Doctoral Thesis

Beneficial effects of *Aspergillus*-derived protease preparations on colonic luminal environment

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Chapter 1

Introduction

1.1 *Aspergillus*

Aspergillus is a fungal genus contains a large number of species found in various ecological niches. Some species belong to this fungus are invasive pathogens which can cause serious diseases in human and animals. Among them, *Aspergillus fumigatus* is the most prevalent and is largely responsible for the increased incidence of invasive aspergillosis in the immunocompromised patient population (Dagenais and Keller 2009). On the other hand, several *Aspergillus* species have been used in traditional fermentation foods and pharmaceuticals due to their rich enzymatic profile. For instance, *A. niger* is used for the industrial production of amylases, pectinases, phytases, proteases, and citric acid; *A. terreus* is used for the cholesterol-lowering drug lovastatin; and *A. oryzae* is used for the fermentation of soybeans and rice into soy sauce and sake, respectively.

A. oryzae, in particular, has established its reputation for its long history applications in fermentation industries. In Asian, *A. oryzae* is widely used for the large-scale production of traditional food products, including soy sauce, sake, miso, rice vinegars, and huangjiu. Due to the vital role of *A. oryzae* in Japanese food culture, *A. oryzae* has been proposed as the national microorganism in 2006 (Ichishima 2018). The long history of extensive use of *A. oryzae* in food fermentation industries prompted it serve as a well-known industrial strain, and this fungus has been listed as "Generally Recognized as Safe (GRAS)" by the Food and Drug Administration (FDA) in the USA and its safety was supported by the World Health Organization (WHO) (Taylor and Richardson 1979; Abe et al. 2006). In addition, a molecular study of *A. oryzae* indicated that the homologous gene cluster for aflatoxin biosynthesis was not expressed in *A. oryzae* even under conditions that are favorable to aflatoxin expression in *A. flavus* (Tominaga et al. 2006).

According to the sequencing data by Machida et al., *A. oryzae* has 37-megabase (Mb) genome and is a bigger genome size than that of its counterparts *A. nidulans* and *A. fumigatus* which contain 30-Mb genome (Machida et al. 2005). Additionally, *A. oryzae* contains 2000-3000 more genes than the other two species, which are the

increasing gene number mainly derived from the gene expansion of metabolic genes (Machida et al. 2005). Accordingly, it is easy to understand in genome level why *A. oryzae* has a wide variety of hydrolytic enzymes and a strong capacity for degrading various materials.

1.2 Prebiotics and *Aspergillus oryzae*

Prebiotics have a great potential to improve or maintain a balanced intestinal microflora to enhance health and well-being. The concept of prebiotics was first defined as a 'non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health (Gibson and Roberfroid 1995). The first common prebiotic property is to stimulate the probiotic strains, such as *Bifidobacterium spp.* and *Lactobacillus spp.* (Su et al. 2007; Vitali et al. 2012). Another important prebiotic property is high efficiency in inhibiting intestinal pathogens such as *Escherichia coli, Salmonella spp.* and *Clostridium spp.* (Gibson et al. 2005). Moreover, prebiotics show additional beneficial health effects in the host, such as the prevention of inflammatory bowel disease and bowel cancer (Rafter 2002; Szilagyi et al. 2002), mineral absorption improvement (Scholz-Ahrens et al. 2007), and regulation of lipid metabolism (Daubioul et al. 2000). Among the current prebiotics, inulin and fructooligosaccharide (FOS) were most well-studied in the past few years (Kaur and Gupta 2002; Kolida et al. 2002). In addition, galactooligosaccharides (GOS), lactulose and polydextose are also well-known as the established prebiotics, whereas isomaltooligosaccharides, xylooligosaccahrides (XOS), and lactitol are categorized as emerging prebiotics which are proved to have huge applications (Patel and Goyal 2012).

Many fungus species were identified to have potential of secretion of various enzymes for prebiotics production. The first fungus reported to achieve a high yield of FOS production was described in by Hidaka et al. (Hidaka et al. 1988). This study showed that they obtained high-conversion of FOS from sucrose by using *A. niger*-derived fructosyl transferase. Subsequently, another fungus *A. japonicus* was discovered to produce fructosyltransferase that catalyzes FOS synthesis (Cruz et al. 1998). Remarkably, *A. oryzae* has revealed its powerful strength in the production of prebiotics. There are a large number of studies have shown the β-galactosidase derived

from *A. oryzae* is very helpful for the production of GOS from the substrate lactose (Albayrak and Yang 2002; Vera et al. 2012). Additionally, *A. oryzae* is able to secret endoxylanase to catalyze xylan to XOS which exhibits a range of biological activities including gut modulation, antioxidant activity, anti-allergy, antimicrobial, anti-infection and anti-inflammatory properties, immunomodulatory action, and a variety of other properties (Moure et al. 2006; Aachary and Prapulla 2009).

