Identification of pyroglutamyl peptides with anti-colitic activity in Japanese rice wine, sake, by oral administration in a mouse model

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ARTICLE INFO

Article history:
Received 20 July 2016
Received in revised form 17 September 2016
Accepted 13 October 2016
Available online

ABSTRACT

Two pyroglutamyl peptides with anti-colitic activity were identified in Japanese rice wine, sake, by oral administration of a small dose (0.1–1.0 mg/kg body weight) and in vivo activity-guided fractionation. Compounds in sake were fractionated by preparative isoelectric focusing followed by preparative reversed phase-liquid chromatography. Anti-colitic activity was evaluated using a dextran sulphate sodium (DSS)-induced colitis mouse model. The final active fraction contained three 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). Orally administrating artificially synthesized pyroGlu-Tyr (1.0 mg/kg body weight) and pyroGlu-Asn-Ile (0.1 and 1.0 mg/kg body weight) exhibited a significant protective effect against colitis in mice, whereas pyroGlu-Asn-Ile-Asp-Pro showed no significant effect. Additionally, administrating pyroGlu-Asn-Ile normalized colitis-induced colonic dysbiosis, whereas pyroGlu-Tyr did not. These results suggest that identified pyroglutamyl peptides exhibited an anti-colitic activity via different mechanisms.

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Keywords:
Pyroglutamyl peptide
Colitis
Sake
Japanese rice wine
Microbiota

1. Introduction

Inflammatory bowel disease (IBD) is a chronic disorder of the gastrointestinal tract and includes ulcerative colitis and Crohn’s disease (Podolsky, 2002). Patients suffering from IBD show symptoms characterized by diarrhoea, bloody stools, and weight loss. IBD is generally treated with a combination of anti-inflammatory and immunomodulatory drugs. In some cases, these therapies exert limited efficacy and have a risk of adverse
higher doses than normally present (15% alcohol content) made from rice polished to Saccharomyces cerevisiae by oral administration in small doses. The sake L. <Aspergillus oryzae was concentrated (5-fold) by a rotary evaporator. Activity-guided fractionation can identify really active peptide pyroglutamyl peptides (15 mg/L of pyroGlu-Leu, and eighteen other short chain pyroglutamyl peptides with protective effect against colitis et al., 2015; Lee et al., 2009a, 2009b) have been demonstrated in animal models. In those studies, decrease and/or down regulation of pro-inflammatory cytokines and enzymes involved in inflammation such as inducible nitric oxide synthase and cyclooxygenase-2 have been demonstrated. Based on these findings, anti-inflammatory activity in colon has been suggested to be the mechanism for moderation of IBD by such food compounds. The IBD symptoms can be alleviated by pre-and pro-biotics through improvement of colonic microbiota and metabolites of microbiota such as short chain fatty acids (Damaskos & Kolios, 2008; Hijova & Soltesova, 2013; Osman, Adawi, Ahrné, Jeppsson, & Molin, 2008), which can consequentially suppress colonic inflammation. Then apparent anti-inflammatory response by ingestion of these foods does not always mean that compounds in the food directly suppress inflammatory action of host cells in colon. The identification of active compound in the food, which can ameliorate IBD by oral administration, is crucial to elucidate the underlying mechanism of anti-colitic activity. The response of specific cells, including host and exogenous cells, can be elucidated by using compounds isolated from food. It has been demonstrated that food compounds with some beneficial in vitro activity such as the anti-oxidant activity also exert anti-colitic activity (Larrosa et al., 2009; Osman et al., 2008; Yoda et al., 2014). However, in most studies considerably higher doses than normally present in foods were needed to exert beneficial effects against colitis in animal models. Therefore, the beneficial effects of the plant extract could not solely be attributed to the suggested compounds and their in vitro activities. Similarly, some amino acids, derived from dietary proteins, also improve the symptoms of IBD; however, the effective doses are higher than the content in the protein. There is a possibility that specific peptides, which could be derived from proteins, might be responsible for suppression of IBD, however, there are few data indicating such active peptide in food. Recently, we found that pyroglutamyl-leucine (pyroGlu-Leu), which was identified in wheat gluten enzymatic hydrolysate as hepatoprotective peptide by oral administration (Sato et al., 2013), also attenuates dextran sulphate sodium (DSS)-induced colitis and normalizes the colonic dysbiosis in mice upon oral administration at 0.1 mg/kg body weight (Wada et al., 2013). Pyroglutamyl peptide, wherein the pyroglutamic acid residue is generated from amino terminus glutaminyl residue, is found in food protein hydrolysates (Sato et al., 1998; Suzuki, Motoi, & Sato, 1999) and fermented foods (Kaneko, Kumazawa, & Nishimura, 2011; Kiyono et al., 2013). We previously reported that Japanese rice wine, sake, a fermented alcohol beverage made of water and rice inoculated with Aspergillus oryzae and Saccharomyces cerevisiae, contains 12–15 mg/L of pyroGlu-Leu, and eighteen other short chain pyroglutamyl peptides (Kiyono et al., 2013). In the context of IBD, the objective of the present study was to identify other pyroglutamyl peptides with protective effect against colitis present in sake by oral administration in small dose. The in vivo activity-guided fractionation can identify really active peptide by oral administration compared to the conventional in vitro activity-guided fractionation without considering bioavailability. However, it has been difficult to fractionate samples to obtain enough amounts of fractions for animal experiment. To solve this problem, the present authors have developed large-scale preparative ampholyte free isoelectric focusing referred to as autofocusing (Hashimoto, Sato, Nakamura, & Ohtsuki, 2005). By using this technique, two anti-colitic peptides by oral administration of small dose (<1 mg/kg body weight) were identified in the present study.

