Beneficial effects of protease preparations derived from *Aspergillus* on the colonic luminal environment in rats consuming a high-fat diet

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Abstract. The present study investigated the effects of the dietary addition of the protease preparations derived from *Aspergillus* on the colonic luminal environment. Rats were fed a 30% beef tallow diet with or without the protease preparations, including Amano protease (protease A 'Amano SD', neutral proteases from *Aspergillus* spp.) or orientase (orientase AY, acid proteases from *Aspergillus niger*) at the dose of 0.2% for 3 weeks. Cecal *Bifidobacterium* was significantly elevated in the dietary Amano protease group (194-fold, P<0.05), but not in the orientase group. *Lactobacillus* was elevated in the two groups (P<0.05). Cecal *n*-butyrate, propionate and lactate were higher in the Amano protease and orientase groups compared with the controls (P<0.05). Fecal immunoglobulin A and mucins were elevated in the Amano protease group (P<0.05). These results suggest the potential effect of the consumption of *Aspergillus*-derived protease preparations that are favorable for the colonic luminal environment in rats fed a high-fat diet.

Introduction

Prebiotics, such as dietary fiber and oligosaccharides, contribute to the improvement of human health. Numerous attempts to promote health by prebiotics aim to selectively increase colonic bacterial groups, mainly *Bifidobacterium*. Increasing evidence indicates that *Bifidobacterium* improve the colonic luminal environment. Elevated levels of colonic *Bifidobacterium* are associated with increased colonic production of organic acids, such as butyrate and propionate (1); butyrate is an important energy source in the colonic epithelium that regulates cell growth and differentiation in healthy colon cells, and increases mucin production, which contributes to intestinal barrier function and induces apoptosis in tumor cells (2,3). Furthermore, the administration of certain *Bifidobacterium* species, such as *Bifidobacterium bifidum* (*B. bifidum*), can enhance intestinal immunoglobulin A (IgA), which is an indicator of intestinal immune function (4).

Our previous study developed a method for producing *Aspergillus awamori*-fermented burdock. Burdock is traditionally consumed as a root vegetable or herbal medicine in Asia, Europe and North America. It is rich in dietary fibers and has been studied extensively due to its prebiotic effects (5). A high-fat (HF) diet decreases the levels of colonic probiotics and organic acids, and it is believed to elevate the risk of colon cancer (6). Notably, our previous study reported that the consumption of dietary *Aspergillus awamori*-fermented burdock markedly increased the levels of cecal *Bifidobacterium* and organic acids, including lactate, propionate, acetate and butyrate, and fecal IgA and mucins (index of colonic barrier function) compared to burdock powder in rats fed an HF diet (7). Our previous study indicated that consumption of the water-soluble fraction of *Aspergillus awamori*-fermented burdock markedly increases cecal *Bifidobacterium* levels (unpublished data); this fraction of *Aspergillus awamori*-fermented burdock contains extracellular neutral and acid proteases of *Aspergillus awamori*. Accordingly, we hypothesize that the beneficial effect of *Aspergillus awamori*-fermented burdock on colonic health is derived from the proteases of *Aspergillus per se*. This hypothesis will aid in the clarification of the possible mechanism by which *Aspergillus awamori*-fermented burdock increased the cecal *Bifidobacterium* and organic acids, and fecal IgA and mucins. Our preliminary study investigated the effect of the consumption of several food-processing proteases derived from *Aspergillus* on colonic luminal microflora. Among them, two enzyme preparations, protease A 'Amano SD' and orientase AY, were notably identified to cause a marked increase in the colonic levels of *Bifidobacterium* and

Lactobacillus in rats fed an HF diet. Accordingly, the present study analyzed the effects of these enzyme preparations on colonic variables, including microflora, fermentation, IgA, mucins and gene expression in rats fed an HF diet.

Materials and methods

Animals and diets. A total of 27 male Sprague-Dawley rats (4-week-old) were purchased from the Hiroshima Laboratory Animal Center (Hiroshima, Japan) and were maintained according to the 'Guide for the Care and Use of Laboratory Animals' established by Hiroshima University. The study was approved by the Ethics Committee of Hiroshima University. The rats were housed individually in an air-conditioned room at 23-24°C under a 12-h light/dark cycle (lights on from 08:00 a.m. to 20:00 p.m.). Following acclimatization with a non-purified commercial rodent diet (moderate fat diet; Oriental Yeast Co., Tokyo, Japan) for 7 days, the rats (mean body weight, 108 g) were divided into 3 groups with 9 rats in each.

