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

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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.

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

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effects (Bernstein, 2015). Several efforts have been focused towards the discovery of natural compounds from foods that display protection against IBD. Some plant extracts rich in phytochemicals (Cazarin et al., 2015; Jia et al., 2014; Zielinska et al., 2015; Zorrilla et al., 2014) and some proteins (Kobayashi et al., 2015; Lee et al., 2009a, 2009b) have been demonstrated to moderate IBD 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 consequently 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 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. L-pyroglutamic acid was purchased from Nacalai Tesque (Kyoto, Japan). 9-Fluorenylmethoxycarbonyl (Fmoc)-Tyr(tBu)-Wang resin, Fmoc-Ile-Wang resin, H-Pro-2-chlorotritylchloride 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