2. Materials and methods

2.1. Samples

Bottled sake (15% alcohol content) made from rice polished to 60% was commercially obtained from Shotoku Brewery (Kyoto, Japan). Sake was concentrated (5-fold) by a rotary evaporator at 60 °C and used for subsequent experiments.

2.2. Reagents

Lipopolysaccharide (LPS) from Escherichia coli, O111 B4, was obtained from Sigma (Saint Louis, MO). Foetal bovine serum (FBS) and Dulbecco’s modified Eagle’s medium (DMEM) were obtained from JR Scientific (Woodland, CA) and Thermo Fisher Scientific (Waltham, MA), respectively. Dextran sulphate sodium (DSS; average molecular weight, 8000) was purchased from Seikagaku (Tokyo, Japan). Pyrococcus furiosus pyroglutamate amniopeptidase 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. 1-pyroglutamic acid was purchased from Nacalai Tesque (Kyoto, Japan). 9-Fluorenylmethoxycarbonyl (Fmoc)-Tyr(tBu)-Wang resin, Fmoc-Ile-Wang resin, H-Pro-2-naphthylchloride resin, and Fmoc-amino acids for peptide synthesis were purchased from HiPep Laboratories (Kyoto, Japan). Other reagents used were of analytical or higher grade.

2.3. DSS-induced colitis in mice

Animal experiments were conducted according to a previously described procedure (Wada et al., 2013). Seven-week-old male C57BL/6 mice were purchased from Shimizu Laboratory Supplies (Kyoto, Japan). The mice (n = 6 or 7) were housed in a room under controlled conditions of 18–24 °C, 40–70% relative humidity, and a 12 h light/dark cycle. Mice were allowed free access to food CRF-1 (Oriental Yeast, Tokyo, Japan) and drinking water during a one-week acclimatization period. All animals were treated and cared for in accordance with the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals, 8th Edition. 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 oral administration of 2.5 or 3.0% (w/v) DSS dissolved in drinking water for 7 days. The working concentration of DSS (2.5% or 3.0%) was determined


613
by preliminary experiments by using the same batch of animals to induce an identical grade of inflammation (more than disease activity index score 2) as described in a previous study (Wada et al., 2013). Mice were allowed free access to drinking water either containing DSS (DSS+) or without DSS (DSS−). Samples were dissolved in distilled water and administered by gavage in aliquots of 200 μL once a day during the entire induction period. The vehicle group of mice received an identical amount of distilled water. The body weight of each mouse was measured on days 0, 3, 5, and 7. Mice were sacrificed on day 7 and the entire colon was removed. Immediately after colon resection, the colon length was measured.