The composition of the experimental diets (Table I) was based on an HF diet (30% beef tallow). The group of rats were randomly assigned to 1 of 3 diets: A control diet and experimental diets containing protease A 'Amano SD' [neutral proteases and peptidase from *Aspergillus* spp., containing 70% (w/w) dextrin, protease activity at pH 6.0; 50,000 U/g; Amano Enzyme Co., Ltd., Nagoya, Japan], or orientase AY [acid proteases from *Aspergillus niger*, containing 20% (w/w) dextrin, protease activity at pH 4.0; 200,000 U/g; HBI Enzymes Inc., Shisou, Japan]. Amano protease (protease A 'Amano SD') or orientase (orientase AY) was added to the experimental diet at 0.2% (w/w) (Table I). Equal amounts of the experimental diets were incorporated daily into food cups at 19:00 p.m. (9, 10, 12, 14 and 15 g on days 1, 2-4, 5-7, 8-13 and 14-21, respectively) to prevent differences in food intake. All the food was consumed each day until the food was served on the following day. The weight of any spilled food was recorded daily and was accounted for in the calculation of food intake. Feces were collected during the last 3 days, stored at -30°C, freeze-dried and milled. At the end of the feeding period, the rats were sacrificed by decapitation under diethyl ether anesthesia. Blood was collected and serum was separated by centrifugation at 2,000 x g for 20 min and stored at -80°C. The cecum was excised and its contents were immediately collected, weighed and stored at -80°C until subsequent analysis. The colonic mucosa was scraped with a sterilized glass slide and used for RNA extraction.

Measurements. Bacterial genomic DNA was isolated from the cecal digesta using the UltraClean™ Fecal DNA extraction kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. Bacterial groups were quantified by quantitative polymerase chain reaction (qPCR) using a LightCycler 480 System II (Roche Applied Science, Indianapolis, IN, USA). The group-specific primers for qPCR are shown in Table II. qPCR was performed in a reaction volume of 20 μl containing 10 μl SYBR qPCR mix (Toyobo Co., Ltd., Osaka, Japan), 200 nM each of the forward and reverse primers (8-13), and 2 μl cecal DNA samples. The reaction conditions were 95°C for 30 sec followed by 40 cycles

Table I. Composition of experimental diets.

Characteristics	Control (%, w/w)	Amano protease (%, w/w)	Orientase (%, w/w)
Beef tallow	30.00	30.00	30.00
Casein[a]	20.00	20.00	20.00
L-cystine	0.30	0.30	0.30
Vitamin mixture[b]	1.00	1.00	1.00
Mineral mixture[b]	3.50	3.50	3.50
Cellulose	5.00	5.00	5.00
Sucrose	20.00	20.00	20.00
Corn starch	20.20	19.53	19.95
Amano protease[c]		0.67	
Orientase[d]			0.25

[a]Casein: Net protein content, 87% (w/w). [b]American Institute for Nutrition (AIN-93). [c]Protease A 'Amano SD': This powder contains 70% (w/w) dextrin. [d]Orientase AY: This powder contains 20% (w/w) dextrin.

at 95°C for 5 sec, 55°C for 15 sec and 72°C for 30 sec. The fluorescent products were detected at the last step of each cycle. Melting curve analysis was performed following amplification to distinguish the targeted PCR product from the non-targeted PCR product. Data were analyzed by the second derivative maximum method of the LightCycler 480 Basic Software. The relative abundances of the microbial populations are expressed as the proportions of total bacterial 16S rDNA gene.

The pH of cecal digesta was measured directly by a compact pH meter (B-212; Horiba, Ltd., Kyoto, Japan). Cecal organic acids were measured according to the internal standard method using high-performance liquid chromatography (HPLC) (L-2130; Hitachi, Tokyo, Japan) equipped with an Aminex HPX-87H ion exclusion column (7.8 mm i.d. x 30 cm; Bio-Rad, Richmond, CA, USA) (14). Briefly, 500 mg of cecal digesta was homogenized in 5 ml 50 mmol/l H_2SO_4 containing 10 mmol/l 2,2-dimethyl butyric acid as an internal standard and subsequently centrifuged at 17,000 x g at 2°C for 20 min. The supernatant was ultrafiltered and the filtrate was applied to the HPLC. Cecal ammonia levels were measured according to the method of Lin and Visek (15).

Total IgA concentrations in feces were measured by using an ELISA quantitation kit (Bethyl Laboratories, Montgomery, TX, USA). Mucins were extracted as described by Bovee-Oudenhoven *et al* (16) and quantitated by a fluorometric assay (17).

