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Ingestion of Low Dose Pyroglutamyl Leucine Improves Dextran Sulfate Sodium-Induced Colitis and Intestinal Microbiota in Mice

Sayori Wada,[†] Kenji Sato,[†,*] Ryoko Ohta,[†] Eri Wada,[†] Yukiho Bou,[†] Miki Fujiwara,[†] Tamami Kiyono,[†] Eun Young Park,[†] Wataru Aoi,[†] Tomohisa Takagi,[‡] Yuji Naito,[‡] and Toshikazu Yoshikawa[‡]

[†]Division of Applied Life Sciences, Graduate School of Life and Environmental Sciences, Kyoto Prefectural University, Shimogamo, Kyoto, 606 8522, Japan

[‡]Molecular Gastroenterology and Hepatology, Kyoto Prefectural University of Medicine, Kajii-cho, Kyoto, 602 8566, Japan

ABSTRACT: Inflammatory bowel diseases (IBD) are based on chronic inflammation in the gastrointestinal tract. We previously found anti-inflammatory peptide pyroGlu-Leu in the enzymatic hydrolysate of wheat gluten. The objective of present study is to elucidate improvement of colitis by oral administration of pyroGlu-Leu in an animal model. Acute colitis was induced by dextran sulfate sodium (DSS), and various concentrations of pyroGlu-Leu were administrated by oral gavage for 7 days. A dose of 0.1 mg/kg body weight/day showed the most significant improvement. The pyroGlu-Leu concentration was significantly increased 24 h after oral administration both in the small intestine and the colon compared with the baseline. It was 20-fold higher in the small intestine than the colon. Administration of pyroGlu-Leu normalized population of *Bacteroidetes* and *Firmicutes* in the colon. These results indicate that pyroGlu-Leu has a potential therapeutic effect against IBD at a practical dose.

KEYWORDS: *IBD, pyroGlu-Leu, gluten peptide, microbiota, colitis*

INTRODUCTION

Inflammatory bowel diseases (IBD) are relapsing-emitting conditions characterized by chronic inflammation in the gastrointestinal tract, which result in diarrhea and abdominal pain. The major types of IBD are Crohn's disease (CD) and ulcerative colitis (UC). UC is limited to the colon. On the other hand, CD can occur in any parts of the gastrointestinal tract.[1,2] IBD affects people of all ages, but usually begins before age 30.[3] In the U.S., the mean annual costs for CD and UC are estimated as $8,265 and $5,066 per patient, respectively.[4]

It has been suggested that early appendectomy[5] reduces the risk of IBD, and the use of some nonsteroidal anti-inflammatory drugs (NSAIDs)[6] increases the risk. A smoking habit also increases the risk of CD, but has a protective effect on the development of UC.[7−9] The incidence of IBD in Asia is far less than in Western Europe and North America.[10] However, the incidence and prevalence of IBD in Asia has been increasing in the last two decades.[11,12] In the same period, eating habits in Asia have changed. For example, the consumption of traditional fermented food, such as soy sauce and soy paste, have decreased in Japan.[13] These facts suggest that in addition to genetic and environmental factors, life-style might affect the development of IBD. In fact, it has been reported that a high consumption of sweets increases the risk of both UC and CD.[14] On the other hand, increasing numbers of studies have suggested that some nutrients or food components exert beneficial effects on IBD beyond their conventional nutritional values. Epidemiological studies have demonstrated that the consumption of foods rich in vitamin C decreases the risk of IBD.[14] It has been also demonstrated that the supplementation of some amino acids, such as Gln, Cys, Ser, Thr, and Pro attenuate IBD in animal models.[15,16] In addition, the supplementation of cheese whey protein,[16] casein macropeptide,[17] lysozyme,[18] and soy protein hydrolysate[19] also attenuate IBD in animal models. These data indicate that specific food proteins or peptides have a potential therapeutic effect against IBD, possibly due to the provision of protective intestinal amino acids or the direct modulation of pro-inflammatory cytokines. However, a relatively large dose of these protein/peptides is required to exert any beneficial activity against IBD in animal models. In addition, some human trials revealed that a Gln-enriched polymeric diet offers no advantage over a standard low-Gln diet in the treatment of active CD.[20]