The recent increase in the demands of nutrition in the food industry has led to the search for the specific nutritional value-addition in the current foodstuffs. *A. oryzae* has attracted much attention because it has ability to increase biological and functional characters of foods and it is extensively used in the application of functional food production. A recent study showed *A. oryzae* is used in the prebiotic synthesis from rice and rice bran with solid state fermentation (Sawangwan and Saman 2016). After 7 days fermentation, *A. oryzae* increased the alpha-glucosidase activity and several reducing sugars which are considered as prebiotic compounds. The prebiotic properties of the fermented products were also demonstrated for their growth stimulation on two probiotic strains (*Lactobacillus plantarum* and *Lactobacillus acidophilus*) and the inhibition of two pathogens (*Escherichia coli* and *Salmonella paratyphi*) (Sawangwan and Saman 2016). Moreover, another study indicated *A. oryzae*-fermented brown rice had a suppressive effect on the dextran sulfate sodium (DSS)-induced colitis and suggested *A. oryzae*-fermented brown rice mediated modification of colonic microbiota (Kataoka et al. 2008). Several studies demonstrated that Japanese koji (rice fermented with *A. oryzae* or *A. luchuensis*) contains abundant glycosylceramide which is composed of a sugar moiety, fatty acid moiety, and sphingoid base moiety (Hirata et al. 2012; Takahashi et al. 2014). Hamajima et al. reported that the dietary of 1% purified koji glycosylceramides with mice caused an increase of intestinal *Blautia coccoides* which has beneficial effects on health (Hamajima et al. 2016). In 2008, Park et al. isolated an extracellular antimicrobial protein from *A. oryzae*, which inhibited pathogenic microbial strains, including pathogenic fungi, *Fusarium moniliform* var. *subglutinans* and *Colletotrichum coccodes*, and showed antibacterial activity against bacteria, including *E. coli* and *Staphylococcus aureus* (Park et al. 2008). The knowledge of fungus *A. oryzae* in the utilization of functional food production suggests *A. oryzae* is a suitable fungus species for prebiotics production.

1.3 Key factors associated with intestinal health

Recently, with the change in eating habits, increasing dietary fat intake leads to many metabolic diseases, including gastrointestinal (GI) disorders. Colorectal cancer, one of advanced GI disorders, has become the third most common cancer worldwide and the fourth most common cause of death (Siegel et al. 2017). The GI tract is necessary in the maintenance of health and well-being and is described as a complex and dynamic ecosystem. In such complex ecosystem, there are various elements which including internal and external factors can influence on the GI functions. Herein, several external key factors playing important roles in the intestinal health will be discussed in the following sections.

1.3.1 Gut microbiota

There are many factors can affect the intestinal health. Among them, gut microbiota is considered to have a profound effect on intestinal health. Human beings, live in a co-evolutionary association with huge numbers of microorganisms that resident on the exposed and internal surfaces of our bodies. The human GI tract contains approximately 10^{14} bacteria belonging to approximately 1000 species (Neish 2009). It is estimated that commensal microbiota outnumbers at least 100-folds more than the human somatic genome (Belkaid and Naik 2013). The healthy adult GI tract is most dominated by *Bacteroidetes* and *Firmicutes* phyla (both account for up to 70–90% of total bacteria), followed by *Actinobacteria, Proteobacteria,* and *Verrucomicrobia* (Donaldson et al. 2015). The symbiotic microbiota dwelling in the gut have long been appreciated for the various beneficial effects they offered to the host. Normally, they provide essential nutrients by metabolizing indigestible dietary compounds, defend against opportunistic pathogen colonization by nutrient competition and antimicrobial substance production, and contribute to intestinal epithelial barrier via other means (Round and Mazmanian 2009). Moreover, the studies on the immune defects in germ-free (GF) mice have suggested that gut microbiota were essential to the host immune system (Falk et al. 1998; Macpherson and Harris 2004).

