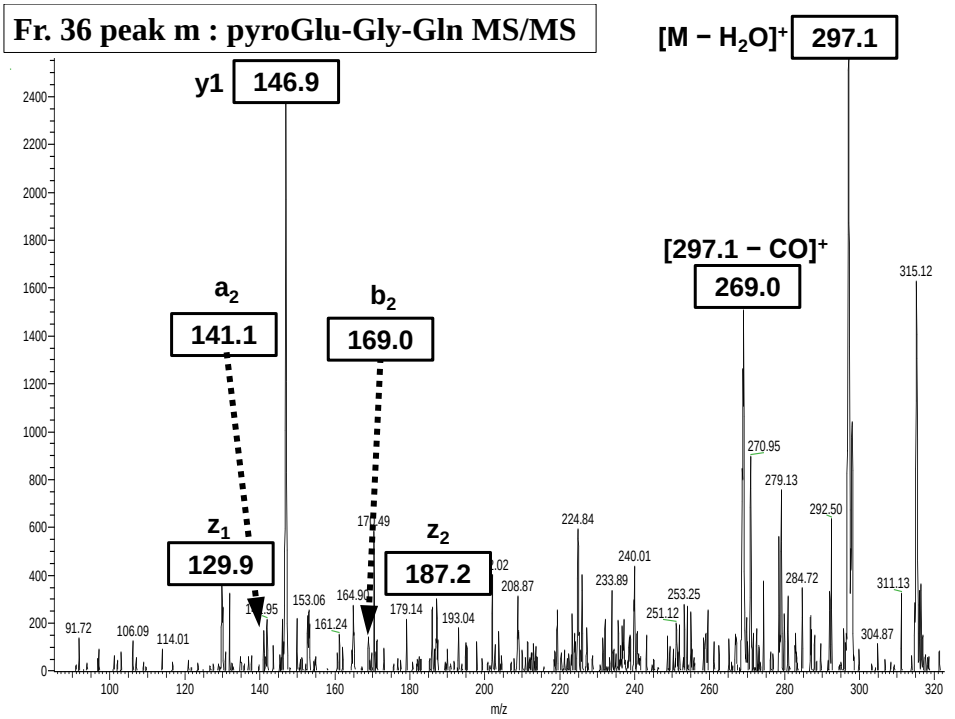

**Fr. 36 peak j : pyroGlu-Gln MS/MS**

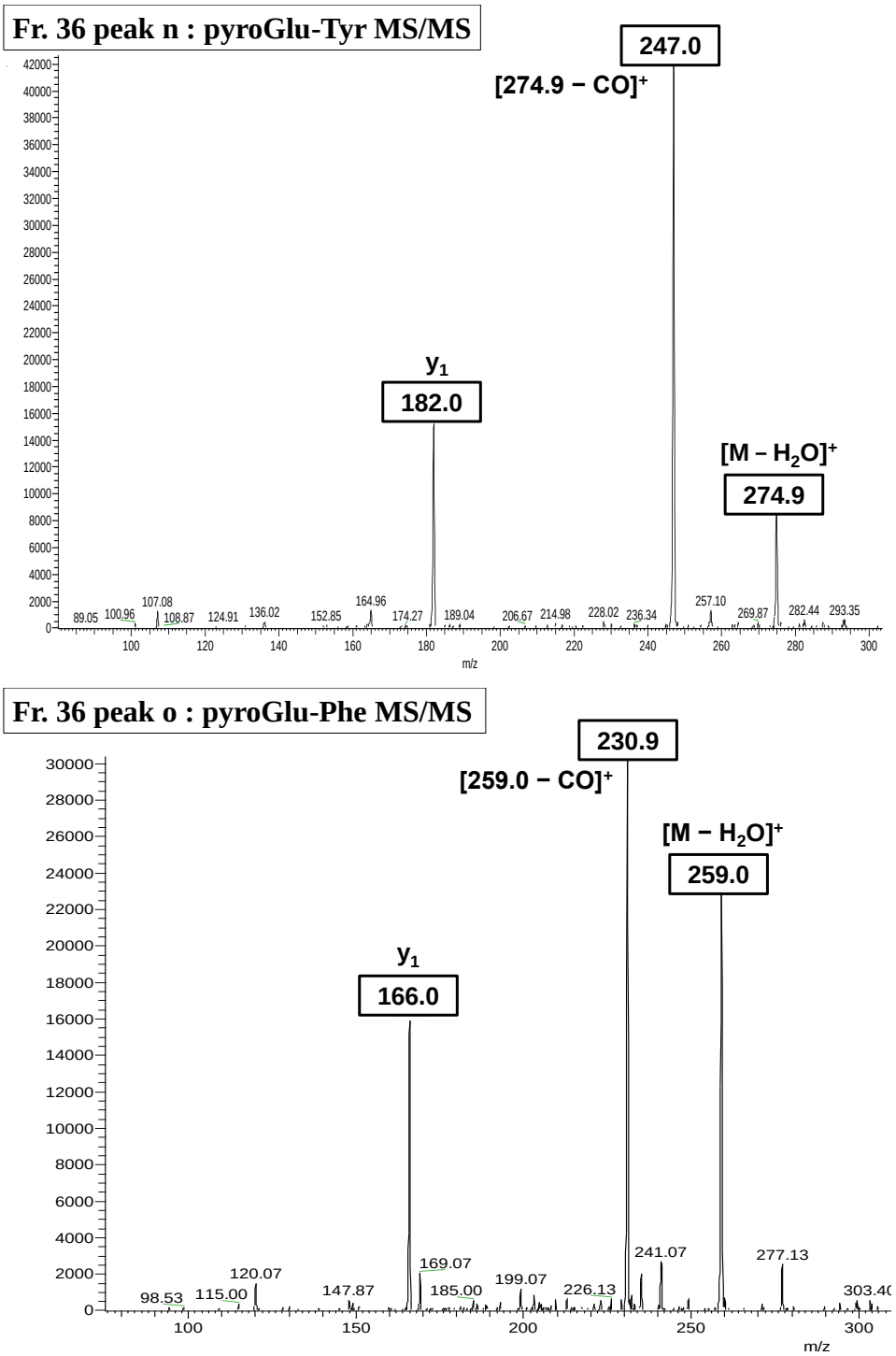


**Fr. 36 peak k : pyroGlu-Ser-Gln MS/MS**



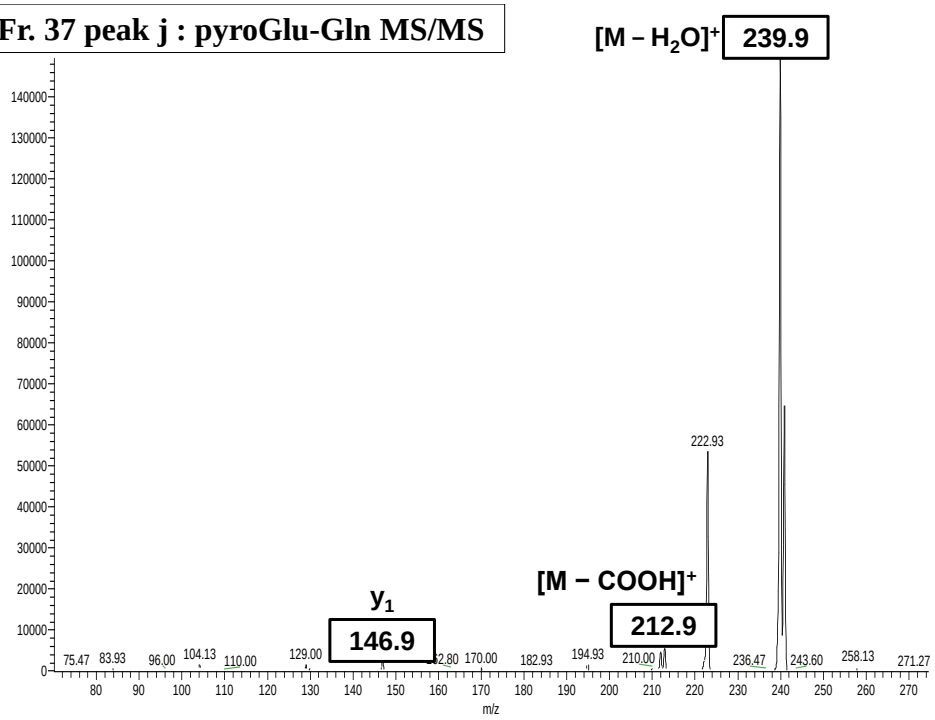

**Fr. 36 peak m : pyroGlu-Gly-Gln MS/MS**



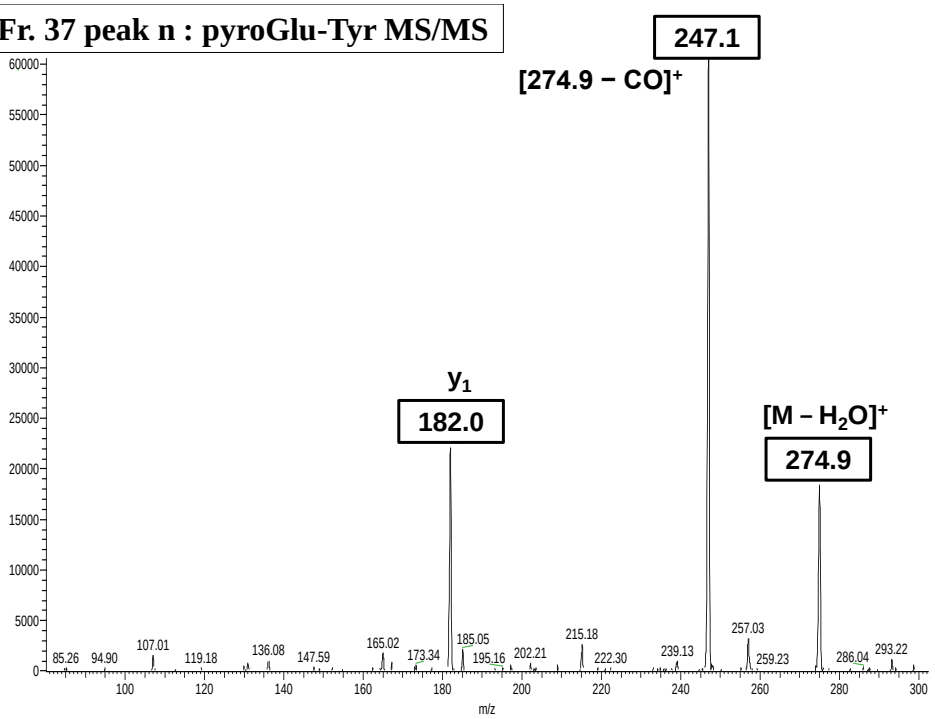



Supplement figure 6. ESI-MS/MS spectra of the peaks i, j, k, m, n, and o in the MS spectrum of SEC Fr. 36. Fragment ions of the sequences indicated on the spectra were observed.