Alternatively, the present authors reported that the ingestion of an enzymatic hydrolysate of wheat gluten can attenuate chronic and acute hepatitis in rat models[21,22] and also that it can decrease serum amino transferase activity in patients suffering from hepatitis.[23] Very recently, a peptide, pyroglutamyl leucine (pyroGlu-Leu), was identified as one of the hepatoprotective peptides in the wheat gluten hydrolysate.[23] Pyroglutamyl peptides are induced from peptides with glutaminyl residue at the amino terminal position in the water phase during food processing and are resist to digestion by amino peptidase, due to the lack of an amino-group.[24] As well as IBD, inflammation plays a significant role in the development of hepatitis. Therefore, we hypothesized that pyroGlu-Leu might have a positive effect on colitis.

The objective of the present study was to elucidate the potential of pyroGlu-Leu to control IBD by using dextran sulfate sodium-induced colitis in mice.

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MATERIALS AND METHODS

Reagents. Dextran sulfate sodium (DSS, average mol. wt. 8000) was purchased from Seikagaku (Tokyo, Japan). Pyroglutamic acid (pyroGlu) and acetonitorile (HPLC grade) were obtained from Wako Pure Chemicals (Osaka, Japan). The resin, which was linked to 9-fluorenylmethoxycarbonyl (FMOC)-leucine, was obtained from HiPep Laboratories (Kyoto, Japan).

Preparation of PyroGlu-Leu. PyroGlu-Leu was synthesized by a manual liquid-phase method as described previously.[22] In some cases, it was also synthesized by an automatic solid-phase method employing a 9-fluorenylmethoxycarbonyl (FMOC) strategy using a PSSM-8 peptide synthesizer (Shimadzu, Kyoto, Japan). Free pyroGlu was reacted to FMOC-Leu-resin.

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 food and drinking water, and fed a certified CRF-1 diet (Oriental Yeast, Kyoto, Japan) during a one-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 oral administration of 2.5 or 3.0% (w/v) DSS dissolved into drinking water, for 7 days according to the method employed in previous studies.[25,26] The concentration of DSS (2.5% or 3.0%) was determined by preliminary experiments for the induction of the same grade of inflammation in each individual experiment, as the severity of DSS-induced colitis in mice depended on the animal lots. The mice were divided into eight groups: a sham group (without DSS, no pyroGlu-Leu; sham 0), a sham group treated with 0.1 mg/kg pyroGlu-Leu (without DSS; sham 0.1), a DSS-induced colitis group as a control, and a DSS-induced colitis group treated with 0.01, 0.05, 0.1, 0.5, and 1.0 mg/kg pyroGlu-Leu (Table 1). Synthetic pyroGlu-Leu dissolved in PBS was administered to the mice by oral gavage during the entire colitis induction period. For the DSS-induced colitis groups, same experiments were carried out 2−6 times.

Table 1. Treated Condition of DSS and Pyroglu-Leu in Each Group[a]

group	DSS	pyroGlu-Leu (mg/kg/day)	*n*
sham 0	-	0	17
sham 0.1	-	0.1	6
0	+	0	43
0.01	+	0.01	13
0.05	+	0.05	19
0.1	+	0.1	44
0.5	+	0.5	32
1.0	+	1.0	18

[a]DSS: dextran sulfate sodium.

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. The inner contents of the colon from three mice in each group were collected by washing with 500 μL of ice-cold PBS per mice, and three samples in the same group were mixed together for microbiota analysis. Then, colon tissue samples were fixed in 10% buffered formalin for histological examination.

Evaluation of Colitis Severity. We evaluated the colitis severity on the basis of body weight, colon length, and the macroscopic and microscopic observations of stool and colon, respectively. The disease activity index (DAI) score was determined by the method employed in previous studies,[26,27] 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. Colon sections were prepared and stained with hematoxylin and eosin (H&E).