The beneficial partnership between gut microbiota and the host immune system greatly contributes to the normal host homeostasis during life. Disruption of a balanced composition of gut microbiota can cause immunological dysregulation that may underlie several inflammatory disorders, such as irritable bowel syndrome and

inflammatory bowel disease or even several kinds of cancer (Round and Mazmanian 2009). Moreover, altered gut microbiota also affects microbiota-derived products and metabolites, including pro- and anti-inflammatory materials, which in turn can influence development or composition of the gut microbiota (Lopez et al. 2014). However, some probiotics, such as *Bifidobacterium* and *Lactobacillus*, may re-establish the composition of the gut microbiota and exert benefits to gut microbial communities, leading to amelioration or prevention of gut inflammation and other intestinal or systemic diseases (Hemarajata and Versalovic 2013).

1.3.2 Short chain fatty acids

Short chain fatty acids (SCFAs) are volatile fatty acids produced by the gut microbiota in the GI tract as fermentation products from food components that are unabsorbed/undigested in the small intestine; they are characterized by containing fewer than six carbons, existing in straight, and branched-chain conformation. Acetic acid (C2), propionic acid (C3), and butyric acid (C4) are the most abundant, representing 90–95% of the SCFAs present in the colon (Ríos-Covián et al. 2016). SCFAs have distinct physiological effects: they contribute to shaping the gut environment, influence the physiology of the colon, they also can be used as energy sources by host cells and the intestinal microbiota and also participate in different host-signaling mechanisms (Ríos-Covián et al. 2016).

SCFAs, particular butyrate, are well established for their important role in maintaining the colonic mucosal health. Butyrate can be directly used as an energy source by colonocytes, and it has been shown to enhance intestinal barrier function through increased expression of claudin-1 and Zonula Occludens-1 and occludin redistribution; proteins which are critical components of the tight junction assembly (Wang et al. 2012). In addition, butyrate has a role as an anti-inflammatory agent, primarily via inhibition of nuclear factor κB (NF-κB) activation in human colonic epithelial cells, which may result from the inhibition of histone deacetylase (Inan et al. 2000). Taken together, butyrate exerts beneficial effects on GI tract mainly through ameliorating mucosal inflammation and oxidative status, reinforcing the epithelial defense barrier, and modulating intestinal motility (Canani et al. 2011).

1.3.3 Other factors

In addition to the gut microbiota and its fermented product SCFAs, the other factors, including mucins and immunoglobulin A (IgA), also play important roles in modulation of GI tract homeostasis. Commonly, the epithelial lining of GI tract is exposed to luminal contents that contain proteases, bile acids, ingested toxins, and substantial numbers of gut microbiota. In order to protecting the intestinal epithelium from chemical and bacterial hazards, the intestinal epithelium is covered by a thick mucus layer, which partly consists of secreted mucins. This relatively protease-resistant viscous coating provides a physical barrier that restricts damage to the epithelium and attenuates activation of innate and adaptive immune responses. A large number of studies indicated mucins could suppress inflammation in the GI tract and thereby inhibit the development of intestinal tumors (Van der Sluis et al. 2006; Kufe 2009). Thus, mucins have been identified as markers of adverse prognosis and as attractive therapeutic targets.

IgA is the most abundant immunoglobulin in humans, and is mainly found in mucosal areas, such as the gut, respiratory tract and urogenital tract (Kerr 1990). Secretory IgA has been recognized as a first line of defense in protecting the intestinal epithelium from gut pathogens and toxins. In addition, IgA is demonstrated to play critical roles in the maintenance of mucosal immunity and intestinal homeostasis. IgA imposes on the composition of the gut microbiota, down-regulates pro-inflammatory responses normally associated with the uptake of highly pathogenic bacteria and potentially allergenic antigens, and promotes the retro-transport of antigens across the intestinal epithelium to dendritic cell subsets in the gut-associated lymphoid tissue (Mantis et al. 2011).