For the identification of active fraction, the severity of colitis was evaluated by a disease activity index (DAI) score as previously reported (Wada et al., 2013). The DAI score was determined by scoring of weight loss, stool consistency and bleeding as described previously (Table 1) (Murano et al., 2000; Naito et al., 2006).

### Table 1 – Scoring system of disease activity index (DAI) .

<table>
<thead>
<tr>
<th>Scoring</th>
<th>Weight loss (%)</th>
<th>Stool consistency</th>
<th>Stool bleeding</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None</td>
<td>Normal</td>
<td>Negative</td>
</tr>
<tr>
<td>1</td>
<td>1–5</td>
<td>Mild loose</td>
<td>Light</td>
</tr>
<tr>
<td>2</td>
<td>5–10</td>
<td>Loose</td>
<td>Mild</td>
</tr>
<tr>
<td>3</td>
<td>10–20</td>
<td>Mild bleeding</td>
<td>Severe</td>
</tr>
<tr>
<td>4</td>
<td>&gt;20</td>
<td>Diarrhoea</td>
<td>Entire</td>
</tr>
</tbody>
</table>

a DAI = (Combined scores of weight loss, stool consistency and bleeding)/3.

b Normal, formed pellets; Loose, pasty stools that do not stick to the anus; diarrhoea, liquid stools that stick to the anus.

2.4. Fractionation of compounds in sake by preparative isoelectric focusing

For animal experiments, the concentrated compounds in sake were fractionated by preparative isoelectric focusing based on the amphoteric nature of the sample according to a previously described method (Hashimoto et al., 2005) with slight modifications (Sato et al., 2013). This method has been referred to as autofocusing. An autofocusing apparatus with 12 compartments (75 mm in length × 80 mm in width × 85 mm in height for each compartment), separated by a thin agarose gel layer, was prepared. Phosphoric acid at 0.03 M concentration and 0.1 M sodium hydroxide were loaded in the compartments at either ends of the tank, which were used as anode and cathode, respectively. Ten compartments in the centre of the tank were used as sample compartments and were numbered from the anode side (No. 1) to the cathode side (No. 10). Sample compartments numbers 8 and 9 were each filled with 500 mL of the 5-fold sake concentrate. The other sample compartments were filled with deionized water. Autofocusing was performed at constant voltage mode at 500 V for 24 h. Following this, all fractions were collected and their pH was adjusted to 7.0 by addition of 1 M HCl or sodium hydroxide, and used for subsequent experiments.

2.5. Fractionation of compounds by preparative reversed phase-liquid chromatography

The fraction obtained after autofocusing, which attenuated DSS-induced colitis in mice, was subjected to a second fractionation step using preparative reversed phase-liquid chromatography (RP-LC). The RP-LC system consisted of a column (160 × 15 mm, Millipore, Billerica, MA) 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 a flow rate of 10 mL/min. Following this, 80 mL of the autofocusing fraction, diluted to 3.0 mg peptide/mL for animal experiments, was loaded to the column using the pump. Elution was performed using a binary linear gradient consisting of 10 mM HCl (solvent A) and 10 mM HCl containing 60% (v/v) acetonitrile (solvent B). Gradient profile was 0% B for 1 min followed by 0–100% B over 20 min and 100% B for 2 min. Fractions were collected every 0.5 min. Elution was monitored by absorbance at 214, 230, and 254 nm using UV/Vis spectrometer (DU640, Beckman Coulter, Brea, CA).