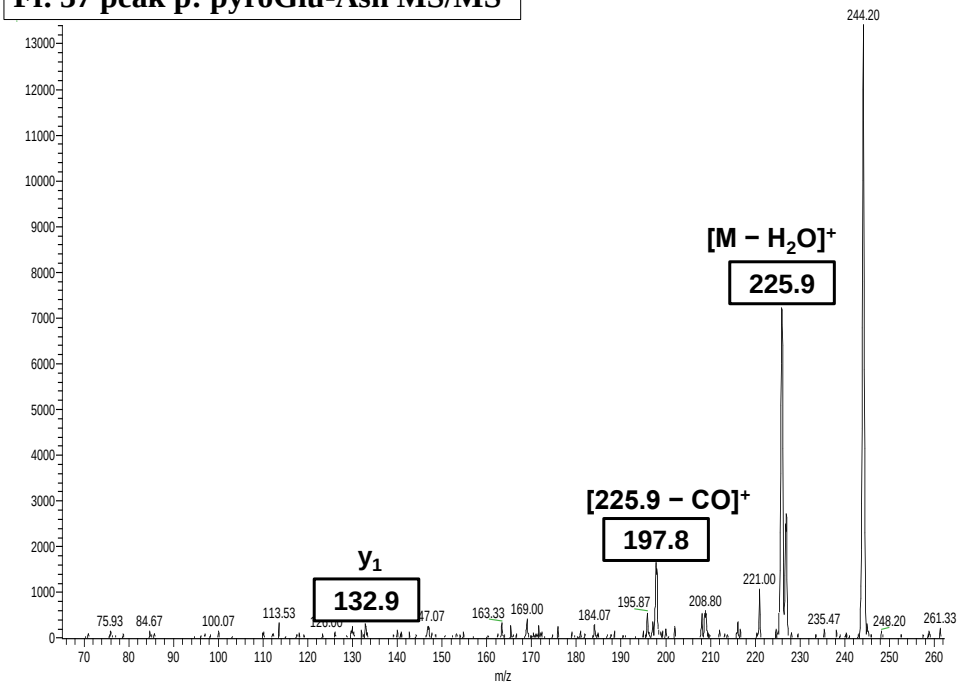
**Fr. 37 peak j : pyroGlu-Gln MS/MS**



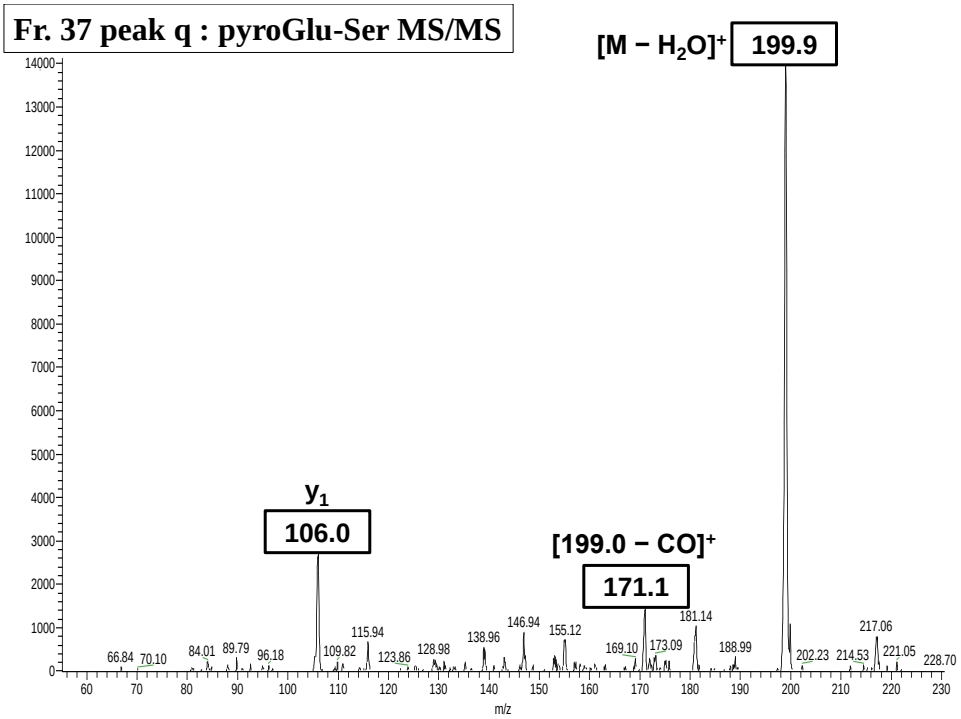

**Fr. 37 peak n : pyroGlu-Tyr MS/MS**

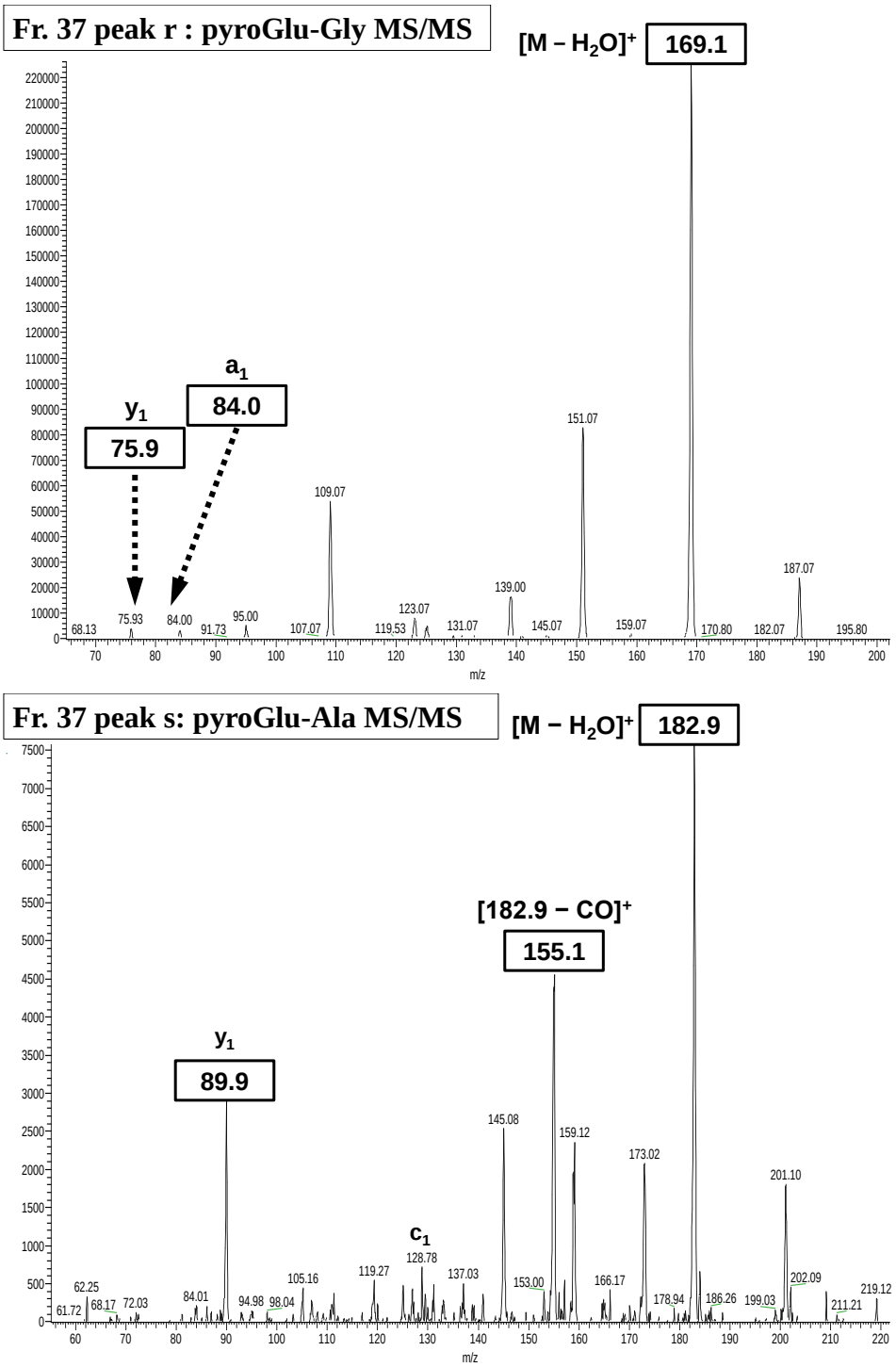


**Fr. 37 peak p: pyroGlu-Asn MS/MS**




**Fr. 37 peak q : pyroGlu-Ser MS/MS**






Supplement figure 7. ESI-MS/MS spectra of the peaks j, n, p, q, r, and s in the MS spectrum of SEC Fr. 37. Fragment ions of the sequences indicated on the spectra were observed.

# Chapter 3

# Identification of pyroglutamyl peptides with protective effect against dextran sulfate sodium (DSS)-induced colitis in mice from sake

## Introduction

Inflammatory bowel disease (IBD) is chronic disorder of gastrointestinal tract, including ulcerative colitis (UC) and Crohn's disease (CD). UC is limited to the colon, whereas CD can occur in any parts of the gastrointestinal tract (Xavier et al., 2007). IBD patients suffer from diarrhea, bloody stool, and body weight loss, and etc. To treat IBD, anti-inflammatory medicine, enteral nutrient, and severe dietary limitation are generally used. However, these therapies exert limited efficacy and sometimes induce undesirable side effects. Therefore, many efforts have been focused to find natural compounds with protective effect against IBD from foods and food compounds (Camuesco et al., 2005; Osman et al., 2008; Larrosa et al., 2009; Shizuma et al., 2011; Young et al., 2012; Yoda et al., 2014). However, in most of these studies, high doses of the compounds are needed to exert beneficial effects in animal colitis models.

Recently, Wada et al. (2013) have reported that pyroGlu-Leu, which had been first identified from wheat gluten hydrolysate, attenuate dextran sulfate sodium (DSS)-induced colitis in mice by oral administration of low dose (0.1 mg/kg body weight). As shown in chapter 2, unexpectedly, pyroGlu-Leu is contained in *sake* as a major pyroglutamyl peptide. In addition to pyroGlu-Leu, sake contains nearly 20 pyroglutamyl peptides with different structures. These pyroglutamyl peptides in sake may also have the protective effect against colitis as pyroGlu-Leu. Therefore, the

objective of study in chapter 3 was to identify new bioactive pyroglutamyl peptides from sake.

## Materials and methods

### 1. Samples.

A bottled Japanese rice wine, sake, which was made of rice polished to 60% and contains 15% (v/v) of alcohol, was commercially obtained from Shotoku Brewery (Kyoto, Japan), which had been prepared by the method as described in chapter 2. The sake was concentrated to 5-fold by a rotary evaporator at 60°C and used for following fractionation.

### 2. Regents.

Dextran sulfate sodium (DSS, average molecular weight 8,000) was purchased from Seikagaku (Tokyo, Japan). *Pyrococcus furiosus* pyroglutamate aminopeptidase was purchased from Takara Bio (Otsu, Japan). Acetonitrile (HPLC grade), trifluoroacetic acid (TFA), and phenyl isothiocyanate (PITC) were purchased from Wako Chemicals (Osaka, Japan). Triethylamine (TEA) was purchased from Thermo Fisher Scientific (Waltham, MA). Free pyroglutamic acid and D-(+)-glucose (anhydrous) was purchased from Nacalai Tesque (Kyoto, Japan). 9-fluorenylmethoxycarbonyl (Fmoc)-Tyr(tBu)-wang resin, Fmoc-Ile-wang resin, H-Pro-2-chlorotritylchloride resin, Fmoc-Asn(Trt)-OH, Fmoc-Ile-OH, Fmoc-Asp(OtBu)-OH for peptide synthesis were purchased from HiPep Laboratories (Kyoto, Japan). Other reagents used were of analytical grade or better.

### 3. DSS-induced colitis in mice.

Seven-week-old male C57BL/6 mice were purchased from Shimizu Laboratory

Supplies (Osaka, Japan). The mice were caged, 6–7 animals in each cage, in a room kept at 18–24°C and 40–70% relative humidity, with a 12 h light/dark cycle. The mice were allowed free access to drinking water and standard diet (CRF-1, Oriental Yeast, Kyoto, Japan) during a 1 week acclimatization period. All animals were treated and cared for in accordance with the National Institutes of Health's (NIH) guidelines for the use of experimental animals. All experimental procedures were approved by the Animal Care Committee of Kyoto Prefectural University of Medicine (M23–37, M24–25, Kyoto, Japan).

Acute colitis was induced by addition of DSS into drinking water to give 2.5 or 3.0% (w/v) for 7 days according to the method employed in previous studies (Naito et al., 2006; Takagi et al., 2011; Wada et al., 2013). The concentration of DSS (2.5% or 3.0%) was determined by preliminary experiments for the induction of the same grade (more than disease activity index score 2) of inflammation in each individual experiment as described in the previous paper (Wada et al., 2013), as the severity of DSS-induced colitis in mice depended on the animal lots. Samples were dissolved into distilled water and administered at 200 μL to the mice by oral gavage once per day during the entire colitis induction period. DSS-induced colitis mice with administration of 200 μL of distilled water were considered as vehicle group (DSS +). Mice without DSS solution were also administered 200 μL of distilled water, which was considered as normal group (DSS −). The body weight of each mouse was measured on days 0, 3, 5, and 7. The mice were sacrificed on day 7 and the entire colon was removed from the cecum to the anus. The colon length was measured as an indirect marker of inflammation, immediately after colon resection.

Colitis severity was evaluated on the basis of body weight, colon length, and the

macroscopic observations of stool and colon. The disease activity index (DAI) score was determined by the method employed in previous studies (Murano et al., 2000; Naito et al., 2006; Wada et al., 2013) using five grades of weight change (0, no loss or weight gain; 1, 1–5% loss; 2, 5–10% loss; 3, 10–20% loss; and 4, more than 20% loss), stool consistency (0, normal; 1, mild loose; 2, loose; 3, mild diarrhea; and 4, diarrhea), and stool bleeding (0, negative; 1, light bleeding; 2, mild bleeding; 3, severe bleeding; and 4, entire bleeding). The DAI score was obtained using an average of each criterion.

**4. Fractionation of sake compounds by preparative isoelectric focusing.**

For the animal experiment, the compounds in the sake concentrate were first fractionated by preparative isoelectric focusing based on the amphoteric nature of the samples by the method of Hashimoto et al. (2005) with slight modification (Sato et al., 2013). This method has been referred to as autofocusing. 0.1 M phosphoric acid and 0.1 M sodium hydrate were loaded in the compartments at either ends of the tank, which were used as the anode and cathode compartments, respectively. 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 8 and 9 were filled with 500 mL of the 5-fold sake concentrate. The other sample compartments were filled with deionized water. Autofocusing was performed in constant voltage mode at 500 V for 24 h. All autofocusing fractions were collected and adjusted to pH 7.0 by addition of 1 M HCl or NaOH solutions and used for following experiments.

**5. Fractionation of sake compounds by preparative reversed-phase liquid chromatography (RP-LC).**

The autofocusing fraction that exerted the protective effect against the DSS-induced colitis in mice was subjected to second fractionation using preparative reversed-phase liquid chromatography (RP-LC). The RP-LC system consisted of the column (161 mm × 15 mm i.d., Millipore, Billerica, MA, Division of Merck, Darmstadt, Germany), which was packed with YFLC Gel ODS (50 μm particle size, 120 Å pore size, Yamazen, Osaka, Japan), a pump (FL600A, Yamazen), a gradient controller (Gradicon III AC-5900, Atto, Tokyo, Japan), and a gradient mixer (AC-5905, Atto). The column was equilibrated with 10 mM HCl at flow rate of 10 mL/min. Then, eighty milliliters of the autofocusing fraction that had been diluted to 3.0 mg peptides (total amino acids)/mL was loaded to the column using the pump. Elution was performed using a binary linear gradient that consisted of 10 mM HCl (solvent A) and 10 mM HCl containing 60% (v/v) (solvent B) acetonitrile. The gradient profile was B 0% for 1 min and followed by B 0–100% over 20 min and then B 100% for 2 min. Fractions were collected every 0.5 min from sample injection corresponding Fr. 1–31.