Accumulating evidence has suggested *Aspergillus spp.*-derived enzymes have been extensively used in the modern food industry. However, there is limited study *in vivo* have examined the potentially physiological functions of *Aspergillus spp.*-derived enzymes. In particular, *A. oryzae*-derived amylase, has established its reputation for using as GI drugs (digestive enzyme preparations). In my study, I investigated the potential positive effects of *Aspergillus spp*.-derived protease preparations in rats. Firstly, I conducted the screening of several protease preparations derived from *Aspergillus spp.* on the colonic luminal environment of rats. Among them, two kinds of protease preparation (Amano protease derived from *A. oryzae* and Orientase derived

from *A. niger*) were found have positive and powerful prebiotics-like effects on the colonic luminal environment of rats fed a high fat diet. The Amano protease preparation contains several digestive enzymes, including acid protease, alkaline protease, and amylase. In order to find which enzyme containing in the Amano protease preparation responsible for the prebiotic-like effects, I investigated whether purified acid protease have such effect in the chapter 3. Further, I found protein sources, such as casein, soy protein and rice protein, combined with Amano protease have no difference in the promotion of *Bifidobacterium*, suggesting that there is no specific protein required for bifidogenic effect. In order to know the potential mechanism of beneficial effects of Amano protease on colonic environment, I hypothesized that some digestive enzymes in the protease preparation may successfully pass through the tough condition of stomach, and subsequently influence the protein and amino acids metabolism. Accordingly, in the chapter 4, I investigated the free amino acids profile alteration in the cecum contents of rats fed Amano protease supplemented high fat diet.

Chapter 2 Beneficial effects of protease preparations derived from *Aspergillus* on the colonic luminal environment in rats consuming a high- fat diet

2.1 Brief introduction

The previous study in my laboratory 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 (Li et al. 2008). Notably, the previous study reported that the consumption of dietary *A. 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 a high-fat (HF) diet (Okazaki et al. 2013). The previous study indicated that consumption of the water-soluble fraction of *A. awamori*-fermented burdock markedly increases cecal *Bifidobacterium* levels (unpublished data); this fraction of *A. awamori*-fermented burdock contains extracellular neutral and acid proteases of *A. awamori*. Accordingly, I hypothesize that the beneficial effect of *A. 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 *A. awamori*-fermented burdock increased the cecal *Bifidobacterium* and organic acids, and fecal IgA and mucins. The 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, Amano protease and Orientase, 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, and mucins in rats fed an HF diet. The study was conducted with the rats fed HF diet, which has been reported to alter the microflora composition and is considered to be a risk factor for colon diseases (Wu and Chen 2011; Parnell and Reimer 2012; Serino et al. 2012; Shen et al. 2014).

2.2 Methods and materials

2.2.1 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 2-1) 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 Amano protease [neutral proteases and peptidase from *A. oryzae*, containing 70% (w/w) dextrin, protease activity at pH 6.0; 50,000 U/g; Amano Enzyme Co., Ltd., Nagoya, Japan], or Orientase [acid proteases from *A. niger*, containing 20% (w/w) dextrin, protease activity at pH 4.0; 200,000 U/g; HBI Enzymes Inc., Shisou, Japan]. Amano protease or Orientase was added to the experimental diet at 0.2% (w/w) (Table 2-1). Equal amounts of the experimental diets were incorporated daily into food cups at 18:00 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.

2.2.2 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 2-2. 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 (Bartosch et al. 2004; Matsuki et al. 2004; Ahmed et al. 2007; Delroisse et al. 2008; Sonoyama et al. 2010; Parnell and Reimer 2012), and 2 μL cecal DNA samples. The reaction conditions were 95˚C for 30 sec followed by 40 cycles 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 as described by previous study (Carberry et al. 2012) according to the following equation: relative quantification = $2^{-(\text{CT-target} - \text{CT-total bacteria})}$, where C_T represents the threshold cycle.

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) (Okazaki et al. 2011). 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 through a microconcentrator with a molecular-mass cutoff of 3000 Da, and the filtrate was applied to the HPLC. Cecal ammonia levels were measured according to the method of Lin and Visek (Lin and Visek 1991).

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 (Bovee-Oudenhoven et al. 1997) and quantitated by a fluorometric assay (Crowther and Wetmore 1987).

2.2.3 Statistical analysis

Data are expressed as mean ± standard error (SE). 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 statistically significant difference.

2.3 Results

2.3.1 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 weights did not differ significantly among the groups (data not shown).

2.3.2 Proportions of bacteria

The proportions of *Bifidobacterium, Lactobacillus, Clostridium, Akkermansia muciniphila, Enterobacteriaceae*, and *Bacteroides* in cecal digesta are shown in Table 2-3. The proportions of cecal microflora were markedly different in the Amano protease and Orientase 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 *A. 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 ($P < 0.05$).

2.3.3 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 2-4). 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 acids 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 acids 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 three groups.

2.3.4 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 2-5). 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 ($r = -0.466$, $P < 0.05$).