2.6. Identification of pyroglutamyl peptides

Identification of pyroglutamyl peptides was performed by using methods described previously (Kiyono et al., 2013) with slight modifications. About 4 mL aliquots of the preparative RP-LC fraction, which attenuated DSS-induced colitis in mice, was dried under vacuum and dissolved in 400 μL of 0.1% (v/v) TFA containing 30% acetonitrile. This sample was clarified by passing through a spin column (15 × 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 solvent. Sample was eluted from the spin column by centrifugation at 7000 rpm for 1 min after which the spin column was washed with 50 μL of the same solvent, and the effluents were combined (450 μL of total volume). A 200-μL aliquot of the clarified sample was loaded onto a size exclusion chromatography (SEC) column (Superdex Peptide 10/30 GL, GE Healthcare), pre-equilibrated with 0.1% TFA containing 30% acetonitrile at a flow rate of 0.5 mL/min. Fractions (21–45 in number) were collected at 1 min interval for 20–45 min. This procedure was performed in duplicate.

The SEC fractions 31–45 above were dried under vacuum, dissolved in 200 μL of distilled water, and subjected to solid phase extraction using a strong cation exchange resin (AG50W-x8, hydrogen form, 100–200 mesh, Bio-Rad Laboratories, Hercules, CA) to separate the pyroglutamyl peptides from peptides bearing amino groups. The sample (200 μL) was loaded onto the spin column packed with the resin, which was pre-equilibrated with 10 mM HCl containing 50% (v/v) methanol, and eluted by centrifugation at 5000 × g for 1 min. The columns were washed twice with 100 μL of 50% methanol. Unabsorbed effluents were combined and dried under vacuum, and then dissolved in 200 μL of distilled water. Unabsorbed samples (80 μL) were injected to a reversed phase-high performance liquid chromatography (RP-HPLC) column (Inertsil BDS-5, 250 × 4.6 mm, 5 μm, GL Science, Tokyo, Japan) pre-equilibrated with 0.1% formic acid at a flow rate of 1.0 mL/min. Elution was
performed using a binary linear gradient consisting 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% B from 0–30 min; 30–100% B from 30–35 min; 100% B from 35–40 min; 100–0% B from 40–40.1 min; 0% B from 40.1–50 min. Column temperature was maintained at 45 °C. Elution was monitored at 214 nm and peaks were collected. Two sets of sample aliquots (100 µL) were transferred to 1.5-mL centrifuge tubes and dried under vacuum. One set was used for pyroglutamate aminopeptidase digestion and another set was used as blank. One hundred microlitres of 50 mM sodium phosphate buffer, pH 7.0, containing 10 mM dithiothreitol and 1 mM ethylenediaminetetraacetic acid, was added to the blank tubes. Eighty microlitres of the same buffer and 20 µL of pyroglutamate aminopeptidase solution (0.4 mU/20 µL of the same buffer) were added to the tubes used for the digestion. The enzymatic reaction was carried out at 60 °C for 1 h. The reaction was terminated by drying under vacuum. The amino groups liberated upon digestion from the pyroglutamyl peptide underwent a reaction with FITC and the resulting phenyl thiocarbamyl (PTC) derivatives were resolved by RP-HPLC as previously described (Kiyono et al., 2013). Peaks appearing exclusively in the enzyme digests were collected and dried under vacuum, following which a ‘re-drying solution’ (20 µL) consisting of methanol, water, and TEA (7:1:2) was added and the contents were re-dried under vacuum to remove ammonia. The residual contents were dissolved in 30% methanol and applied to an automatic peptide sequencer operating based on Edman degradation (PSSM-8, Shimadzu, Kyoto, Japan). The peptide sequencer was reprogrammed to begin with TFA cleavage reaction.

Aliquots of the RP-HPLC fractions without pyroglutamate aminopeptidase treatment were subjected to direct infusion electrospray ionization-tandem mass spectrometry (ESI-MS/MS) analysis using 3200 QTRAP System (AB SCIEX, Framingham, MA).