**6. Identification of pyroglutamyl peptides.**

Identification of pyroglutamyl peptides in the preparative RP-LC fraction was performed as described in chapter 2 with slight modification. Aliquot (4 mL) of the preparative RP-LC fraction that had exerted protective effect against the DSS-induced colitis in mice was dried under vacuum and dissolved in 400 μL of 0.1% (v/v) TFA containing 30% acetonitrile. The sample was clarified by passing through a spin column (15 mm × 7 mm i.d., 5 μm pore size, Ultrafree-MC, Millipore) packed with Sephadex

G-25 fine grade (GE Healthcare, Buckinghamshire, UK), which was pre-equilibrated with the same solution. After passing the sample, the spin column was washed with 50 μL of the same solution, and the effluents were combined (450 μL of total volume). Aliquot (200 μL) of the clarified sample 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 20–45 min corresponding to fractions 21–45. This fractionation was performed twice.

Aliquots of the SEC fractions 31–45 were dried under vacuum and dissolved with 200 μL of distilled water. The SEC fractions were subjected to solid phase extraction with a strong cation exchange resin (AG50W-x8, hydrogen form, 100–200 mesh, Bio-Rad Laboratories, Hercules, CA) to separate pyroglutamyl peptides from peptides with amino group as described in chapter 2. The samples (200 μL) were loaded onto spin columns packed with the resin, which had been equilibrated with 10 mM HCl containing 50% methanol, and eluted by centrifugation at 7,000 rpm for 1 min. The columns were washed with 100 μL of 50% methanol (twice). Unabsorbed effluents were combined and dried under vacuum, and then dissolved in 200 μL of distilled water.

These unabsorbed samples (80 μL) were injected to a reversed-phase high performance liquid chromatography (RP-HPLC) column, Inertsil ODS-3 (250 × 4.6 mm, 5 μm pore size, GL Science, Tokyo, Japan), which had been equilibrated with 0.1% (v/v) formic acid at flow rate of 1.0 mL/min. Elution was performed using a binary linear gradient which consisted of 0.1% formic acid (solvent A) and 0.1% formic acid containing 80% acetonitrile (solvent B). The gradient profile was as follows: 0–30 min, B 0–30%; 30–35 min, B 30–100%; 35–40 min, B 100%; 40–40.1 min, B 100–0%;

40.1–50 min, B 0%. The column was maintained at 45°C. Elution was monitored at 214 nm. Peaks were collected. The collected fractions were dried under vacuum and dissolved in 200 μL of 0.1% formic acid containing 30% acetonitrile.

Two sets of aliquots (100 μL) of the samples 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 the 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 ethylendiaminetetraacetic 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 enzyme reaction was carried out at 60°C for 1 h. The reaction was terminated by drying under vacuum. The amino groups liberated by the digestion were reacted with PITC. The resultant phenyl thiocarbamyl (PTC) derivatives were resolved by RP-HPLC as described in chapter 2. Peaks that appeared only in the enzyme digest were collected and dried under vacuum. A “redrying solution” (20 μL) consisting of methanol, water, and TEA (7:1:2) was added to the PTC derivatives, and redried under vacuum to remove ammonia. Then, the residues were dissolved in 30% methanol and applied to an automatic peptide sequencer that operated on 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 (Sato et al., 1999).

**7. Synthesis of pyroglutamyl peptides.**

Pyroglutamyl-tyrosine (pyroGlu-Tyr), pyroglutamyl-asparaginyl-isoleucine (pyroGlu-Asn-Ile), and pyroglutamyl-asparaginyl-isoleucyl-asparagyl-asparaginyl-proline

(pyroGlu-Asn-Ile-Asp-Asn-Pro) were synthesized by an automatic solid-phase peptide synthesizer (PSSM-8, Shimadzu) according to the protocol provided by the supplier. The synthesized pyroglutamyl peptides were purified by RP-HPLC using an Inertsil ODS-3 (250 × 10 mm, 5 μm, GL Science) using acetonitrile/10 mM HCl solvent system. The content of these peptides were determined by amino acid analysis after HCl hydrolysis as described below.

**8. Amino acid analysis.**

Samples were hydrolyzed with 6 M HCl vapor at 150°C using the method as described in chapter 2. The resultant amino acids were reacted with PITC, and the PTC amino acids were resolved using the same conditions for identification of pyroglutamyl peptides as described above. The contents of free amino acids were determined by amino acid analysis without HCl hydrolysis.

**9. Sugar analysis.**

Samples were appropriately diluted with distilled water to give absorbance within standard curve. Concentrated sulfuric acid (150 μL) and 10% phenol (15 μL) were added to samples (30 μL) in 1.5-mL centrifugal tube and kept for 10 min. Then, the tubes were mixed and kept for additional 30 min. The reaction mixtures were transferred to a 96 well plate and monitored at 490 nm. The sugar contents of samples were presented as glucose equivalent.

**10. Determination of pyroglutamyl peptides.**

The contents of pyroGlu-Leu, pyroGlu-Tyr, pyroGlu-Asn-Ile, and

pyroGlu-Asn-Ile-Asp-Asn-Pro in sake concentrate, autofocusing Fr. 8, and RP-LC Fr. III were determined using liquid chromatography-tandem mass spectrometry (LC–MS/MS) as described in chapter 2 with slight modification. These samples were diluted at 10-folds with ultrapure water, and clarified by passing through a filter (Cosmonice filter W, 4 mm i.d., 0.45 μm pore size, Nacalai Tesque). The LC–MS/MS system was consisted of the Prominence 20A HPLC system (Shimadzu, Kyoto, Japan), the API 3200 (AB Sciex, Foster City, CA), and an Inertsil ODS-3 column (250 × 2.1 mm i.d., 5 μm, GL Science, Tokyo, Japan). 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 liner gradient consisting of 0.1% formic acid containing 5% acetonitrile (solvent A) and 0.1% formic acid containing 80% acetonitrile (solvent 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 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).

**11. Microbiota analysis.**

The inner contents of the colon were collected from mice in each group with single pyroglutamyl peptide ingestion. Population of phylum *Bacteroidetes* and *Firmicutes* were quantified on the basis of amplification of genome DNA coding 16S rRNA by polymerase chain reaction by using group-specific primers (Guo et al., 2008; Tannock et al., 2000). For detection of *Bacteroidetes*, Bact934F (GGARCATGTGGTTTAATTCGATGAT) and Bact1060R (AGCTGACGACAACCATGCAG) were used. For *Firmicutes*, Firm934F

(GGAGYATGTGGTTTAATTCGAAGCA) and Firm1060R (AGCTGACGACAACCATGCAC) were used. DNA was extracted using by QIAamp DNA StoolMini Kit (Qiagen, Venlo, Netherland) according to the instruction manual. Real-time PCR was done using a LightCycler 480 (Roche Applied Science, Manheim, German) according to SYBR Green I Master Protocol. PCR analysis was carried out in Primary Cell Division of Cosmo Bio (Sapporo, Japan).

**12. Statistical analysis.**

The values of body weight change, colon length, and DAI score of DSS-induced colitis mice were presented as mean ± standard error. The values of population of *Firmicutes* and *Bacteroidetes* in feces of mice and the rate of two microorganisms (*Firmicutes*/*Bacteroidetes*) were presented as mean ± standard error. Data were subjected to one-way ANOVA with Dunnett's multiple comparison of means test. Differences showing $p < 0.05$ were considered significant. Statistical analysis was performed using Ekuseru-Toukei 2010 Version.1.11 (Social Survey Research Information, Tokyo, Japan).

## Results

### 1. Improvement of DSS-induced colitis by sake autofocusing fractions.

After autofocusing of 5-fold sake concentrate, autofocusing fraction (Fr.) 1–8 showed approximately pH 3 and sharp pH gradient from 3 to 12 was formed between Fr. 8–10 (Figure 1A). Approximately 80% of total peptides were recovered in Fr. 8 and 9 (Figure 1B). As shown in Figure 1C, peptides in the acidic fractions (Fr. 1–8) consisted of higher levels of acidic amino acids such as glutamic acid after HCl hydrolysis, and peptides in the basic fractions (Fr. 10) consisted of higher levels of basic amino acids such as lysine compared with the other fractions, which indicate that the peptides in sake were fractionated on the basis of their isoelectric points. To make flat pH region (Fr. 1–8), high amounts of non-peptide acidic compounds, such as organic acids, might be present in the sample. Approximately 60% of sugars were also distributed in Fr. 8 and 9. On the basis of these results, autofocusing Fr. 1–5 and 6–7 were mixed and used for animal experiment, respectively. Fr. 8, 9, and 10 were individually used for the animal experiment. Autofocusing fractions were mixed with ethanol to give 75% (v/v) to remove agarose that was derived from the separator of autofocusing. The supernatants were collected and concentrated by a rotary evaporator to remove ethanol. The concentrates were diluted with distilled water to give 3.0 mg peptides (total amino acids)/mL (30 mg/kg body weight per day) for the animal experiment.

As shown in Figure 2, DSS treatment induced colitis as indicated by significant increase of DAI score. Administration of Fr. 6–7, 8, and 9 significantly moderated body weight loss. Only Fr. 8 significantly decreased the DAI score. There was no significant difference in colon length among all groups. On the other hands, crude sake concentrate

exerted no significant protective effect against DSS-induced colitis in mice.

**2. Improvement of DSS-induced colitis by RP-LC fractions.**

Compounds in autofocusing Fr. 8 (3.0 mg peptides (total amino acids)/mL, 80 mL) were further fractionated by RP-LC. Elution profile is shown in Figure 3. After elution of non-absorbed compounds in the RP-LC column, absorbed compounds were eluted by increasing acetonitrile concentration. Most of free amino acids and sugars are eluted in the non-absorbed fraction. Peptide content can be estimated by subtracting free amino acids from total amino acids in HCl hydrolysate. Peptides were eluted in both non-absorbed and absorbed fractions. As shown in Figure 3C, UV absorbance peak was observed at 20–21 min. On the basis of these results, four fractions (I–IV) were collected as follows; Fr. I (Fr. 0.5–14), II (Fr. 14.5–17), III (Fr. 17.5–19.5), and IV (Fr. 20–31). Each fraction was freeze dried to remove acetonitrile and then dissolved into 80 mL of distilled water. Two hundred microliters of the Fr. I–IV were administered to mice with DSS-induced colitis.

As shown in Figure 4, administration of Fr. III significantly moderated body weight loss compared with DSS + control group. DAI scores of Fr. I–IV groups had no significant difference compared with DSS + control group, whereas administration of crude Fr. 8 significantly decreased the DAI score. There was no significant difference in colon length among all groups. On the basis of these results, Fr. III was used for identification of constituting pyroglutamyl peptides.

**3. Identification of pyroglutamyl peptides in Fr. III and their protective effect against DSS-induced colitis.**

Peptides in the Fr. III were first resolved by SEC. As shown in Figure 5A, approximately 75% of peptides were eluted in Fr. 31–36. Pyroglutamyl peptides in these fractions were separated by the solid phase extraction and resolved by RP-HPLC.

As shown in Figure 5B, major peaks marked a, b, and c by RP-HPLC were collected. The pyroglutamyl peptides in Fr. a, b, and c were digested with pyroglutamate aminopeptidase. The digests and non-digests were reacted with PITC. The resultant PTC derivatives were resolved by second RP-HPLC. Peaks marked with a', b', and c' were appeared in the only enzyme digest of Fr. a, b, and c (Figure 5C). These PTC derivatives were identified by Edman degradation as follows; peak a', pyroglutamyl-tyrosine (pyroGlu-Tyr), peak b', pyroglutamyl-asparaginyl-isoleucine (pyroGlu-Asn-Ile), and peak c', pyroglutamyl-asparaginyl-isoleucyl-asparagyl-asparaginyl-proline (pyroGlu-Asn-Ile-Asp-Asn-Pro). Peaks marked with asterisks in Figure 5B and C did not yield significant sequence by Edman degradation even after pyroglutamate aminopeptidase digestion.

Three identified pyroglutamyl peptides were chemically synthesized and administered to DSS-induced colitis mice. As shown in Figure 6, administration of these pyroglutamyl peptides did not significantly moderate the body weight loss compared with DSS + control group. On the other hand, administration of pyroGlu-Tyr and pyroGlu-Asn-Ile significantly moderate the colon shortening at 1.0 mg/kg body weight ($p < 0.05$ and $p < 0.01$, respectively). pyroGlu-Asn-Ile significantly decreased DAI score compared with DSS + control group in dose dependent manner. On the other hand,

pyroGlu-Asn-Ile-Asp-Asn-Pro did not exert any protective effects on the colitis.