2.4 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 (Brownawell et al. 2012). 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 (Hoshi et al. 1994), 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 (Wu and Chen 2011). Propionate and n-butyrate are also considered to have important roles in colonic health (Topping and Clifton 2001). In particular, butyrate is shown to modulate cell proliferation, apoptosis and activity of immune cells in the gut epithelial layer (Scheppach 1994; Tang et al. 2011). 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 (Han et al. 2014). Thus, supplementation of 0.2% Amano protease may not alter the ammonia level by modulating the amount of ammonia-producing bacteria in the colon.

The high production of intestinal IgA and mucins is associated with a lower risk of colon cancer (Velcich et al. 2002; Ito et al. 2009). 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 (Park et al. 2002). Elevated IgA levels may be associated with elevated *Bifidobacterium* levels. The decrease in colonic pH due to increased organic acids production may be responsible for increased mucin production (van den Abbeele et al. 2011). A study using a rat colon model shows that acetate and butyrate stimulate mucin release, although the underlying mechanism remains unknown (Barcelo et al. 2000). 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. The increasing evidence from animal and human studies suggest that *A. muciniphila* has been inversely associated with obesity, diabetes, inflammation, and metabolic disorders (Roopchand et al. 2015; Zhou 2017). Currently, I do not know why the abundance of this bacterium decreased by Amano protease supplementation. The future study should

be addressed this issue. In this study, the proportion of *A. muciniphila* 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 *A. muciniphila*. Mitsuoka (Mitsuoka 2014) 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.

2.5 Summary

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.

2.6 Tables

Table 2-1. Composition of experimental diets

Ingredient	Control (%, w/w)	Amano protease (%, w/w)	Orientase (%, w/w)
Beef tallow	30.00	30.00	30.00
Casein[1]	20.00	20.00	20.00
L-Cystine	0.30	0.30	0.30
Vitamin mixture[2]	1.00	1.00	1.00
Mineral mixture[2]	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[3]		0.67	
Orientase[4]			0.25

[1]Casein: net protein content, 87% (w/w).

[2]American Institute for Nutrition (AIN-93G).

[3]Amano protease: This powder contains 70% (w/w) dextrin.

[4]Orientase: This powder contains 20% (w/w) dextrin.

Table 2-2. Target bacteria group, primers sequence and product size for quantitative PCR

Target bacteria group	Sequence (5' to 3')	Product size (bp)	Reference
Total bacteria	F: ACTCCTACGGGAGGCAG R: GTATTACCGCGGCTGCTG	200	(Parnell and Reimer 2012)
Bifidobacterium spp.	F: CGCGTCYGGTGTGAAAG R: CCCCACATCCAGCATCCA	244	(Delroisse et al. 2008)
Lactobacillus spp.	F: GAGGCAGCAGTAGGGAATCTTC R: GGCCAGTTACTACCTCTATCCTTCTTC	126	(Delroisse et al. 2008)
Clostridium coccoides	F: AAATGACGGTACCTGACTAA R: CTTTGAGTTTCATTCTTGCGAA	440	(Matsuki et al. 2004)
Clostridium leptum	F: GCACAAGCAGTGGAGT R: CTTCCTCCGTTTTGTCAA	239	(Matsuki et al. 2004)
Akkermansia muciniphila	F: CAGCACGTGAAGGTGGGGAC R: CCTTGCGGTTGGCTTCAGAT	327	(Sonoyama et al. 2010)
Enterobacteriaceae	F: CATTGACGTTACCCGCAGAAGAAGC R: CTCTACGAGACTCAAGCTTGC	195	(Bartosch et al. 2004)
Bacteroides	F: GTCAGTTGTGAAAGTTTGC R: CAATCGGAGTTCTTCGTG	127	(Ahmed et al. 2007)

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

Table 2-3. Effects of Amano protease and orientase on the profile of microflora in the cecal digesta of rats fed an HF diet

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

Mean ± standard error (n = 9). ^{abc} Significantly different by Tukey's multiple-range test ($P < 0.05$). HF, high-fat.