Determination of PyroGlu-Leu. For detemination of pyroGlu-Leu, small intestine and colon were collected day 7 from the normal mice and DSS-induced colitis mice that received vehicle or 0.1 mg/kg pyroGlu-Leu (n = 3 for each group). To examine time course of absorption of pyroGlu-Leu, the normal mice were administered with 0.1 mg/kg of synthetic pyroGlu-Leu dissolved in PBS by oral gavage after one night fasting, and then sacrificed before and 0.5, 1, 3, 6, and 24 h after the administration, respectively (n=3 except baseline which were n = 4). The small intestine and colon were thoroughly washed 3−5 times by PBS, and added to the same amounts of PBS and homogenized. Then, a 2-fold amount of 10% TCA was added, and the samples were rehomogenized, followed by centrifugation at 13 000 rpm. The supernatants were collected. PyroGlu-Leu in the TCA extract was separated from the amino acids and other peptides having an amino group by solid phase extraction using a strong cation exchanger (AG50W- × 8, hydrogen form, 100−200 mesh, Bio-Rad Laboratories, Hercules, CA).[22] The nonabsorbed fraction was collected. Before injection to LC-MS/MS, samples were clarified by passing through a filter (0.45 μm, column guard, Millipor, Billerica, MA). PyroGlu-Leu was determined by LC-MS/MS in multiple reaction monitoring (MRM) mode using the Q-TRAP 3200 (AB SCIEX, Foster City, CA) as described previously.[22]

Microbiota Analysis. The inner contents of the colon were collected from three mice in each group and then it was mixed together for microbiota analysis. Population of *Bacteroiditis* and *Firmicutes* were quantified on the basis of amplification of genome DNA coding 16S rRNA by polymerase chain reaction by using group-specific primers.[28−30] 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. DNA extraction and PCR analysis were carried out in Primary Cell Division of Cosmo Bio (Sapporo, Japan).

Statistical Analysis. After assignment to the eight groups, the homogeneity of variances and the mean of the body weight were confirmed by Bartlett's test and one-way ANOVA, respectively. Results were presented as mean ± SEM. For body weight change and DAI score, we compared the values between groups using Kruskal−Wallis analysis. Statistical significance (P < 0.05) was analyzed by one-way analysis of variance followed by Steel's test for multiple comparisons. For the concentration of pyroGlu-Leu, data were subjected to one-way ANOVA with Dunnett's multiple comparison of means test. Differences showing P < 0.05 were considered significant. Statistical analyses were performed using Excel 2010 (Microsoft, Redmond, WA) with the add-in software Ekuseru-Toukei 2010 (Social Survey Research Information, Tokyo Japan).

RESULTS

Improvement of DSS-Induced Colitis by PyroGlu-Leu. There were no significant differences in the consumption of the water containing DSS among the groups. DSS treatment induced significant weight loss, shortened colon length, diarrhea, and rectal bleeding, and an increase in the DAI score, compared with the non-DSS treatment group (Figure 1A, B). As shown in Figure 2, DSS treatment induced extensive infiltration of inflammatory cells reaching the muscularis mucosa and thickening of the mucosa with edema. Severe