Total RNA was extracted from colonic mucus using QIAzol lysis reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Isolated RNA was purified using the RNeasy® Lipid Tissue Mini kit (Qiagen). cDNA was synthesized from total RNA using the Revertrace reverse transcription-PCR kit (Toyobo Co., Ltd.). qPCR was performed on an Opticon 2 system (Bio-Rad) using the SYBR qPCR mix (Toyobo Co., Ltd.) and the following primers: Fasting-induced adipose factor (FIAF) forward, GACTGCCAGGAACTCTT TCA and reverse, TGTGATGCTGTGCATCTTCT;

Table II. Target bacteria group, primers sequence and product size for quantitative PCR.

Target bacteria group	Sequence (5'-3')	Product size, bp	Reference
Total bacteria	F: ACTCCTACGGGAGGCAG	200	(8)
	R: GTATTACCGCGGCTGCTG		
Bifidobacterium spp.	F: CGCGTCYGGTGTGAAAG	244	(9)
	R: CCCCACATCCAGCATCCA		
Lactobacillus spp.	F: GAGGCAGCAGTAGGGAATCTTC	126	(9)
	R: GGCCAGTTACTACCTCTATCCTTCTTC		
Clostridium coccoides group	F: AAATGACGGTACCTGACTAA	440	(10)
	R: CTTTGAGTTTCATTCTTGCGAA		
Clostridium leptum subgroup	F: GCACAAGCAGTGGAGT	239	(10)
	R: CTTCCTCCGTTTTGTCAA		
Akkermansia muciniphila	F: CAGCACGTGAAGGTGGGGAC	327	(11)
	R: CCTTGCGGTTGGCTTCAGAT		
Enterobacteriaceae	F: CATTGACGTTACCCGCAGAAGAAGC	195	(12)
	R: CTCTACGAGACTCAAGCTTGC		
Bacteroides	F: GTCAGTTGTGAAAGTTTGC	127	(13)
	R: CAATCGGAGTTCTTCGTG		

PCR, polymerase chain reaction; bp, base pairs; F, forward; R, reverse.

Table III. Effects of Amano protease and orientase on the profile of microflora in the cecal digesta of rats fed an HF diet[a].

Bacteria	Control (% of total bacteria)	Amano protease (% of total bacteria)	Orientase (% of total bacteria)
Bifidobacterium spp.	0.016±0.002[b]	3.105±0.758[c]	0.882±0.203[b,c]
Lactobacillus spp.	3.5±0.5[b]	26.7±3.0[d]	13.7±2.1[c]
Clostridium			
Clostridium coccoides group	6.60±0.07[b,c]	3.88±0.85[b]	9.48±1.32[c]
Clostridium leptum group	0.263±0.071[b]	0.004±0.001[c]	0.034±0.006[c]
Akkermansia muciniphila	1.332±0.512[b]	0.0009±0.0004[c]	0.601±0.278[b]
Enterobacteriaceae	0.21±0.09[b]	8.24±0.84[c]	6.57±1.70[c]
Bacteroides	14.0±1.79[b]	1.10±0.69[c]	3.57±1.65[c]

[a]Mean ± standard error (n=9). [b-d]Significantly different by Tukey's multiple-range test (P<0.05). HF, high-fat.

proglucagon forward, GGTTGATGAACACCAAGAGG and reverse, GAAGGATCCATCAGCATGTC; *GAPDH* forward, TGACAACTCCCTCAAGATTGTCA and reverse, GGCATGGACTGTGGTCATGA. The expression of the target genes, FIAF and proglucagon, was normalized to that of *GAPDH*, the endogenous control gene (18).

Statistical analysis. Data are expressed as mean ± standard error. Statistical analysis was performed by one-way analysis of variance and Tukey's post hoc test (Excel Statistics 2010 for Windows; Social Survey Research Information, Tokyo, Japan). P<0.05 was considered to indicate a statisically significant difference.

Results

Characteristics of the groups. The final body weights of the control, Amano protease and orientase groups were 254±2, 246±2 and 252±2 g, respectively (P>0.05). In addition, food intake, epididymal and perirenal adipose tissue weight did not differ significantly among the groups (data not shown).

Proportions of bacteria. The proportions of *Bifidobacterium*, *Lactobacillus*, *Clostridium*, *Akkermansia muciniphila*, *Enterobacteriaceae*, and *Bacteroides* in cecal digesta are shown in Table III. The proportions of cecal microflora were markedly different in the Amano protease and orientase

Table IV. Effects of Amano protease and orientase on the cecal organic acids in rats fed an HF diet[a].