Table 2-4. Effects of Amano protease and orientase on the cecal SCFAs in rats fed an HF diet

	Control	Amano protease	Orientase
Cecal digesta (g)	2.21 ± 0.10[a]	7.02 ± 0.29[c]	4.10 ± 0.33[b]
pH of cecal digesta	7.16 ± 0.10[a]	5.32 ± 0.12[b]	5.46 ± 0.11[b]
Organic acids (μmol/g wet cecal digesta)			
n-Butyrate	18.6 ± 1.8[a]	78.9 ± 7.9[b]	58.9 ± 7.2[b]
Propionate	10.9 ± 0.9[a]	35.5 ± 4.2[b]	28.7 ± 3.6[b]
Lactate	4.7 ± 0.6[a]	38.7 ± 3.5[b]	31.9 ± 6.2[b]
Acetate	31.2 ± 3.8[a]	19.4 ± 1.7[b]	24.2 ± 3.9[ab]
Succinate	18.3 ± 3.5[a]	13.6 ± 2.6[ab]	7.9 ± 1.5[b]
Total organic acids	84 ± 7.0[a]	186 ± 12[c]	152 ± 14[b]
Ammonia (μmol/g wet cecal digesta)	3.84 ± 0.28	3.38 ± 0.21	4.25 ± 0.36

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

Table 2-5. Effect of Amano protease and Orientase on fecal parameters of rats fed a HF diet

Variables	Control	Amano protease	Orientase
Dry wt (g/3 days)[1]	3.39 ± 0.07[a]	4.13 ± 0.26[b]	2.91 ± 0.17[a]
Mucin (mg/g dry wt)	0.44 ± 0.03[a]	4.55 ± 0.45[b]	1.11 ± 0.23[a]
Mucin (mg/3 days)[1]	1.48 ± 0.09[a]	18.85 ± 2.26[b]	3.35 ± 0.85[a]
IgA (mg/g dry wt)	0.267 ± 0.025[a]	0.634 ± 0.057[b]	0.500 ± 0.092[a]
IgA (mg/3 days)[1]	0.90 ± 0.09[a]	2.37 ± 0.15[b]	1.21 ± 0.19[a]

[1]Fecal collection for final 3 days.

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

Chapter 3

Effects of consumption of an acid protease derived from *Aspergillus oryzae* on cecal microflora in rats

3.1 Brief Introduction

Recently, I demonstrated that compared with the consumption of burdock root powder, the consumption of the *A. awamori*-fermented burdock root powder caused a marked elevation in cecal *Bifidobacterium* in rats that were fed a HF diet (Okazaki et al. 2013). A subsequent study found that the addition of 0.2% Amano protease preparation markedly elevated cecal *Bifidobacterium* levels (Yang et al. 2015). The protease preparation contains several digestive enzymes, including acid protease (AcP), alkaline protease, and amylase (Fig. 3-1A). In the present study, I hypothesized that the digestive enzymes in the protease preparation are responsible for the bifidogenic effect. The purpose of this study was to test this hypothesis by investigating whether cecal *Bifidobacterium* levels are elevated by the addition of the purified AcP at the dose equivalent to the level found in the 0.1% Amano protease diet, which is considered the effective dose for generating a bifidogenic effect.

3.2 Methods and materials

3.2.1 Purification of acid protease from Biozyme A

AcP from *A. oryzae* was purified from Biozyme A (derived from *A. oryzae*, Amano Enzyme Inc. Nagoya, Japan) containing the same AcP as Amano protease. The AcP contained in the Biozyme A is much easier to purified than that contained in the Amano protease. Eighty grams of Biozyme A was dissolved in 800 mL of 20 mM bis-Tris buffer (pH 6.0). The enzyme solution was dialyzed at 4°C overnight in 20 mM bis-Tris buffer (pH 6.0) and filtered through a 0.45-μm membrane filter. The filtrate was applied to a DEAE-Toyopearl 650 M column (ø 5 × 20 cm) equilibrated with 20 mM bis-Tris buffer (pH 6.0). The column was washed with 600 mL of the same buffer (flow rate, 6 mL/min) and eluted with a linear gradient of 0–0.2 M NaCl. Collected fractions were analyzed by SDS-PAGE, and those containing acid protease were pooled. The enzyme solution was concentrated to a volume of 5 mL with a centrifugal ultrafiltration device (cutoff: 5 kDa) at 4,000 × *g*. The concentrate was applied to a HiLoad 26/60 Superdex

200 pg (ø 26 × 60 cm) equilibrated with 20 mM acetate buffer (pH 5.0) containing 0.3 M NaCl. The column was eluted with the same buffer (flow rate, 1.5 mL/min). The fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and those containing acid protease were mixed. The enzyme solution was concentrated to a volume of 5 mL as above. The concentrate was applied to a HiPrep 26/10 Desalting column (ø 26 × 10 cm) equilibrated with ultrapure water. Fractions were analyzed by SDS-PAGE and those including acid protease were mixed. The enzyme solution was freeze-dried for 2 days, and the dried product was pulverized in a mortar.