2.7. Synthesis of pyroglutamyl peptides

Pyroglutamyl-tyrosine (pyroGlu-Tyr), pyroglutamyl-asparaginyl-isoleucine (pyroGlu-Asn-Ile), and pyroglutamyl-asparaginyl-isoleucyl-asparagyl-asparagyl-proline (pyroGlu-Asn-Ile-Asp-Asn-Pro) were synthesized by Fmoc solid phase synthesis using an automatic peptide synthesizer (PSSM-8, Shimadzu) according to the supplier’s protocols. The synthesized pyroglutamyl peptides were cleaved from the resin using TFA and dried under vacuum. These peptides were then purified using RP-HPLC (Cosmosil 5C18-MS-II, 250 × 10 mm, 5 µm, Nacalai Tesque) using an acetonitrile/10 mM HCl solvent system.

2.8. Amino acid analysis

Peptide content was determined by amino acid analysis after HCl hydrolysis. For this purpose, samples were hydrolysed using 6 M HCl vapour at 150 °C as described previously (Bidlingmeyer, Cohen, & Tarvin, 1984). The amino acids were reacted with FITC and the resulting PTC amino acids were resolved using the same conditions used for isolation of PTC-peptides in pyroglutamate aminopeptidase digest as described previously (Kiyono et al., 2013).

2.9. Sugar analysis

Samples were diluted with distilled water to result in absorbance values within the standard curve range. Concentrated sulphuric acid (150 µL) and 10% (w/v) phenol (15 µL) were added to samples (30 µL) in 1.5 mL centrifuge tubes and incubated for

Fig. 1 – Fractionation of sake concentrate by ampholyte-free preparative isoelectric focusing (autofocusing). pH value (A), total peptides content (total constituent amino acids after HCl hydrolysis) (B), ratio of the basic, neutral, and acidic amino acids to the total peptide contents (C), and sugar content as glucose equivalent (D) of each autofocusing fraction are shown.
10 min. The tubes were then vortexed and incubated further for 30 min. Reaction mixtures were transferred to a 96 well plate and absorbance was monitored at 490 nm. The sugar contents of samples are presented as glucose equivalents.

2.10. Detection of pyroGlu-Leu

PyroGlu-Leu in preparative RP-LC fractions was detected by LC-MS/MS analysis as described previously (Wada et al., 2013).

2.11. Microbiota analysis

DNA was extracted from mice faeces by using a QIAamp DNA Stool Mini Kit (Qiagen, Venlo, Netherlands). Analysis of faecal bacterial flora in the extracted DNA was outsourced to Primary Cell, Division of Cosmo Bio (Sapporo, Japan). The population of phyla Bacteroidetes and Firmicutes in mice faeces were evaluated as the ratio of each phylum to all bacteria by genomic DNA coding 16S rRNA. The DNA amplification was carried out by real-time polymerase chain reaction (RT-PCR). HDA1-GC (5′CGCCCGGGCGCGCCGGCGGGCGGGCGGGCGGGCACGGGGGGACCTCTACGGGAGGCAGCAGT3′) and HDA2 (5′GTATTACCGCGGCTGCTGGCAC3′) were used for detection of all bacteria (Tannock et al., 2000). Bact934F (5′GGARCATGTGGTTTAATTCGATGAT3′) and Bact1060R (5′AGCTGACGACAACCATGCAC3′) were used for detection of Bacteroidetes. Firm934F (5′GGAGYATGTGGTTTAACTCGAAGCA3′) and Firm1060R (5′AGCTGACGACAACCATGCAC3′) were used for detection of Firmicutes (Guo et al., 2008). Real-time PCR was performed using a LightCycler 480 (Roche Applied Science, Mannheim, Germany) according to the SYBR Green I Master Protocol.