Variables	Control	Amano protease	Orientase
Cecal digesta, g	2.21±0.10[b]	7.02±0.29[d]	4.10±0.33[c]
pH of cecal digesta	7.16±0.10[b]	5.32±0.12[c]	5.46±0.11[c]
Organic acids, μmol/g wet cecal digesta			
n-Butyrate	18.6±1.8[b]	78.9±7.9[c]	58.9±7.2[c]
Propionate	10.9±0.9[b]	35.5±4.2[c]	28.7±3.6[c]
Lactate	4.7±0.6[b]	38.7±3.5[c]	31.9±6.2[c]
Acetate	31.2±3.8[b]	19.4±1.7[c]	24.2±3.9[b,c]
Succinate	18.3±3.5[b]	13.6±2.6[b,c]	7.9±1.5[c]
Total organic acids	84±7[b]	186±12[d]	152±14[c]
Ammonia, μmol/g wet cecal digesta	3.84±0.28	3.38±0.21	4.25±0.36

[a]Mean ± standard error (n=9). [b-d]Significantly different by Tukey's multiple-range test (P<0.05). HF, high-fat.

Table V. Effects of Amano protease and orientase on the fecal parameters of rats fed an HF diet[a].

Variables	Control	Amano protease	Orientase
Dry wt, g/3 days[b]	3.39±0.07[c]	4.13±0.26[d]	2.91±0.17[c]
IgA, mg/g dry wt	0.267±0.025[c]	0.634±0.057[d]	0.500±0.092[c]
IgA, mg/3 days[b]	0.90±0.09[c]	2.37±0.15[d]	1.21±0.19[c]
Mucins, mg/g dry wt	0.44±0.03[c]	4.55±0.45[d]	1.11±0.23[c]
Mucins, mg/3 days[b]	1.48±0.09[c]	18.85±2.26[d]	3.35±0.85[c]

[a]Mean ± standard error (n=9). [b]Fecal collection for final 3 days. [c,d]Significantly different by Tukey's multiple-range test (P<0.05). HF, high-fat; IgA, immunoglobulin A.

Table VI. Effects of Amano protease and orientase on the colonic gene expression in rats fed an HF diet[a].

	Relative expression		
Genes	Control	Amano protease	Orientase
FIAF	1.00±0.22	0.96±0.12	0.73±0.14
Proglucagon	1.00±0.18[b]	3.80±0.43[c]	2.17±0.45[d]

[a]Mean ± standard error (n=9). [b-d]Significantly different by Tukey's multiple-range test (P<0.05). HF, high-fat; FIAF, fasting-induced adipose factor.

groups compared to those in the control group. The proportion of *Bifidobacterium* spp. was significantly higher in the Amano protease group compared to the control group (194-fold greater, P<0.05). The proportions of *Lactobacillus* spp. were also markedly higher in the Amano protease and orientase groups compared to the control group (7.6- and 3.9-fold, respectively, P<0.05). There were no significant changes in the proportion of *Clostridium cocoides* with protease supplementation. However, the proportion of *Clostridium cocoides* was significantly higher in the orientase group compared to the Amano protease group (P<0.05). The proportions of *Clostridium* leptum were significantly lower in the protease groups compared to the control group. The proportion of *Akkermansia muciniphila* was markedly lower in the Amano protease group compared to the control group (P<0.05). The proportions of *Enterobacteriaceae* were significantly higher in the two protease groups compared to the control group (P<0.05). The proportions of *Bacteroides* were significantly lower in the two protease groups compared to the control group (P<0.05).

Cecal organic acids. Compared to the control group, the cecal digesta weights were significantly greater in the Amano protease and orientase groups (3.2- and 1.9-fold greater, respectively, P<0.05) (Table IV). The pH of the cecal digesta was significantly lower in the two protease-treated groups compared to the control group (P<0.05). Total organic acid concentration was significantly higher in the Amano protease group compared to the control group; compared to the control group, *n*-butyrate, propionate and lactate concentrations were 4.2-, 3.3- and 8.2-fold greater (P<0.05), respectively, whereas acetate concentration was significantly lower (P<0.05). Similarly, total organic acid concentrations were also significantly higher in the orientase group, and compared to the control group, *n*-butyrate, propionate and lactate concentrations were 3.2-, 2.6- and 6.8-fold greater (P<0.05), respectively, whereas succinate concentration was significantly lower (P<0.05). Cecal ammonia content did not differ significantly among the 3 groups.

Fecal IgA and mucins. Fecal dry weight was significantly greater in the Amano protease-treated group compared to the other groups (P<0.05; Table V). IgA and mucins were measured in the fecal samples as indices of colonic immune and barrier functions. As a result, fecal IgA and mucin levels were also significantly higher in the Amano protease-treated group compared to the other groups (P<0.05). The proportion of *Akkermansia muciniphila*, a mucin-degrading commensal bacterium, was inversely correlated with mucin release (r=-0.466, P<0.05).