The contents of pyroGlu-Leu, pyroGlu-Tyr, pyroGlu-Asn-Ile, and pyroGlu-Asn-Ile-Asp-Asn-Pro in samples for animal experiment were determined using LC-MS/MS. Doses of these peptides for DSS-induced colitis mice are shown in Table 1. When sake concentrate and autofocusing Fr. 8 were administered to mice, the doses of pyroGlu-Leu were higher than 0.1 mg/kg body weight. On the other hand, it was less than 0.01 mg/kg body weight, when Fr. III was used. Doses of pyroGlu-Tyr and pyroGlu-Asn-Ile-Asp-Asn-Pro were higher than 0.1 mg/kg body weight, only when autofocusing Fr. 8 was used. In all cases, doses of pyroGlu-Asn-Ile were less than 0.1 mg/kg body weight.

**4. Effects of pyroglutamyl peptides in sake on enteric microbiota.**

DSS treatment increased population of *Firmicutes* and decreased that of *Bacteroidetes* in the inner content of the colon compared to DSS – normal group. Consequently, ratio of *Firmicutes* to *Bacteroidetes* significantly increased. Administration of pyroGlu-Asn-Ile significantly normalized the ratio, whereas pyroGlu-Tyr did not significantly change the ratio.

## Discussion

While crude sake without fractionation did not show any significant protective effects against DSS-induced colitis, some autofocusing fractions exerted beneficial and negative effects by oral administration, respectively. These facts suggest presence of some compounds which have positive and negative effects on the colitis in sake. In order to identify pyroglutamyl peptides with protective activity against acute colitis, active autofocusing fraction was further fractionated by using RP-LC. pyroGlu-Tyr, pyroGlu-Asn-Ile, and pyroGlu-Asn-Ile-Asp-Asn-Pro were identified in the active fraction. Chemically synthesized pyroGlu-Tyr and pyroGlu-Asn-Ile significantly attenuated colitis-induced shortening of colon. pyroGlu-Asn-Ile also significantly decreased DAI score, which indicate that these peptides in sake have potential for improvement of acute colitis.

Wada et al. (2013) have demonstrated that pyroGlu-Leu significantly moderates DSS-induced colitis in mice by oral administration at 0.1 mg/kg body weight. However, it did not exert the beneficial activity higher than 0.5 and lower than 0.05 mg/kg body weight, respectively. On the other hand, the present study demonstrates that pyroGlu-Asn-Ile exerts the protective effect in dose dependent manner at least from 0.1 to 1.0 mg/kg body weight. Then, pyroGlu-Asn-Ile shows beneficial effect in the same animal model in wider dose range than pyroGlu-Leu, which might be advantage in practical use to pyroGlu-Leu.

pyroGlu-Leu in sake was recovered in the autofocusing Fr. 8 and RP-LC Fr. IV (data not shown). However, administration of RP-LC Fr. IV (dose of pyroGlu-Leu; 0.31 mg/kg body weight) did not exert the protective effect against the colitis possibly due to

higher dosage of pyroGlu-Leu. On the other hand, administration of autofocusing Fr. 8 (dose of pyroGlu-Leu; 0.46 mg/kg body weight) exerted the protective effect possibly due to presence of other pyroglutamyl peptides such as pyroGlu-Tyr and pyroGlu-Asn-Ile. These facts suggest that pyroGlu-Tyr and pyroGlu-Asn-Ile can modulate biological activity of pyroGlu-Leu or coordinately act with pyroGlu-Leu in this animal model.

Wada et al. (2013) have demonstrated that ratio of *Firmicutes* to *Bacteroidetes*, two major phylums of colonic microbiota, dramatically increases in the mice with DSS-induced colitis, and administration of pyroGlu-Leu normalizes the ratio. In the present study, administration of pyroGlu-Asn-Ile also normalized the colonic microbiota in the DSS-induced colitis mice. On the other hand, pyroGlu-Tyr did not affect the microbiota, while it moderated the colitis-induced shortening of colon. These facts suggest that pyroGlu-Tyr might exert protective effect by different mechanism from pyroGlu-Leu and pyroGlu-Asn-Ile.

Mechanism for the normalization of colonic microbiota in mice with DSS-induced colitis by pyroGlu-Leu and pyroGlu-Asn-Ile have remained to be solved. It is, however, unlikely that these peptides have a direct antimicrobial effect on colonic microbiota, as these peptides modulated microbiota in low doses (0.1–1.0 mg/kg body weight). Alternatively, it could be assumed that pyroGlu-Leu and pyroGlu-Asn-Ile might indirectly normalize colonic microbiota through the modulation of host innate immune or/and inflammatory systems. The further studies on the effect of administration of pyroGlu-Leu and pyroGlu-Asn-Ile on production of host antimicrobial compounds and also immune response under physiological and pathological conditions are in progress.

The present study demonstrated that sake contains compounds with protective and

negative effects on colitis. The preparation with higher protective activity against colitis could be prepared by fractionation of sake as shown in the present study and also by optimizing fermentation process, which will be described in the following chapter. pyroGlu-Leu, pyroGlu-Asn-Ile, and other pyroglutamyl peptides may be used as good markers to predict the efficacy and also to explore the mechanism for the protective effect against colitis.

## Figures and tables

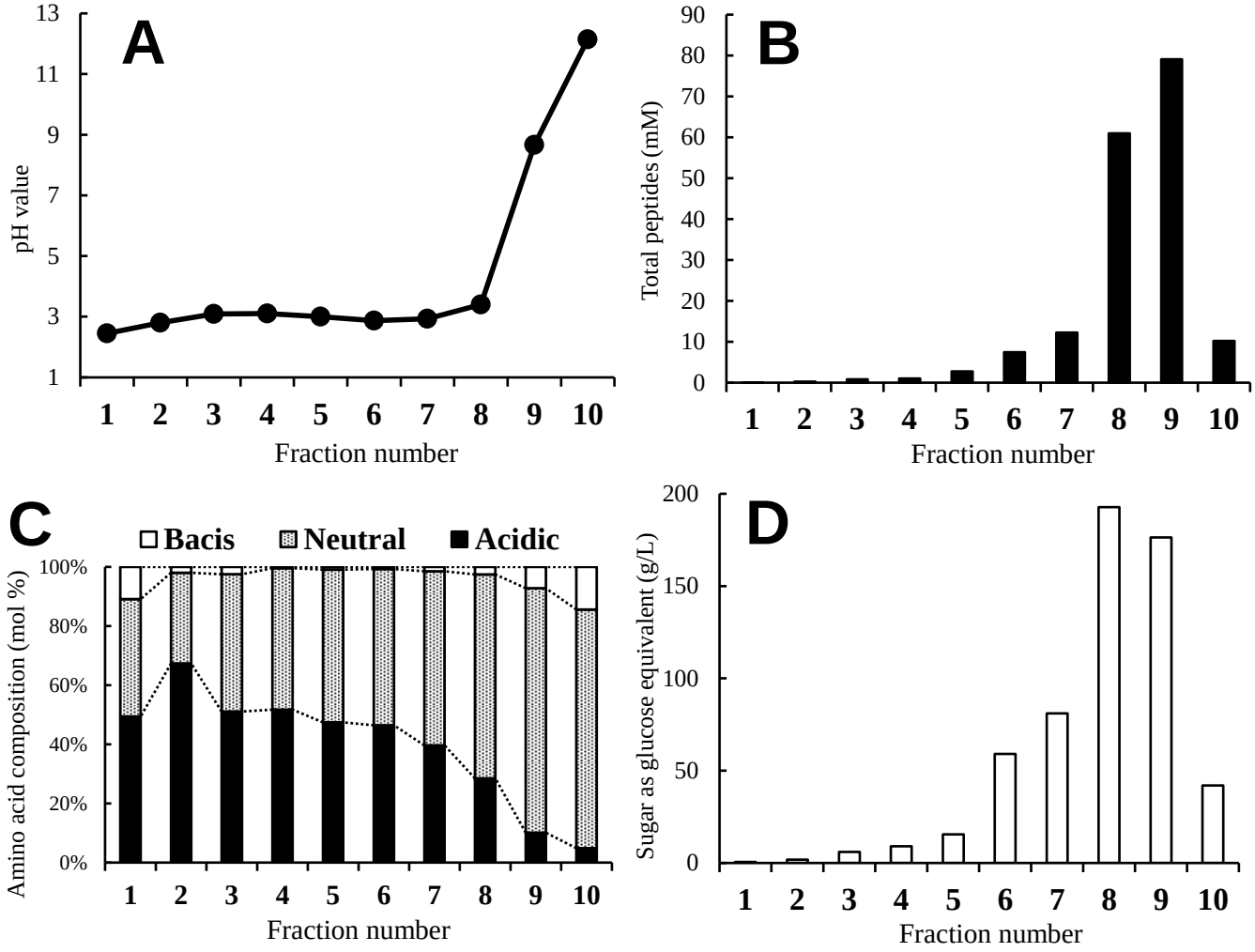



Figure 1. Fractionation of sake concentrate by ampholite-free preparative isoelectric focusing. pH values (A). Contents of total peptides (total constituent amino acids after HCl hydrolysis) (B). Ratio of basic, neutral, and acidic amino acids to the total amino acids (C). Contents of sugar as glucose equivalent (D).

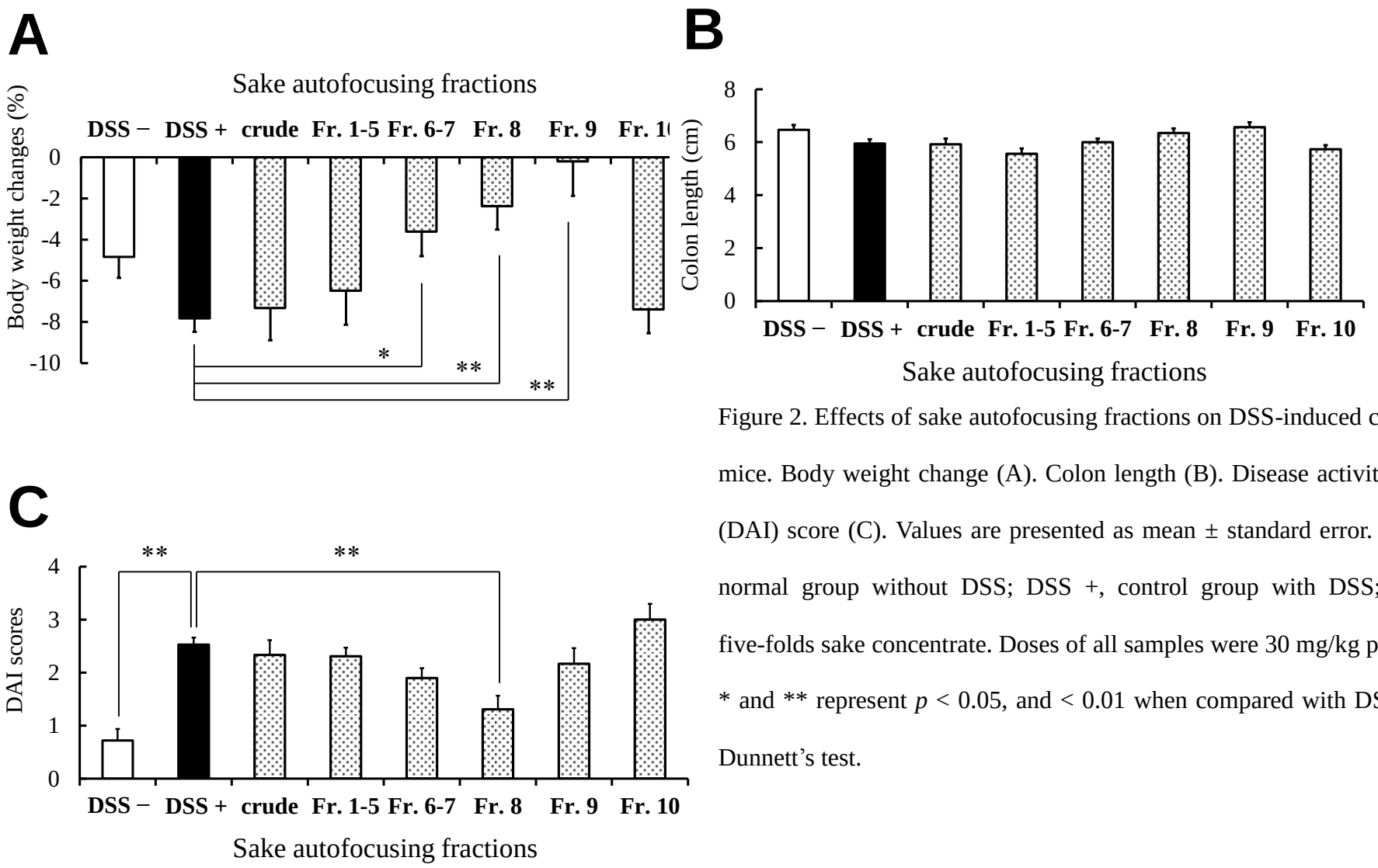



Figure 2. Effects of sake autofocusing fractions on DSS-induced colitis in mice. Body weight change (A). Colon length (B). Disease activity index (DAI) score (C). Values are presented as mean ± standard error. DSS −, normal group without DSS; DSS +, control group with DSS; crude, five-folds sake concentrate. Doses of all samples were 30 mg/kg peptides. * and ** represent $p < 0.05$, and $< 0.01$ when compared with DSS + by Dunnett's test.