2.12. Statistical analysis

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

3. Results

3.1. Fractionation by autofocusing

The pH of the ten fractions (Fr.) produced by autofocusing of the sake sample was about 3.0 between Fr. 1 and 8 then rose sharply in Fr. 9 and 10 to pH 9.0 and 13.0, respectively (Fig. 1A). Approximately 80% of total peptides were recovered in Fr. 8 and 9 (Fig. 1B). Only negligible amounts of peptides were recovered in Fr. 1 and 2. Peptides present in Fr. 3–8 consisted of higher levels of acidic amino acids (such as glutamic acid) after HCl hydrolysis, and peptides in Fr 10 consisted of higher levels of basic amino acids (such as arginine and lysine) compared with the other fractions (Fig. 1C). This charge-based separation of amino acids indicated successful fractionation of peptides contained in sake based on their isoelectric points. A relatively high sugar content (~24%) was recovered in the acidic fractions, particularly Fr. 6 and 7 (Fig. 1D). All autofocusing fractions were mixed with ethanol at end concentrations of 75% (v/v) in order to remove agarose, which was derived from the separator of autofocusing. Based on the results of autofocusing, Fr. 1–5 and 6–7 obtained after autofocusing were pooled into two different fractions and used further for animal experiments. Fr. 8, 9, and 10 were used for animal experiments without pooling. The samples were further concentrated by a rotary evaporator to remove ethanol and then diluted with distilled water to give 3.0 mg/mL peptide (total amino acids) and administered to the mice with DSS-induced colitis at 30 mg/kg body weight a day.

The induction of colitis was confirmed by a significant increase in DAI score observed after treatment with DSS (DSS +), as compared to the mice that did not receive DSS (DSS −) (Fig. 2A). Only the mice receiving autofocusing Fr. 8 showed a

![Fig. 2 – Effect of administrating of autofocusing fractions of sake on DAI scores (A), body weight change (B), and colon length (C) in mice with DSS-induced colitis. Values are presented as mean ± standard error. DSS − and DSS + represent the mice, which were allowed free access to DSS free drinking water and water containing DSS, respectively. Sample doses were adjusted to 30 mg/kg body weight for peptide (total amino acids)/content. ** represents p < 0.01, when compared to the vehicle group (DSS +) by Dunnett’s test.](image-url)
significant decrease in DAI score compared to the vehicle group (DSS +). However, the crude sake concentrate, before undergoing autofocusing, exerted no significant decrease in the DAI score. The mice receiving Fr. 6–7, 8, and 9 significantly attenuate body weight loss (Fig. 2B), and there was no significant difference in colon length among all groups (Fig. 2C). Based on the above data, Fr. 8 was selected for a further fractionation treatment to identify active compounds.

3.2. Fractionation by RP-LC

Fraction 8 (3.0 mg/mL peptide) was subjected to further fractionation by RP-LC. Following the elution of non-absorbed compounds from the RP-LC column, the absorbed compounds were eluted by increasing acetonitrile concentrations (Fig. 3A). Free amino acids and sugars were mostly eluted in the non-absorbed fraction (Fig. 3A and B). The peptide content could be estimated by subtracting free amino acids from total amino acids in HCl hydrolysate. Peptides were eluted in both the non-absorbed and the absorbed fractions. A strong UV absorbance peak was observed at 20–21 min (Fig. 3C). Thus, four fractions were collected and labelled as follows: Fr. I (0–14 min), Fr. II (14–17.5 min), Fr. III (17.5–20 min) and Fr. IV (20–31 min). These fractions were freeze-dried to remove acetonitrile and dissolved into 80 mL of distilled water, which were further used for animal experiments.

Although statically insignificant (p > 0.05), the smallest DAI score was observed in RP-LC Fr. III group (p = 0.08) among the mice receiving Fr. I–IV compared to the vehicle group (DSS +) (Fig. 4A). In addition, administration of Fr. III significantly averted body weight loss compared to the vehicle group (DSS +) (Fig. 4C), and there was no significant difference in colon length among all groups (Fig. 4B). Based on these results, Fr. III was used for further experiments.