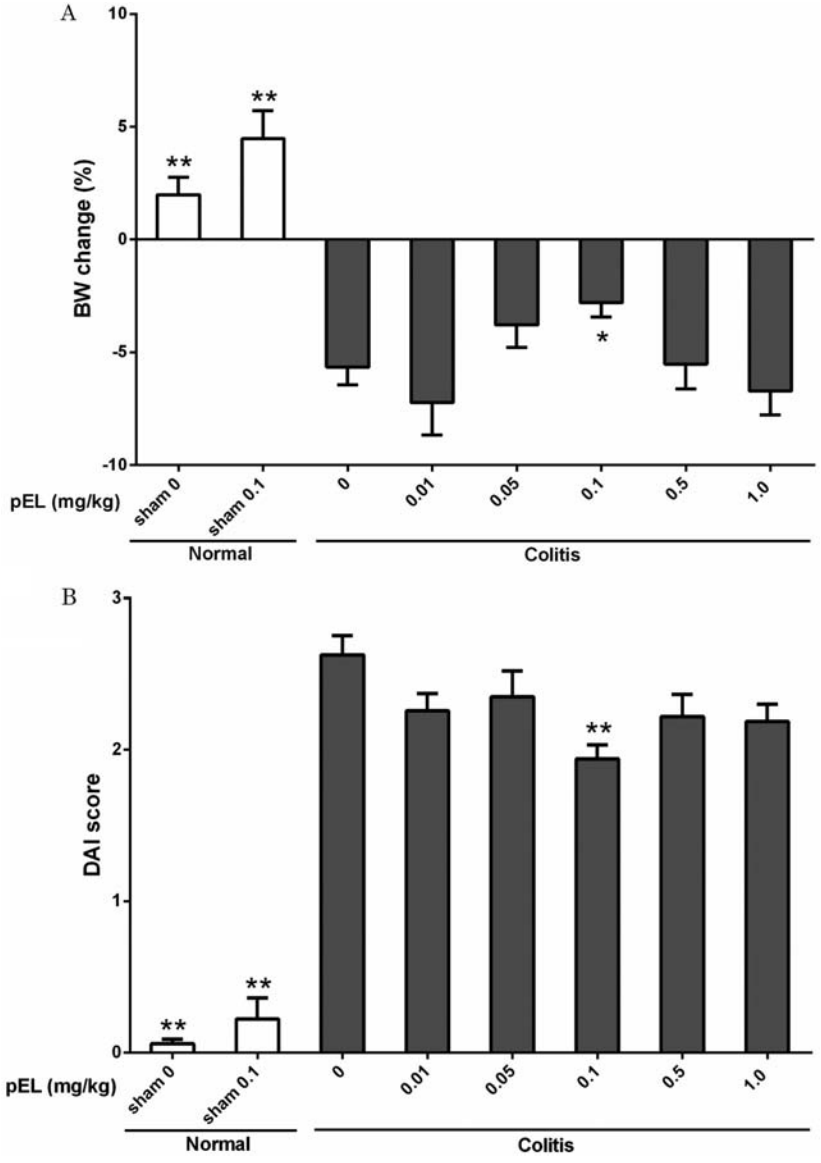

Figure 1. Effects of pyroGlu-Leu (pEL) on body weight change (A) and disease activity index (DAI) score (B) of mice with dextran sulfate sodium (DSS)-induced colitis. Values are presented as mean ± SEM. Open squares indicate the noncolitis inducing groups, and the solid squares indicate the groups induced colitis by DSS. The numbers 0, 0.01, 0.05, 0.1, 0.5, and 1.0 represent the dose of pyroGlu-Leu (mg/kg/day), respectively. Sham: no DSS treatment. The number of experimental animals in each group is listed in Table 1. * and ** represent $P < 0.05$, $P < 0.01$ when compared with control mice receiving DSS solution alone, respectively.

inflammation was then induced by the DSS treatment. The supplementation of pyroGlu-Leu at a dose of 0.1 mg/kg body weight significantly attenuated the DSS-induced weight loss and improved the DAI score. In addition, as shown in Figure 2C, the supplementation of pyroGlu-Leu in this dose improved colonic inflammation. On the other hand, pyroGlu-Leu treatment at higher doses, such as 0.5 mg/kg or 1.0 mg/kg did not show the positive effects on the DSS-induced colitis. The administration of pyroGlu-Leu to normal mice (sham 0.1) did not show any significant effect on the parameters examined in the present study, compared with sham 0 mice (Figure 1B).

PyroGlu-Leu in Intestines. The time course of absorption of pyroGlu-Leu into the intestines was first examined in the normal mice. As shown in Figure 3A, pyroGlu-Leu was detected in the TCA extract of the washed small intestine even before the administration. In all cases, intestines were washed thoroughly with PBS. The final washing effluents contained pyroGlu-Leu less than 10% of that in the TCA extracts of the washed organs (data not shown), which indicates most of free pyroGlu-Leu in the intestinal tract could be washed away. Therefore, the pyroGlu-Leu in the TCA extract can be considered as pyroGlu-Leu in the cell and matrix of intestines. The contents of pyroGlu-Leu in small intestine tended to increase 30 and 60 min after the administration of pyroGlu-Leu at 0.1 mg/kg body weight, but no significant differences were shown. With administration at a higher dose (10 mg/kg body weight), pyroGlu-Leu content in the small intestinal tissue significantly increased at 30 and 60 min, compared to the baseline level (data not shown). Unexpectedly, 24 h after the administration at a dose of 0.1 mg/kg body weight, a significantly higher level of pyroGlu-Leu (2.416 ± 0.143 nmol/g) was observed in the small intestine, compared to the baseline (Figure 3B). The concentrations of pyroGlu-Leu in