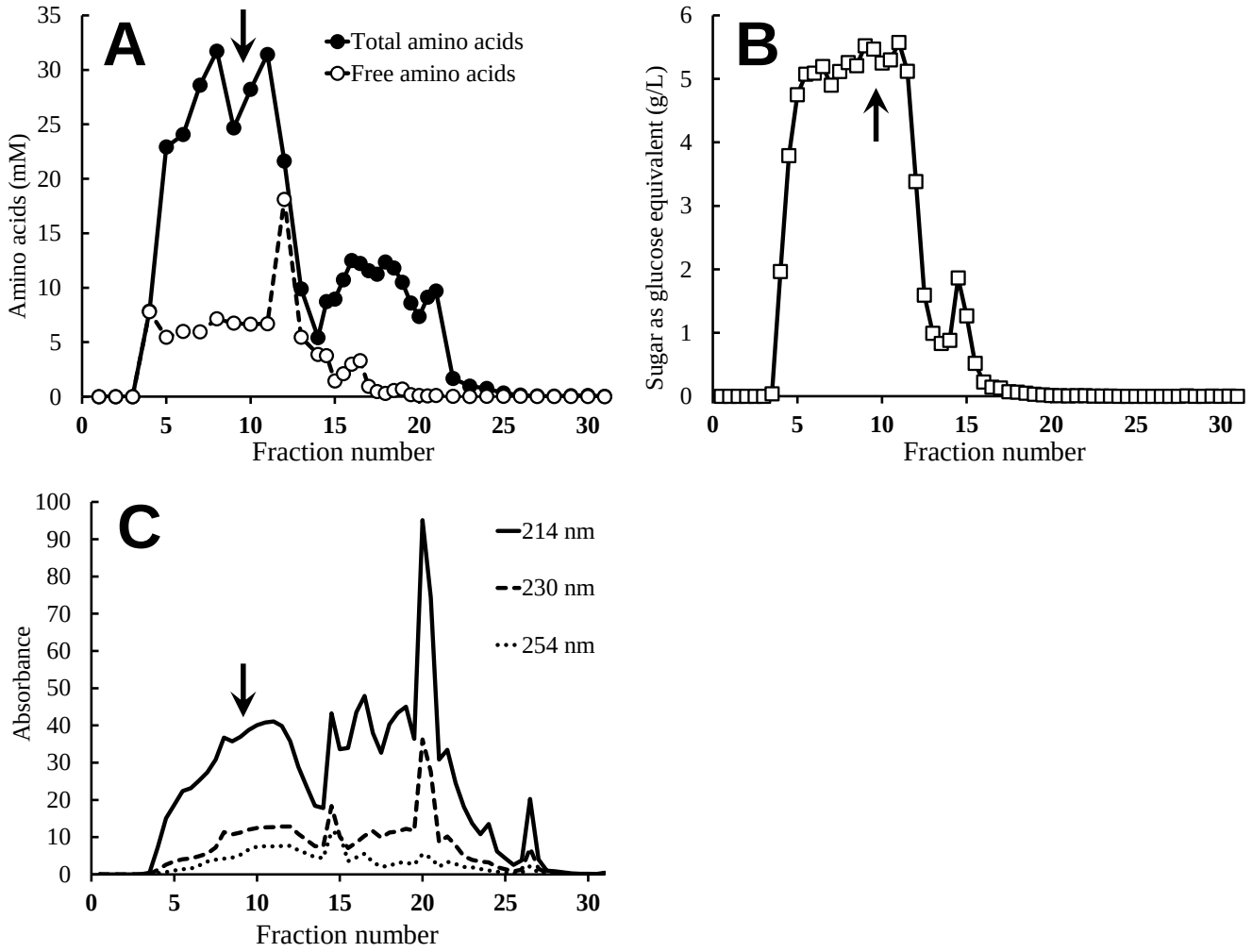



Figure 3. Fractionation of sake autofocusing Fr. 8 by preparative reversed-phase liquid chromatography. Arrows indicate start of acetonitrile gradient. Contents of free amino acids and total constituent amino acids of HCl hydrolysate in each fraction (A). Content of sugar as glucose equivalent (B). UV absorbance of each fraction (C).

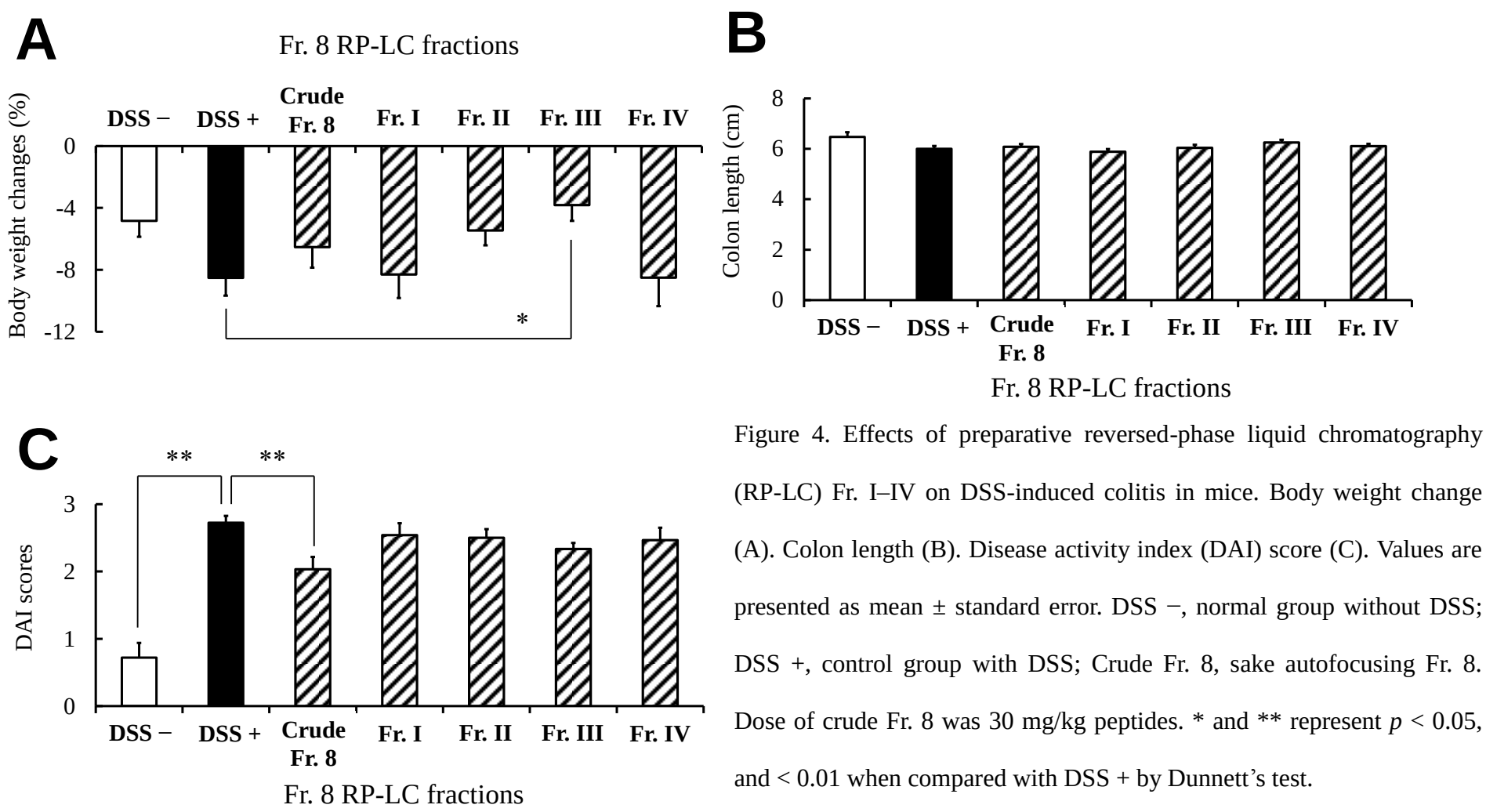



Figure 4. Effects of preparative reversed-phase liquid chromatography (RP-LC) Fr. I–IV on DSS-induced colitis in mice. Body weight change (A). Colon length (B). Disease activity index (DAI) score (C). Values are presented as mean ± standard error. DSS −, normal group without DSS; DSS +, control group with DSS; Crude Fr. 8, sake autofocusing Fr. 8. Dose of crude Fr. 8 was 30 mg/kg peptides. * and ** represent $p < 0.05$, and < 0.01 when compared with DSS + by Dunnett's test.

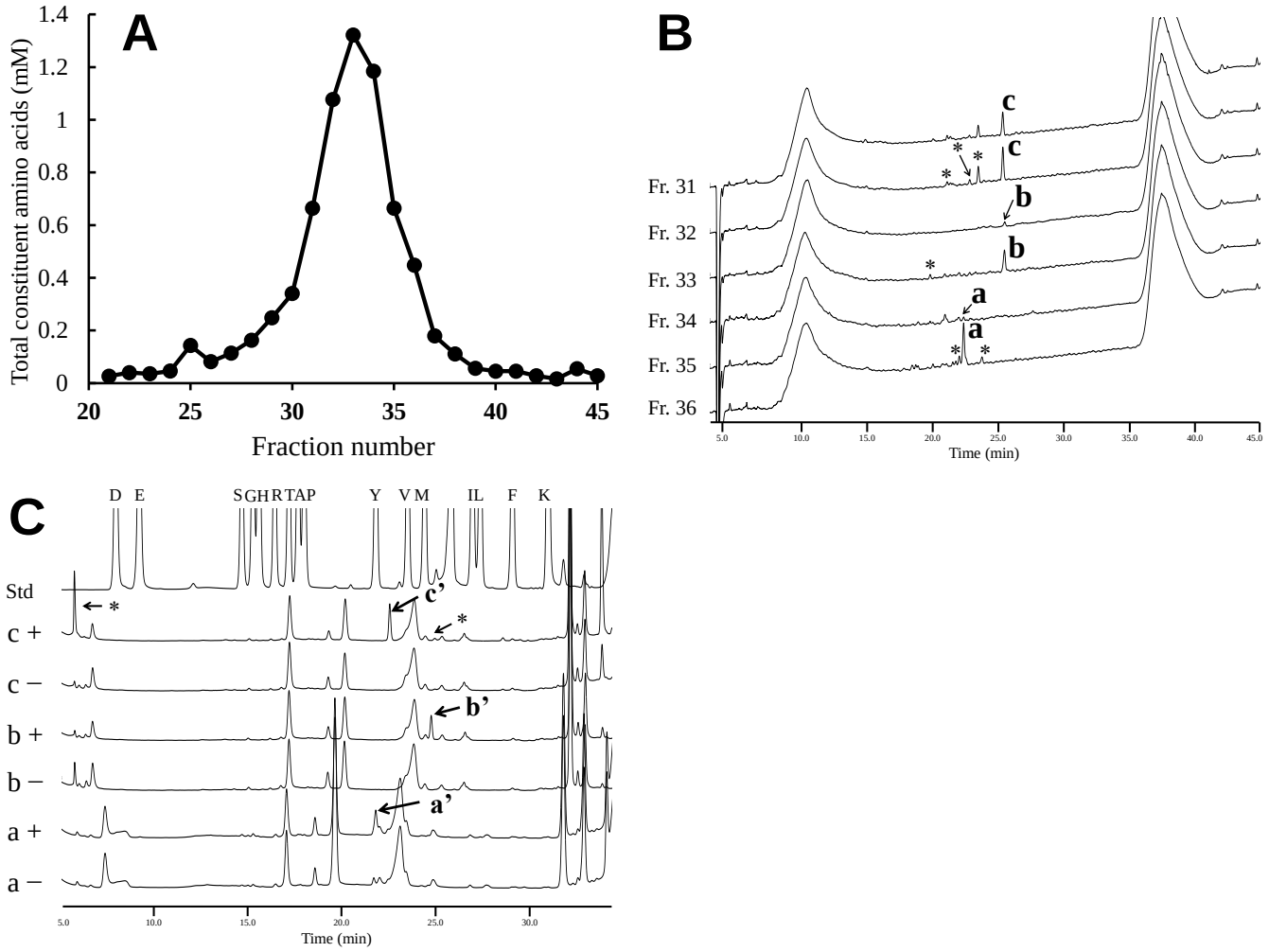
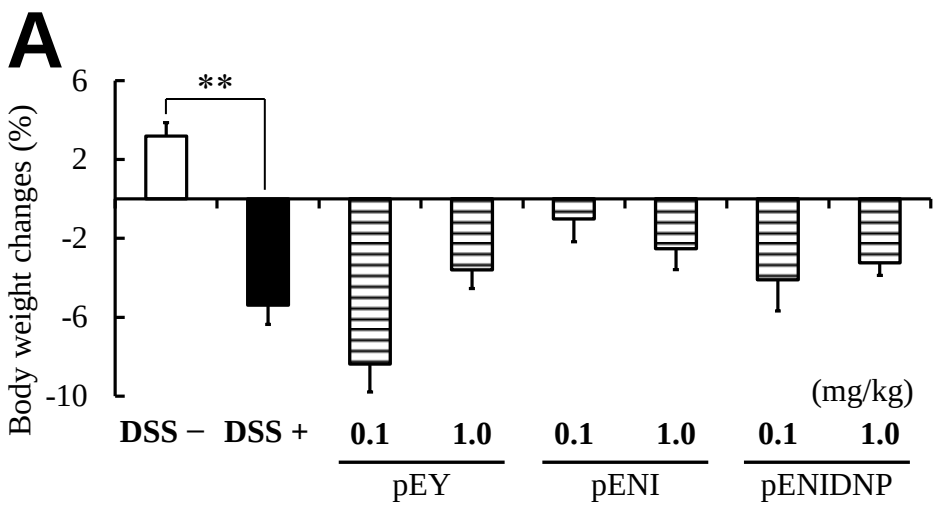
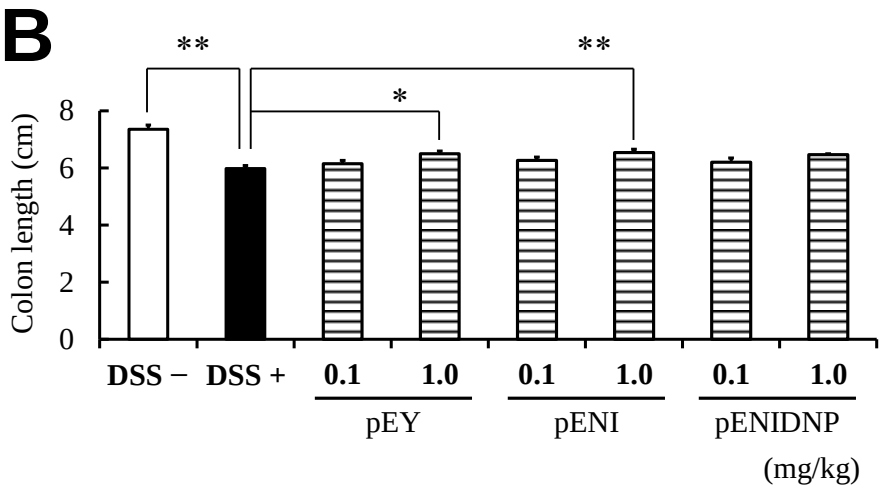