3.3. Identification of pyroglutamyl peptides with protective effect against DSS-induced colitis in RP-LC Fr. III

Peptides contained in Fr. III were initially resolved by SEC. Approximately 75% of the total peptides were eluted between 30 and 36 min corresponding to Fr. 31–36 (data not shown). Pyroglutamyl peptides in Fr. 31–36 were separated from peptides bearing amino groups by solid phase extraction and then resolved by RP-HPLC. The major peaks obtained after RP-HPLC, marked as a, b, and c, were collected (Fig. 5A) and aliquots of the collected fractions were digested with pyroglutamyl aminopeptidase. The digested and nondigested samples were allowed to react with PITC and the resulting PTC derivatives were resolved by a second RP-HPLC separation. Peaks a′, b′, and c′ appeared only in case of enzyme digests (Fig. 5B). These PTC derivatives were identified by Edman degradation as follows; peak a′, tyrosine; peak b′, asparaginyl-isoleucine and peak c′, asparaginyl-isoleucyl-asparagyl-asparaginyl-proline. This fact indicated that peaks a, b, and c in Fig. 5A consisted of pyroglutamyl-tyrosine (pyroGlu-Tyr), pyroglutamyl-asparaginyl-isoleucine (pyroGlu-Asn-Ile), and pyroglutamyl-asparaginyl-isoleucyl-asparagyl-asparaginyl-proline (pyroGlu-Asn-Ile-Asp-Asn-Pro), respectively. ESI-MS and MS/MS analyses of peaks a–c also confirmed the presence of pyroGlu-Tyr, pyroGlu-Asn-Ile, and pyroGlu-Asn-Ile-Asp-Asn-Pro, respectively (Supplemental Figs. S1–S3).

Three identified pyroglutamyl peptides were chemically synthesized and administered to the mice with DSS-induced colitis. Although statistically insignificant (p > 0.05), the administration of these pyroglutamyl peptides showed a tendency towards
reduced body weight loss compared to the vehicle group (DSS +) (Fig. 6A). The pyroGlu-Asn-Ile group (1.0 mg/kg body weight) showed the smallest body weight loss. Administration of pyroGlu-Tyr and pyroGlu-Asn-Ile, at 1.0 mg/kg body weight, significantly reduced colon shortening (p < 0.05 and p < 0.01, respectively) (Fig. 6B). Additionally, pyroGlu-Asn-Ile significantly decreased DAI score compared to the vehicle group (DSS +) in a dose dependent manner (Fig. 6C). However, pyroGlu-Asn-Ile-Asp-Asn-Pro did not significantly attenuate colon shortening and DAI score increasing of DSS-induced colitis mice.

### 3.4. Effects of pyroglutamyl peptides on colonic microbiota

Without administration of pyroglutamyl peptides, DSS treatment resulted in an increase in the population of colonic Firmicutes and decrease in that of Bacteroidetes. Thus, a significantly increased ratio between the colonic populations of Firmicutes to Bacteroidetes by DSS treatment was observed (Fig. 6D). The ratio significantly decreased similar to the level of the normal mice (DSS −) upon administration of pyroGlu-Asn-Ile. On the other hand, pyroGlu-Tyr did not significantly affect the ratio of Firmicutes to Bacteroidetes.

### 4. Discussion

It is known that some crude plant extracts have been demonstrated to have anti-colitic activity in animal models (Cazarin et al., 2015; Jia et al., 2014; Zielinska et al., 2015; Zorrilla et al., 2014). Some compounds with demonstrated in vitro antioxidant, anti-inflammatory and other similar beneficial activities have been selected as candidate anti-colitic compounds. However, relatively large dose of these compounds is necessary for improving colitis in animal model possibly due to low bioavailability and rapid metabolism of these compounds (Larrosa et al., 2009; Shigeshiro, Tanabe, & Suzuki, 2013; Sun et al., 2015).
To address this problem, we used in vivo activity-guided fractionation rather than in vitro activity-guided fractionation for identification of anti-colitic peptides in sake. The present in vivo activity-guided fractionation revealed that pyroGlu-Tyr and pyroGlu-Asn-Ile contained in sake attenuated DSS-induced colitis in mice upon oral administration in relatively low concentrations (1.0 mg/kg body weight for pyroGlu-Tyr, and 0.1 and 1.0 mg/kg body weight for pyroGlu-Asn-Ile). This protective effect exerted by the pyroglutamyl peptides was better at lower concentrations when compared to the dose dependence displayed by other natural compounds reported previously. Therefore, these peptides and food containing these peptides have important clinical relevance to IBD patients. In addition, identification of active peptides allows to explore underlying mechanism by using in vitro assay system.