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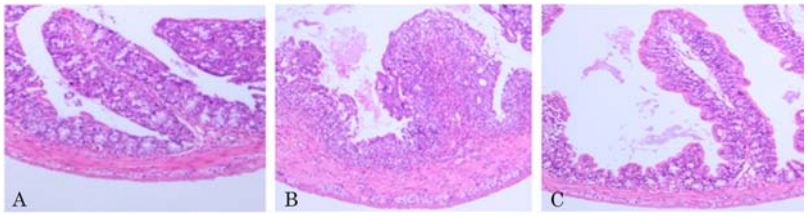

Figure 2. The histological appearance of colonic tissue in sham mice without DSS (A), mice with DSS-induced colitis (B), and mice with DSS-induced colitis treated with 0.1 mg/kg pyroGlu-Leu. Hematoxylin and eosin staining. Magnification, ×400. In B, extensive infiltration reaching the muscularis mucosa and thickening of the mucosa with abundant edema was observed, whereas improvement was shown in C.

colon were approximately 5% of those shown in the small intestinal tissue.

As shown in Figure 3C, similar results were obtained after the 7 days feeding experiment using normal and DSS-induced colitis mice. PyroGlu-Leu was also detected in the intestines from the normal and DSS-induced colitis mice that received vehicle. The content of pyroGlu-Leu in small intestine tended to increase by the administration of pyroGlu-Leu at 0.1 mg/kg body weight for 7 days, whereas the small intestine was collected 24 h after the final administration of pyroGlu-Leu. The contents in the colon were considerably lower than those in the small intestine even after 7 days administration. Administration of pyroGlu-Leu did not affect on the contents in the colon.

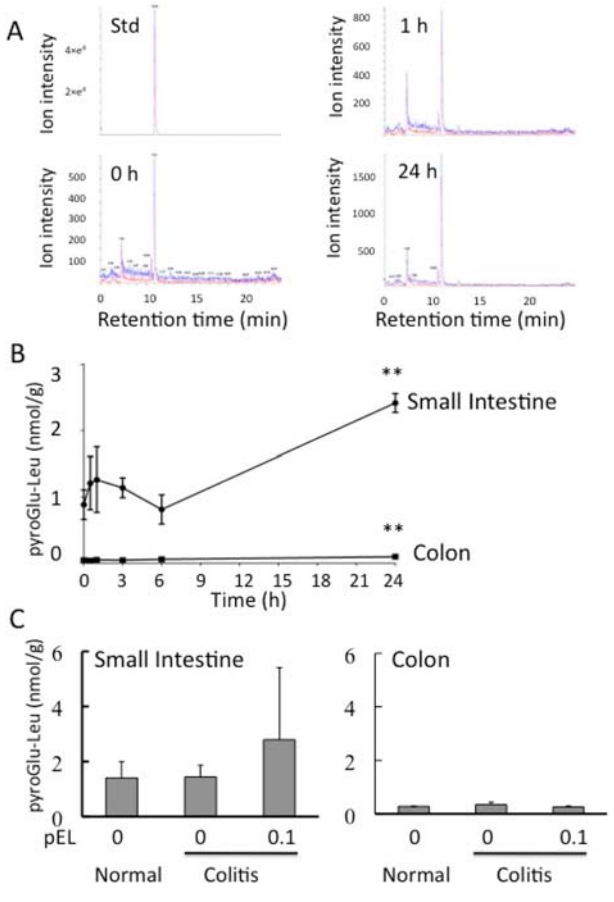

Figure 3. Representative MRM chromatograms of pyroGlu-Leu (m/z 243.1 > 86.1 and m/z 243.1 > 84.0) (A) and pyroGlu-Leu contents in small intestine and colon (B, C). A: Std: authentic pyroGlu-Leu (1 μM), TCA extract of the washed intestine before (0), 1, 24 h after the ingestion of pyroGlu-Leu (0.1 mg/kg body weight). B: Time course of contents of pyroGlu-Leu in the small intestine and colon after oral administration of 0.1 mg/kg body weight of pyroGlu-Leu. Values are presented as mean ± SEM (n = 3 except baseline which were n = 4). ** represents $P < 0.01$ when compared with the baseline. C: Contents of pyroGlu-Leu in the small intestine and colon after the 7 days feeding experiment (n = 3 for each group). Normal and colitis mice received vehicle (0) or pyroGlu-Leu at 0.1 mg/kg/day for 7 days.