Colonic gene expression. Compared to the control group, colonic proglucagon gene expression was 3.8- and 2.2-fold higher in the Amano protease and orientase groups, respectively (P<0.05; Table VI). FIAF expression did not differ among the 3 groups.

Discussion

The present results indicate that the consumption of Amano protease, which consists of neutral proteases derived from

Aspergillus spp., improves the colonic luminal parameters favorable to colonic health. In general, shifts in colonic microflora composition are one of the numerous factors involved in the development of colonic diseases. In particular, *Bifidobacterium* and *Lactobacillus* are considered to have important roles in colonic health (19). In the present study, Amano protease supplementation with an HF diet markedly elevated the proportions of cecal *Bifidobacterium* and *Lactobacillus*, as well as concentrations of organic acids such as *n*-butyrate, propionate and lactate. As lactate is absorbed more slowly in the gut compared to other organic acids (20), the pH of the cecal digesta was considerably lower; such an acidic environment favors acid-resistant bacteria including *Bifidobacterium* and *Lactobacillus*. An HF diet causes lower colonic organic acids, and is considered to increase the risk of colon cancer (21). Propionate and *n*-butyrate are also considered to have important roles in colonic health (22). In particular, butyrate is shown to modulate cell proliferation, apoptosis and activity of immune cells in the gut epithelial layer (23,24). Thus, the present results suggest dietary Amano protease preparation has a favorable effect on cecal fermentation when rats were fed an HF diet.

Some of the end products of protein and amino acids metabolism are considered toxic to the host, such as ammonia and phenolic compounds. However, the present results showed that the cecal ammonia content did not differ significantly among the 3 groups. It has been suggested that elevation in cecal organic acids and a lower pH are associated with suppression of ammonia-producing bacteria (25). Thus, supplementation of 0.2% Amano protease may not alter the ammonia level by modulating the amount of ammonia-producing bacteria in the colon.

Kondo *et al* (26) recently reported that intake of *Bifidobacterium* breve B-3 induces proglucagon expression in the mouse colon. Proglucagon is suggested to be a regulatory signal that controls energy homeostasis. Therefore, whether an increased proportion of *Bifidobacterium* caused by protease treatment increases proglucagon expression was determined in the present study. As expected, the results indicated that Amano protease and orientase treatment significantly increased proglucagon gene expression in the colon.

The high production of intestinal IgA and mucins is associated with a lower risk of colon cancer (27,28). The present study further shows that the consumption of Amano protease increases fecal IgA and mucin levels. The present results suggest that Amano protease may have a favorable effect on colonic immune and barrier functions. The administration of certain *Bifidobacterium* species, such as *B. bifidum*, has been reported to enhance intestinal IgA production (4). Elevated IgA levels may be associated with elevated *Bifidobacterium* levels. The decrease in colonic pH due to increased organic acid production may be responsible for increased mucin production (29). A study using a rat colon model shows that acetate and butyrate stimulate mucin release, although the underlying mechanism remains unknown (30). In the present study, the two proteases decreased pH and increased concentrations of certain organic acids in the cecal digesta. However, only Amano protease significantly increased mucin release; the reason why Orientase did not affect fecal mucin levels is unknown. Notably, Amano protease supplementation markedly decreased the cecal proportion of *Akkermansia muciniphila*, a mucin-degrading commensal bacterium. Furthermore, the proportion of *Akkermansia muciniphila*, a mucin-degrading commensal bacterium, was inversely correlated with mucin release. Therefore, the higher mucin level in the Amano protease group may be, at least in part, associated with the reduction in the proportion of *Akkermansia muciniphila*.

Mitsuoka (31) reported that bacterial extracellular compounds and the metabolites, which are defined as biogenics, may exert beneficial effects on the balance of colonic microflora by elevating the bifidobacteria and/or reducing the harmful bacteria, resulting in improving colon health. The present results suggest that the extracellular products, including several proteases released by *Aspergillus*, may have an important potential as biogenics. However, the study did not indicate any information regarding the underlying mechanism of improved colonic microflora, fermentation, immune and barrier functions by supplemental *Aspergillus*-derived protease preparations. Further study is required to investigate the mechanisms.

In conclusion, the present study suggests that the *Aspergillus*-derived protease preparations may beneficially modify the composition of cecal microflora, organic acids, IgA and mucins in rats fed an HF diet. The powdered protease preparations used contain several proteases and other factors derived from *Aspergillus*. Therefore, further study is in progress to investigate the effects of purified *Aspergillus* proteases on the colonic luminal environment.

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