Figure 5. Identification of pyroglutamyl peptides in RP-LC Fr. III. Elution profile of peptides in Fr. III by size exclusion chromatography (SEC) using Superdex Peptide 10/30 GL (GE Healthcare) (A). Contents of peptide are expressed as the sum of total constituent amino acids in each SEC fraction after HCl hydrolysis. Isolation of pyroglutamyl peptides in SEC Fr. 31–36 by RP-HPLC (B). Fr. a, b, and c in panel B were digested with pyroglutamate aminopeptidase and derivatized with phenyl isothiocyanate. The resultant phenyl thiocarbamyl-amino acids/-peptides were resolved by second RP-HPLC (C). +, pyroglutamate aminopeptidase digests; −, non-digests of each Fr. a, b, and c. Peaks marked with a', b', and c' in panel C yielded phenyl thiohydantoin-amino acids upon Edman degradation. Peaks marked with asterisks in panel B and C did not yield significant sequence by the Edman degradation even after pyroglutamate aminopeptidase digestion.



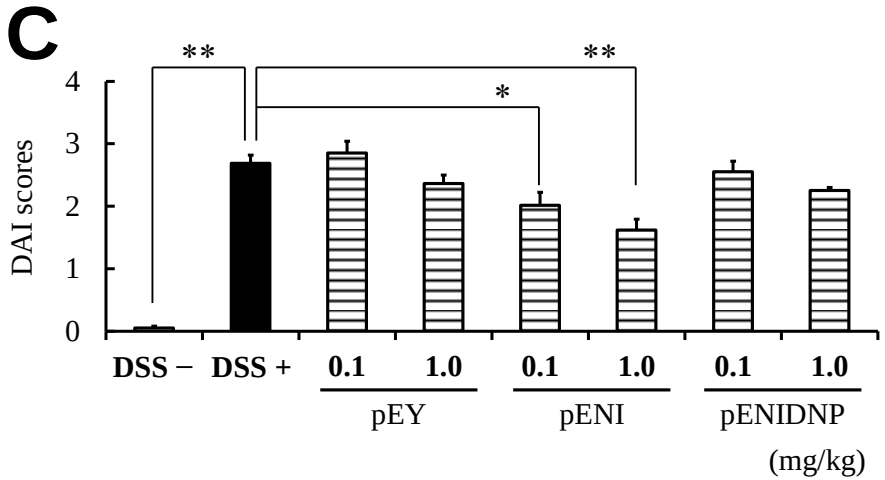




Figure 6. Effects of pyroGlu-Tyr (pEY), pyroGlu-Asn-Ile (pENI), and pyroGlu-Asn-Ile-Asp-Asn-Pro (pENIDNP) on DSS-induced colitis in mice. Body weight change (A). Colon length (B). Disease activity index (DAI) score (C). Values are presented as mean ± standard error. DSS −, normal group without DSS; DSS +, control group with DSS. * and ** represent $p < 0.05$, and < 0.01 when compared with DSS + by Dunnett's test.

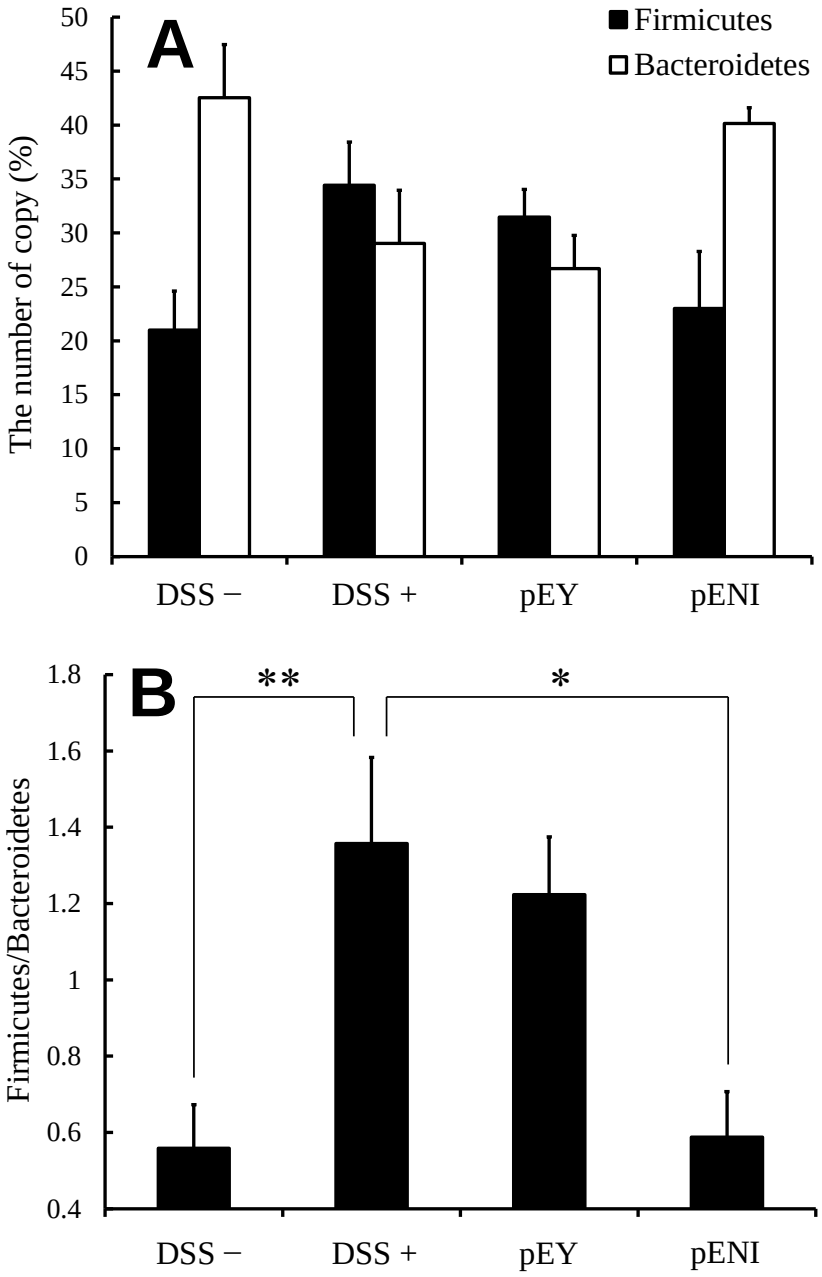



Figure 7. Population of *Firmicutes* and *Bacteroidetes* (A) and the rate of two phylums (*Firmicutes*/*Bacteroidetes*) (B) in the inner content of the colon in DSS-induced colitis mice with administration of chemically pyroGlu-Tyr (pEY) and pyroGlu-Asn-Ile (pENI). Doses of these peptides are 1.0 mg/kg body weight. Values are presented as mean ± standard error. DSS −, normal group without DSS; DSS +, control group with DSS. * and ** represent $p < 0.05$, and < 0.01 when compared with DSS + by Dunnett's test.

Table 1. Doses of pyroglutamyl peptides by administration of crude sake concentrate and fractions to mice

| Samples | Pyroglutamyl peptides (mg/kg body weight) | | | |
|---|---|---|---|---|
| | pEL | pEY | pENI | pENIDNP |
| Sake concentrate | 0.267 | 0.095 | 0.017 | 0.039 |
| Autofocusing Fr. 8 | 0.460 | 0.195 | 0.033 | 0.123 |
| RP-LC Fr. III | 0.003 | 0.011 | 0.014 | 0.040 |

pEL, pyroGlu-Leu; pEY, pyroGlu-Tyr; pENI, pyroGlu-Asn-Ile; pENIDNP, pyroGlu-Asn-Ile-Asp-Asn-Pro.

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# Chapter 4

# Preparation of rice-fermented beverage with protective activity against DSS-induced colitis in mice

## Introduction

It has been demonstrated that intake of some food (Osman et al., 2008; Shizuma et al., 2011), dietary peptides (Young et al., 2012; Lee, et al., 2009), amino acids (Vicario et al., 2007) can attenuate colitis in animal models. However, relatively high doses have been suggested to exert the protective effect against colitis by ingestion of these compounds. Kanauchi et al. have demonstrated that administration of germinated barley foodstuff (GBF) attenuates drug-induced constipation (1998 a) and colitis (1998 b) in animal models. In addition, randomized, open-labeled human study has demonstrated that daily consumption of 20–30 g of GBF for 4 weeks moderates colonic pathology of ulcerative colitis patients, which is one of inflammatory bowel disease (IBD) (Kanauchi et al., 2002). On the basis of these evidence, GBF was certified as a Food for Special Dietary Uses (FOSDU) by Individual Approval System in Japan in 2000, which indicates that IBD can be, at least partially, controlled by taking functional food. However, the GBF has not been popular for IBD patients and has been withdrawn from FOSDU. Therefore, new functional food for IBD patients has been demanded. It should exert beneficial activity preferably by ingestion of small dosage for chronic use.

As shown in previous chapters, *sake* contains pyroglutamyl peptides with the protective activity against DSS-induced colitis in mice; pyroGlu-Leu, pyroGlu-Tyr, and pyroGlu-Asn-Ile. It has been suggested that sake yeast (*Saccharomyces cerevisiae*) does

not play a significant role in production of these peptides. These peptides can be produced by proteolytic digestion of rice proteins by *Aspergillus oryzae* proteases during sake brewing. Therefore, there is a possibility that low-alcohol fermented beverage containing these pyroglutamyl peptides can be produced by fermentation of steamed rice with *A. oryzae*.

The final goal of this study is to develop a low-alcohol functional beverage with the protective activity against IBD by modified sake brewing process. Various types of fermented steamed rice with food-grade *A. oryzae* were prepared and checked for their protective activity against DSS-induced colitis in mouse model.

## Materials and methods

### 1. Food materials.

Food-grade lactic acid was obtained from Musashino Chemical Laboratory (Tokyo, Japan). Dried *kojis*, which is the steamed rice inoculated with *A. oryzae*, was kind gift from Tokushima Seikiku (Tokushima, Japan). The rice used for koji had been polished to 60 and 90% of brown rice. Rice polished to 90% was also used for preparation of steamed rice.

### 2. Reagents.

Dextran sulfate sodium (DSS, average molecular weight 8,000) was purchased from Seikagaku (Tokyo, Japan). Acetonitrile (HPLC grade), phenyl isothiocyanate (PITC) were purchased from Wako Chemicals (Osaka, Japan). Triethylamine (TEA) was purchased from Thermo Fisher Scientific (Waltham, MA). Other reagents used were of analytical grade or better.

### 3. DSS-induced colitis in mice.

The animal experiment of DSS-induced colitis was conducted by the same procedures as described in chapter 3. All animals were treated and cared for in accordance with the National Institutes of Health's (NIH) guidelines for the use of experimental animals. All experimental procedures were approved by the Animal Care Committee of Kyoto Prefectural University of Medicine (M23–37, M24–25, Kyoto, Japan). Supernatants prepared from fermented rice products described below were diluted with distilled water to give 0.3 mg peptides (total amino acids in HCl

hydrolysate)/mL (3 mg/kg body weight), and administered to the mice.

**4. Preparation of fermented rice.**

Fermented rice was prepared by different conditions and from different materials by Shotoku Brewery (Kyoto, Japan). Two types of koji, which were made from rice polished to 60 and 90% of brown rice and referred to koji 60 and koji 90, respectively, were used. In low-temperature fermentation (LTF), koji 60 or koji 90, water, and lactic acid were mixed in a ratio as shown in Table 1. The mixtures were fermented at 10°C or 20°C for 1 week or 2 weeks. In high-temperature fermentation (HTF), the koji 90 was mixed with steamed rice and water as shown in Table 1. The steamed rice was made from the rice polished to 90%. The rice was washed and soaked in water overnight, and then steamed at 120°C for 1 h and cooled until manageable. The mixture was fermented at 55°C for 4 h and followed additional fermentation at 40°C, 50°C, and 55°C, respectively. Aliquots of fermented products were collected 1, 2, and 3 h after the second fermentation.