Effects of PyroGlu-Leu on Microbiota. In normal colon (sham 0 group), the population of *Bacteroidetes* was much higher than that of *Firmicutes*, whereas the population of *Firmicutes* increased in mice with colitis. Administration of pyroGlu-Leu increased and decreased the *Bacteroidetes* and *Firmicutes*, respectively, in a dose dependent manner up to 0.1 mg/kg. Thereafter, these changes reached a plateau (Figure 4).

DISCUSSION

PyroGlu-Leu was first identified in wheat gluten hydrolysate and it was noted that it attenuated D-galactosamine-induced hepatitis at 20 mg/kg body weight.[22] The present study demonstrated that pyroGlu-Leu significantly improved body weight loss and the DAI score of mice with DSS-induced colitis. The effects of pyroGlu-Leu showed a reverse J-curve in body weight change and a J-curve in the DAI score. These results indicated that 0.1 mg/kg is the optimum dose for the moderation of colitis in the present animal model. Improvement of colitis by oral administration of pyroGlu-Leu in this dose was not so high (less than 1 DAI score). However, decrease of 1 DAI score implies significant improvement of quality of life (see criteria of DAI score in the Materials and Methods section). For human application, the dose of pyroGlu-Leu must be carefully considered, since a higher dose not show improving effects in the present animal model. Ingestion of high dose (10 mg/kg body weight) of pyroGlu-Leu tentatively increased its content in the small intestine to approximately 40 nmol/g of wet tissue 30 and 60 min after the ingestion (data not shown). On the other hand, ingestion of low dose (0.1 mg/kg) increased to approximately 2.5 nmol/g unexpectedly 24 h after ingestion by single and daily administrations (Figure 3B and C). Tentative large increase of pyroGlu-Leu by the high dose might be involved in adverse effect. Mechanism for increase of pyroGlu-Leu in small intestinal tissues 24 h after the ingestion of the small dose is unclear. To explain these phenomena, we propose two possibilities. First, pyroGlu-Leu might be absorbed into blood and then accumulated in small intestine by 24 h. However, daily administration of pyroGlu-Leu did not increase small intestine level. Then it is unlikely that food-derived pyroGlu-Leu is specifically accumulated in small intestine. Second, the food-derived pyroGlu-Leu might induce endogenous pyroGlu-Leu in small intestine. PyroGlu-Leu can be produced from parent specific peptides and proteins by protease digestion. In this case, endogenous pyroGlu-Leu