A previous study reported that pyroGlu-Leu, the major pyroglutamyl peptide in sake (12–15 mg/L), (Kiyono et al., 2013) can attenuate DSS-induced colitis upon administration at 0.1 mg/kg body weight, while doses higher than 0.5 mg/kg body weight and lower than 0.01 mg/kg body weight do not exert any protective effects in the same animal model (Wada et al., 2013). This study confirms the previous finding related to changes in colonic microbiota in mice with DSS-induced colitis. Additionally, this study revealed that administration of pyroGlu-Asn-Ile also normalized the colonic dysbiosis in DSS-induced colitis mice, while administration of pyroGlu-Tyr did not affect the colonic microbiota. In contrast, colonic myeloperoxidase activity was decreased by administration of pyroGlu-Tyr compared to that of the vehicle group (DSS +), indicating that colonic inflammation was suppressed by administration of pyroGlu-Tyr (Supplemental Fig. S4). These results indicate that attenuation of colitis does not always result in normalization of colonic microbiota. However, it has been indicated that intestinal dysbiosis plays a significant role in inflammatory bowel disease (Sartor & Mazmanian, 2012). Therefore, normalization of colonic dysbiosis by administration of pyroGlu-Asn-Ile can potentially attenuate colitis.

In addition to pyroGlu-Leu, pyroGlu-Tyr and pyroGlu-Asn-Ile have potential anti-inflammatory activity (data not shown), however, the target cells responding to each pyroglutamyl peptide remain to be solved. In order to identify the target cells of these peptides in the body and to understand the underlying mechanism, contents of inflammatory and anti-microbial peptides responding to supplementation of each peptide should be examined. As mammalian antimicrobial peptides are activated by post-translational modifications (Balducci, Bonucci, Picchianti, Pogni, & Talluri, 2011), a study on the effects of pyroGlu-Tyr, pyroGlu-Asn-Ile, and also pyroGlu-Leu on the proteome of the small intestine and colon of the animals with colitis is under progress.
The present study clearly demonstrates that sake contains at least three pyroglutamyl peptides (pyroGlu-Leu, pyroGly-Tyr, and pyroGlu-Asn-Ile), which can attenuate DSS-induced colitis in mice at doses of 0.1–1.0 mg/kg body weight. However, crude sake did not show anti-colitic activity, rather it has been demonstrated that some organic acids such as lactic acid and succinic acid, which are abundantly present in sake (Tadenuma, 1966), may induce diarrhoea and worsen the symptom of colitis (Tsukahara & Ushida, 2002). Thus sake contains both components with beneficial and adverse effects on colitis and optimizing the contents of these compounds could produce functional beverages against colitis. Currently, studies on producing anti-colitic beverages by optimizing fermentation conditions for sake are in progress.

Acknowledgements

We would like to thank the Kyoto Integrated Science & Technology Bio-Analysis Center for the use of their LC-MS/MS. We also thank Dr. Wataru Aoi, Eun Young Park, and Yasushi Nakamura of Graduate School of Life and Environmental Sciences, Kyoto Prefectural University for their kind assistance. This work was supported by a Japan Society for the Promotion of Science Grant-in-Aid for Scientific Research (C) to S.W. (No. 25450175) and Japan Science and Technology Agency for the Program to support research activities of female researchers to S.W. and grant for Integration Research for Agriculture and Interdisciplinary Fields to K.S.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.jff.2016.10.014.

References