**5. Determination of pyroglutamyl peptides.**

Aliquots of the LTF and HTF products (25 g) were homogenized with 25 mL of distilled water using a glass homogenizer. The supernatants were collected after centrifugation at 2,000 rpm for 10 min and diluted at 100 folds with ultrapure water, and clarified by passing through a filter (Cosmonice filter W, 4 mm i.d., 0.45 μm pore size, Nacalai Tesque, Kyoto, Japan) The contents of pyroGlu-Leu, pyroGlu-Tyr, pyroGlu-Asn-Ile, and pyroGlu-Asn-Ile-Asp-Asn-Pro in these samples were determined using liquid chromatography-tandem mass spectrometry as described in chapter 3.

**6. Amino acid analysis.**

Vapor-phase 6 M HCl hydrolysis and amino acid analysis using PITC derivatization were performed according to the method as described in chapter 2. Peptide content was expressed as sum of total constituent amino acids after HCl hydrolysis.

**7. Statistical analysis.**

The value of body weight change, colon length, and disease activity index (DAI) score of DSS-induced colitis mice were presented as mean ± standard error. Data were subjected to one-way ANOVA with Dunnett's multiple comparison of means test. Differences showing $p < 0.05$ were considered significant. Statistical analysis was performed using Ekuseru-Toukei 2010 Version.1.11 (Social Survey Research Information, Tokyo, Japan).

## Results

### 1. Pyroglutamyl peptides in fermented rice.

Contents of pyroGlu-Leu, pyroGlu-Tyr, pyroGlu-Asn-Ile, and pyroGlu-Asn-Ile-Asp-Asn-Pro in LTF products are shown in Figure 1. Pyroglutamyl di-peptides, pyroGlu-Leu and pGlu-Tyr, increased after longer fermentation period and at 20°C. The contents of these peptides in LTF products from koji 90 were higher than those from koji 60. Content of pyroglutamyl tri-peptide, pyroGlu-Asn-Ile, increased during 2 weeks and were higher by fermentation at 10°C. Polishing rate of rice for koji did not affect the content of pyroGlu-Asn-Ile. On the other hand, content of pyroGlu-Asn-Ile-Asp-Asn-Pro decreased by longer fermentation period at 20°C. At 10°C, pyroGlu-Asn-Ile-Asp-Asn-Pro did not exceed approximately 1 μM.

As shown in Figure 2, HTF products after first fermentation at 55°C contained pyroGlu-Leu and pyroGlu-Tyr at approximately 20 and 4 μM, respectively, which did not increase during second fermentation periods at different temperature. On the other hand, pyroGlu-Asn-Ile and pyroGlu-Asn-Ile-Asp-Asn-Ile were not detected in all HTF samples.

### 2. Improvement of DSS-induced colitis by fermented rice.

LTF products from koji 60 at 10°C for 2 weeks, koji 90 at 10°C and 20°C for 2 weeks, and HTF product without second fermentation step were evaluated for protective activity against colitis. These LTF products are referred to LTF (temperature)-koji (recovery), *ex* LTF 20-koji 90 in the following section. The soluble fractions of these samples were administered (3 mg peptides (total amino acids in HCl hydrolysate)/kg

body weight) for the mice with DSS-induced colitis. As shown in Figure 3, DSS treatment induced colitis as indicated by significant decrease of body weight gain, colon length, and significant increase of DAI score. Administration of LTF 20-koji 60 significantly attenuated body weight loss and decreased DAI score compared with DSS + control group. LTF 20-koji 90 tended to moderate body weight loss ($p$ = 0.08) and significantly decreased DAI score. There was no significant difference in colon length between DSS + control group and each sample group. On the other hand, administration of HTF product showed no significant protective effect against DSS-induced colitis in mice.

Doses of pyroGlu-Leu, pyroGlu-Tyr, pyroGlu-Asn-Ile, and pyroGlu-Asn-Ile-Asp-Asn-Pro by administration of LTF and HTF products to mice are presented in Table 2. Similar doses of pyroGlu-Leu were administered to all groups. The doses of pyroGlu-Try were almost same except for LTF 10-koji 90 group. Doses of the pyroglutamyl tri- and hexa-peptides also did not so change between LTF groups.

## Discussion

While pyroGlu-Leu and pyroGlu-Tyr were presented in all LTF and HTF products, tri- and hexa-pyroglutamyl peptides, pyroGlu-Asn-Ile and pyroGlu-Asn-Ile-Asp-Asn-Pro, were observed only in the LTF products. It has been reported that optimal temperature for extracellular proteases of *A. oryzae* including carboxypeptidase is 50–60°C (Blinkovsky et al., 1999). PyroGlu-Asn-Ile and pyroGlu-Asn-Ile-Asp-Asn-Pro might be degraded by the carboxypeptidase in HTF products. In the LTF products, pyroGlu-Leu and pyroGlu-Tyr increased by longer fermentation period, and higher temperature (20°C). On the other hand, pyroGlu-Asn-Ile and pyroGlu-Asn-Ile-Asp-Asn-Pro did not increase by the higher temperature. Therefore, fermentation temperature is critical to control composition of di-, tri-, and hexa-pyroglutamyl peptides in the products. For production of pyroGlu-Asn-Ile and pyroGlu-Asn-Ile-Asp-Asn-Pro, higher temperature than 20°C is nor preferable.

Three LTF products, LTF 10 and 20-koji 90 and LTF 60-koji 20, which had higher content of pyroGlu-Leu than sake (chapter 2), and one HTF product without second fermentation were examined for the protective effects against DSS-induced colitis in mice. In chapter 3, dose of pyroGlu-Leu by administration of sake concentrate (30 mg peptides (total amino acids)/kg body weight) was higher than 0.1 mg/kg body weight. Synthesized pyroGlu-Leu exerted the protective effect against the colitis in the same animal model by oral administration at 0.1 mg/kg body weight, while higher dose did not show beneficial effect (Wada et al., 2013). Therefore, dose of pyroGlu-Leu should be less than 0.l mg/kg body weight for practical use. Therefore, the LTF products and

HTF product were administered to the mice at 3 mg peptides (total amino acids)/kg body weight, which is 1/10 of the dose in the animal experiments in previous chapter. Doses of four pyroglutamyl peptides by administration of LTF and HTF products are shown in Table 2. The doses of active pyroglutamyl peptides were considerably lower than effective doses evaluated by single administration of synthesized peptides as shown in chapter 3.

As shown in Figure 3, HTF product showed no significant protective effect by the present dose possibly due to the smaller doses of pyroGlu-Leu and pyroGlu-Tyr compared to optimum doses (Table 2). On the other hand, unexpectedly, administration of LTF 20-koji 60 and 90, containing pyroGlu-Asn-Ile and pyroGlu-Asn-Ile-Asp-Asn-Pro in addition to pyroGlu-Leu and pyroGlu-Tyr, significantly moderated DSS-induced colitis in mice, while doses of pyroGlu-Leu and pyroGlu-Tyr by administration of these LTF products were comparable to those by administration of HTF product (Table 2). The previous study (chapter 3) also indicated that administration of pyroGlu-Leu over optimum dose with presence of small doses of pyroGlu-Asn-Ile and pyroGlu-Asn-Ile-Asp-Asn-Pro moderated to the colitis, while in the absence of pyroGlu-Asn-Ile and pyroGlu-Asn-Ile-Asp-Asn-Pro did not show the protective effect. These facts suggest presence of pyroGlu-Asn-Ile and pyroGlu-Asn-Ile-Asp-Asn-Pro in smaller dose, which did not exert the protective effect by single administration of synthesized ones, might cooperatively enhance the protective effect with pyroGlu-Leu and pyroGlu-Tyr. Alternatively, other compounds in the LTF products, such as peptides with amino group, oligosaccharides and etc., might be also involved in the protective effect. Further studies on the protective effect against colitis by combination of pyroGlu-Leu, pyroGlu-Tyr, pyroGlu-Asn-Ile,

pyroGlu-Asn-Ile-Asp-Asn-Pro, and other compounds in the LTF products are in progress.

In the previous study (chapter 3), alcohol-free sake concentrate without fractionation did not exert the protective effect against DSS-induced colitis in mice. The present study demonstrated that some LTF products without purification step exerted the protective effect in small dose corresponding to less than 20 mL per day for human which is simply estimated on the basis of effective dose/kg body weight. The LTF product, which is a low-alcohol fermented food and prepared by using classical method for *Shubo* (sake yeast starter) production, has stronger potential to control human IBD as functional fermented beverage in comparison to sake (Japanese rice wine) or HTF product, which is mimicking Japanese sweet rice-fermented beverage, *amazake*. After identification of active compounds, their effective dose, and elucidation of underlying mechanism, formulated LTF product is necessary for human trial.

## Figures and tables

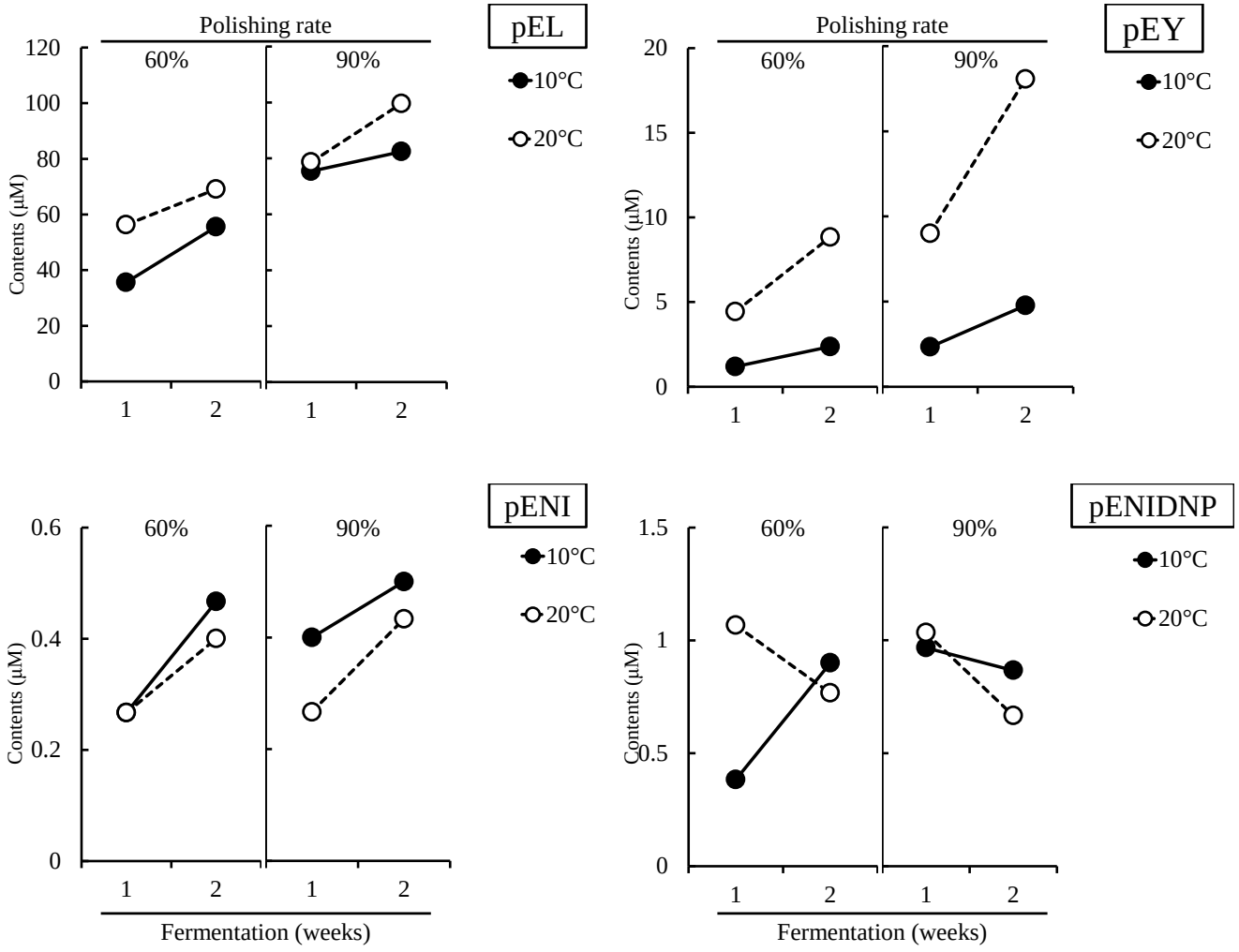


Figure 1. Determination of contents of pyroGlu-Leu (pEL), pyroGlu-Tyr (pEY), pyroGlu-Asn-Ile (pENI), and pyroGlu-Asn-Ile-Asp-Asn-Pro (pENIDNP) in low-temperature fermentation (LTF) products. Koji 60 and 90 were used. Black circles show the fermentation at 10°C, and white circles show at 20°C.