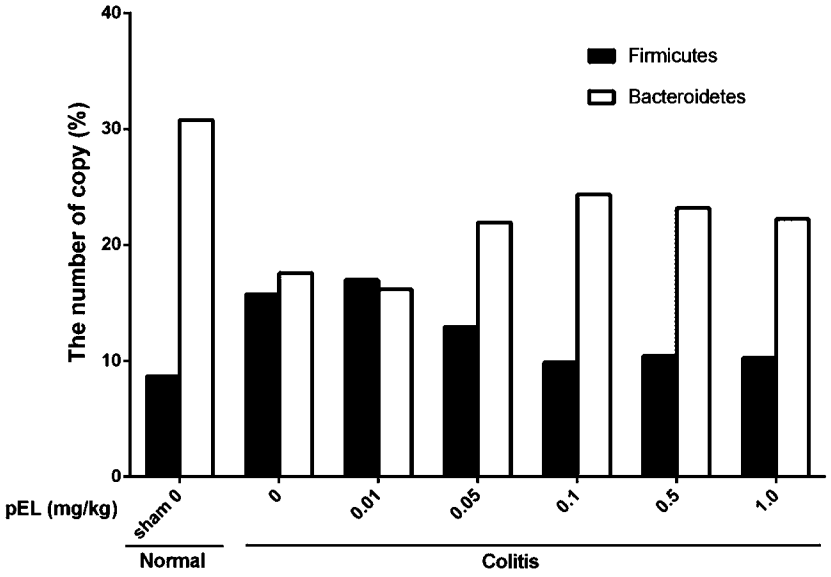

Figure 4. Population of *Firmicutes* and *Bacteroidetes* in the inner content of the colon in each group. The inner contents of the colon were collected from three mice in each group and mixed them together for microbiota analysis. Closed squares indicate *Firmicutes* and open squares indicate *Bacteroidetes*. pEL reperesnts dose of pyroGlu-Leu.

might be involved in communication between cells. Occurrence of pyroGlu-Leu in the mice small intestine before the administration and difference in its level between organs support this speculation. To address these problems, comprehensive metabolome analysis on intestines and other organs using stable isotope-labeled pyroGlu-Leu is necessary.

While the mechanism of IBD remains to be solved, IBD could be induced by a combination of (1) persistent specific infection, (2) dysbiosis (abnormal ratio of beneficial and detrimental commensal microbial agents), (3) defective mucosal barrier function, (4) defective microbial clearance, or (5) aberrant immunoregulation.[31] In the present animal model, severe inflammation of the colon and colonal microbiota change occurred. The administration of pyroGlu-Leu moderated these pathological changes. However, the pyroGlu-Leu level in colonic tissue was low level even by daily administration for 7 days, which was less than values in small intestinal tissue before administration. Then, it is unlikely that the ingested pyroGlu-Leu directly suppresses inflammation in colon. Several studies have suggested that the *Frimicutes/Bacteroidetes* ratio change by progress of CD.[32−34] In the present study, supplementation of pyroGlu-Leu normalized colonic microbiota. Ratio of *Bacteroidetes* to *Firmicutes* in the inner content in colon was highest by the supplementation of pyroGlu-Leu at 0.1 mg/kg body weight/day in the DSS-induced mice, which was closest to that in the normal mice (sham 0). Then it could be speculated that normalizing microbiota might suppress inflammation of colonic cells. PyroGlu-Leu level in the inter content of colon was also low; less than 5 nmol/g of dry matter even after ingestion of higher dose of pyroGlu-Leu (10 mg/kg body weight, data not shown). It is, therefore, also unlikely such low dose of peptide can directly affect growth of bacteria. As the concentrations of pyroGlu-Leu, in the small intestinal tissue were much higher than those shown in the colon. Therefore, we hypothesized that the pyroGlu-Leu might act on small intestinal cells to induce some biologically active compounds, which might modulate colonic microbiota. In fact, it has been demonstrated that intestinal cells produce bactericidal peptides including defensins[35,36] and/or mucin,[16,37] which can modulate colonic microbiota. To confirm the hypothesis, further study on effect of pyroGlu-Leu on production of bioactive compounds including defensin by small intestinal cells are now under progress.

It has been demonstrated that some modified peptides derived from bioactive proteins, such as acetyl-Gln-Ala-Trp from annexin A1 and Lys-D-Pro-Thr from melanocortin-related peptide, improved DSS-induced colitis by oral administration at 5 mg/kg body weight.[38,39] However, these peptides cannot be derived from food protein. For food-derived peptides, Val-Pro-Tyr found in soy protein hydrolysate improved DSS-induced colitis by oral administration at 100 mg/kg/day.[19] Compared to these peptides, pyroGlu-Leu attenuates colitis at an extremely low dose. This is the first report demonstrating that oral ingestion of food-derived short chain peptide can moderate colitis in such low dose. Therefore, pyroGlu-Leu is a potential therapeutic agent against IBD, whereas its mechanism in action is not clear, which might be different from conventional mechanisms; direct anti-inflammatory and/or antimicrobial activities. The present study also encourages us to examine occurrence of other short-chain food-derived peptides, which can moderate colitis by oral ingestion at low dose without adverse effect.

AUTHOR INFORMATION

Corresponding Author

*(K.S.) Phone/Fax: 81 75 723 3503. E-mail: E-mail:k_sato@kpu.ac.jp.

Notes

The authors declare no competing financial interest.

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