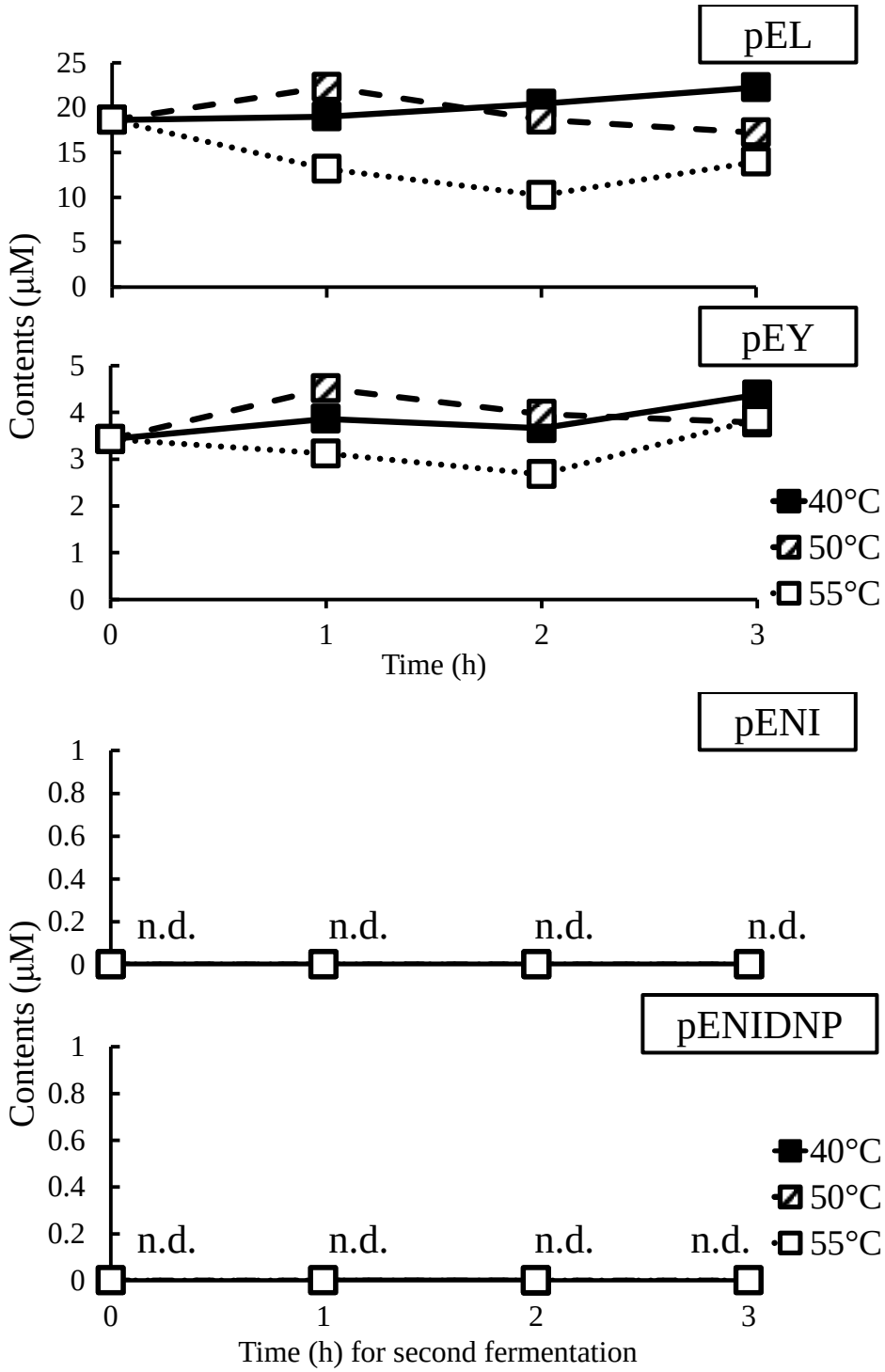


Figure 2. Determination of contents of pyroGlu-Leu (pEL), pyroGlu-Tyr (pEY), pyroGlu-Asn-Ile (pENI), and pyroGlu-Asn-Ile-Asp-Asn-Pro (pENIDNP) in high-temterature fermentation (HTF) products after second fermentation. Time "0 h" is the HTF products 4 h after first fermentation at 55°C. Black squares show the HTF products by additional fermentation at 40°C, shaded squares show at 50°C, and white squares show at 55°C. "n.d." shows no detectable amount.

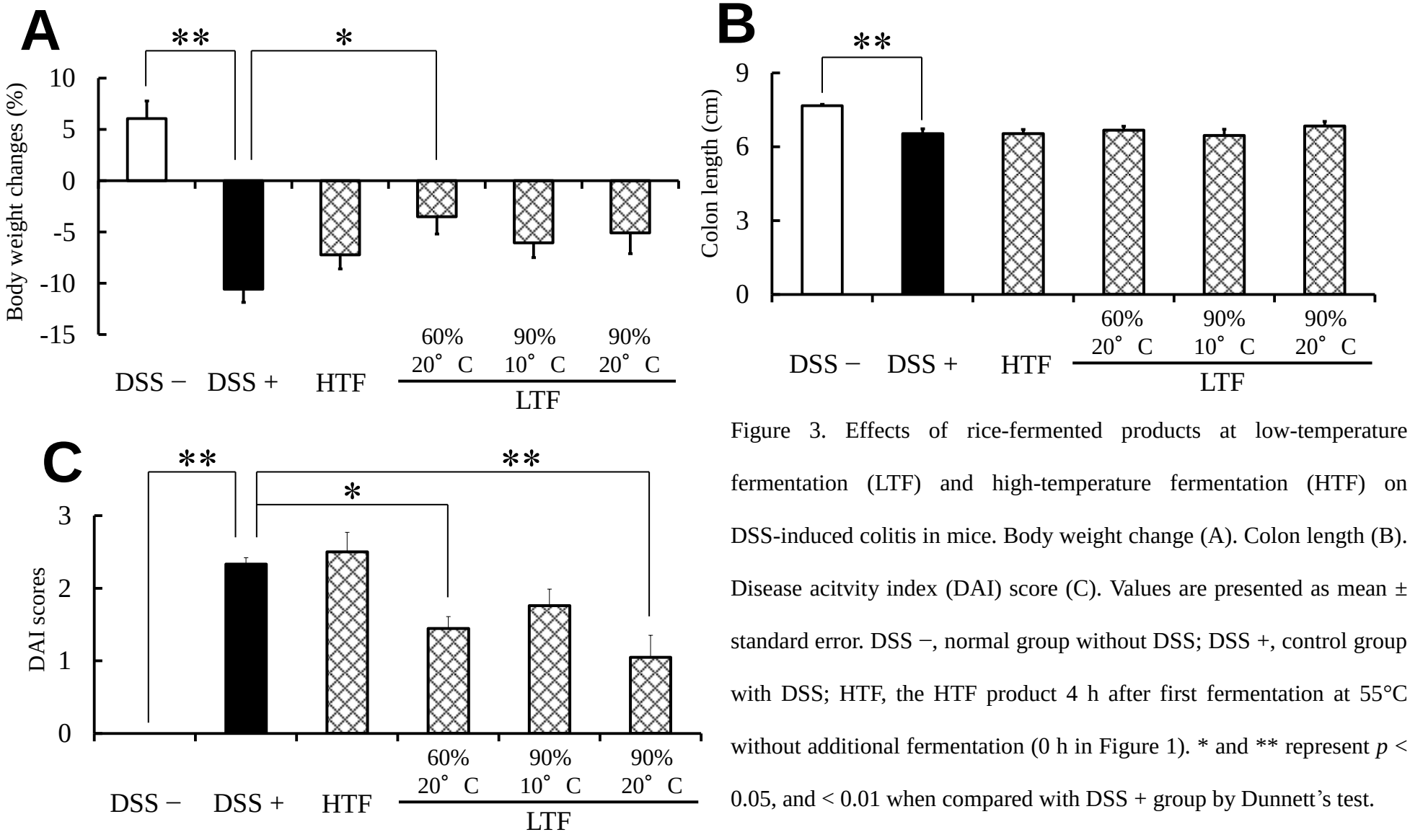


Figure 3. Effects of rice-fermented products at low-temperature fermentation (LTF) and high-temperature fermentation (HTF) on DSS-induced colitis in mice. Body weight change (A). Colon length (B). Disease acitvity index (DAI) score (C). Values are presented as mean ± standard error. DSS −, normal group without DSS; DSS +, control group with DSS; HTF, the HTF product 4 h after first fermentation at 55°C without additional fermentation (0 h in Figure 1). * and ** represent $p < 0.05$, and $< 0.01$ when compared with DSS + group by Dunnett's test.

Table. 1 Composition of materials for rice-fermented products

| Materials | Mixing rate (%) | |
|---|---|---|
| | Low-temperature fermentation | High-temperature fermentation |
| Koji (w/w)* | 44.7 | 31 |
| Steamed rice (w/w)* | 0 | 13.8 |
| Water (v/w) | 55.0 | 55.2 |
| Lactic acid (v/w) | 0.3 | 0 |

*Contents of koji and steamed rice were expressed as the content to polished rice before steaming.

Table 2. Doses of four pyroglutamyl peptides by administration of LTF and HTF products to mice

| (μg/kg | HTF | LTF | | |
|---|---|---|---|---|
| body weight) | 0 h | 60%、20°C | 90%、10°C | 90%、20°C |
| pEL | 4.90 | 4.98 | 4.24 | 4.74 |
| pEY | 1.09 | 0.77 | 0.30 | 1.04 |
| pENI | – | 0.04 | 0.04 | 0.03 |
| pENIDNP | – | 0.16 | 0.13 | 0.09 |

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# Chapter 5

# General discussion and conclusion

In this study, nearly 20 short-chain pyroglutamyl peptides were identified in Japanese rice wine, *sake*, which first provides basic knowledge about the structures of pyroglutamyl peptides in sake. Interestingly, pyroGlu-Leu, which was first identified in wheat gluten hydrolysate as a protective compound against acute hepatitis and colitis in animal models, is one of the major constituents of pyroglutamyl peptides in sake. These findings suggest that sake and its constituents might have potential to exert beneficial activity for human health. In addition, there is a possibility that other pyroglutamyl peptides might have beneficial activities.

The crude alcohol-free sake concentrate did not exert the protective effect against the animal colitis. Fractionation using ampholite-free preparative isoelectric focusing, autofocusing, revealed that sake contains compounds with positive and negative effects on the animal colitis. Active fraction obtained by preparative reversed phase-liquid chromatography with the protective activity consisted of pyroGlu-Tyr, pyroGlu-Asn-Ile, and pyroGlu-Asn-Ile-Asp-Asn-Pro. Chemically synthesized pyroGlu-Tyr and pyroGlu-Asn-Ile significantly attenuated the colitis. The DSS-induced colitis dramatically changes ratio of *Firmicutes* to *Bacteroidetes* in the mouse colon, which are two major phylums of colonic microbiota. PyroGlu-Leu and pyroGlu-Asn-Ile normalized the colonic microbiota in the DSS-induced colitis mice by oral administration of unexpectedly low doses (0.1–1.0 mg/kg body weight). On the other hand, pyroGlu-Tyr did not significantly affect on the microbiota. Therefore, sake contains some protective pyroglutamyl peptides against the colitis based on different

mechanisms. The mechanisms for modulation of colonic microbiota by small doses of pyroGlu-Leu and pyroGlu-Asn-Ile remain to be solved. However, it is unlikely to assume that these peptides directly modulate growth of colonic microorganisms by small dose. Possibly these peptides might affect host innate immune or/and inflammatory responses. Elucidation of the effect of food compounds such as pyroGlu-Leu and pyroGlu-Asn-Ile to host intestinal cells responsible for the innate immune and inflammatory responses might explore a new target for health promotion by food.

The present study demonstrates that proteases from *Aspergillus oryzae* (*koji*) play a significant role in production of pyroGlu-Leu, and sake yeast (*Saccharomyces cerevisiae*) is not significantly involved in production of pyroGlu-Leu. On the basis of these facts, pyroglutamyl peptides were produced by high-temperature fermentation (HTF) and low-temperature fermentation (LTF) of koji. The HTF and LTF of koji correspond to production of Japanese traditional sweet fermented rice beverage, *amazake*, and to classical production of *shubo* (sake yeast starter). PyroGlu-Asn-Ile and pyroGlu-Asn-Ile-Asp-Asn-Pro were observed only in the LTF product, while pyroGlu-Leu and pyroGlu-Tyr were presented in all LTF and HTF products. Although doses of pyroGlu-Leu and pyroGlu-Tyr by administration of HTF and LTF products were lower than optimum dose for each synthesized peptide, the LTF products exerted the protective effect against colitis in mice, while HTF product did not show the effect. These results suggest that presence of pyroGlu-Asn-Ile and pyroGlu-Asn-Ile-Asp-Asn-Pro in smaller dose might cooperatively enhance the protective effect against colitis with pyroGlu-Leu and pyroGlu-Tyr. The identification of pyroglutamyl peptides which have the protective effect against colitis in animal model

with single administration enable to investigate the beneficial effect by combination of these peptides and other food compounds, which would explore how complicated food system exert beneficial effects for human health.

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## List of published paper on the present study

Identification of pyroglutamyl peptides in Japanese rice wine (*sake*): presence of hepatoprotective pyroGlu-Leu.

Tamami Kiyono, Kiyoo Hirooka, Yoshihiro Yamamoto, Sunao Kuniishi, Maho Ohtsuka, Shikou Kimura, Eun Young Park, Yasushi Nakamura, Kenji Sato. *Journal of Agricultural and Food Chemistry*, **2013**, *61*(47), 11660–7. doi:10.1021/jf404381w