3.2.2 Animals and diets

A total of 26 male Sprague–Dawley rats (3 weeks old) were purchased from the Hiroshima Laboratory Animal Center (Hiroshima, Japan) and maintained according to the "Guide for the Care and Use of Laboratory Animals" established by Hiroshima University. This study was approved by the Ethics Committee of Hiroshima University (Ethical approval No. C15-12). The rats were individually housed in an air-conditioned room at 23–24°C under a 12-h light/dark cycle (lights on from 08:00–20:00). Following acclimatization with a non-purified commercial rodent diet (MF, Oriental Yeast, Tokyo) for 7 days, the rats (mean body weight: 110 g) were randomly assigned to one of the four groups (n = 6–8 rats per group).

The composition of experimental diets was based on a HF diet (Table 3-1). The groups of rats were randomly assigned to one of the four diets: a control diet (a HF diet) or experimental diets. The experimental diets included HF diets supplemented with Amano protease at 0.1%, purified AcP at 0.0096% (an equivalent content of AcP in the 0.1% Amano protease diet), purified AcP at 0.0384% (an AcP content 4-fold higher than that in the 0.1% Amano protease diet).

Equal amounts of the experimental diets were incorporated daily into food cups at 18:00 (9, 10, 12, 14, and 15 g on days 1, 2–4, 5–7, 8–12, and 13–14, respectively) to prevent differences in food intake. All food was consumed each day until the next day's food was served. The weight of spilled diet was recorded daily and appropriately incorporated in calculations of food intake. Fecal pellets were collected over the last 2 days, stored at −20°C, and then freeze-dried and milled. The milled samples were stored at −20°C until DNA extraction. At the end of this period, rats were euthanized by

decapitation following anesthesia (13:00–15:00) with inhalation exposure of diethyl ether for about 20–30 sec in total in the desiccator to reduce the suffering. The cecum was immediately excised, and its contents were completely removed, weighed, and stored at −80°C until subsequent analysis of SCFAs. Immediately after collecting the contents, a portion was used for DNA extraction.

3.2.3 Measurements

Bacterial genomic DNA was isolated from the cecal contents and feces using UltraClean™ Fecal DNA extraction kit (MO BIO Laboratories, CA, USA) according to the manufacturer's instructions. Bacterial groups were quantified by Real-time quantitative polymerase chain reaction (qPCR) using StepOne™ Real-time PCR System (Applied Biosystem). The qPCR method and group-specific primers used for amplification were as described in Table 3-2. Real-time 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, and 2 μL cecal or fecal DNA samples. The reaction conditions were 95°C for 30 s followed by 40 cycles at 95°C for 5 s, 55°C for 15 s, and 72°C for 30 s. The fluorescent products were detected at the last step of each cycle. Melting curve analysis was performed after amplification to distinguish the targeted PCR product from the non-targeted PCR product. Data were analyzed by the second derivative maximum method of the StepOne™ Real-time PCR Software. The plasmid copy number/μL was determined for standard plasmid solution (ng of cut standard plasmid mixture/μL • [molecules.bp/1.0 xl0^9 ng] • 1/660 DNA length bp per plasmid = plasmid copies/μL). Real-time qPCR reactions were run on serial dilutions of each standard mixture to relate threshold cycle number to copy numbers of the target sequence and to generate standard curves for quantification in unknown samples. Typically, standard curves were linear across five orders of magnitude ($R^2 > 0.98$). Cecal organic acids were measured as described in Chapter 2.

3.2.4 Statistical analyses

All values are expressed as means with their SE. Values were analyzed by one-way ANOVA, followed by Tukey–Kramer Honestly significant difference (HSD) test (Excel Statistics 2010 for Windows, Social Survey Research Information, Tokyo). For all tests, $P < 0.05$ was considered statistically significant.

3. 3 Results

3.3.1 Purification of acid protease

Analysis of N-terminal amino acid sequence indicated the purified enzyme is an AcP (Gomi K, Arikawa K, Kamiya N, Kitamoto K 1993). Then, the purified AcP (molecular weight: 44 kDa) from *A. oryzae* was examined its effects on cecal *Bifidobacterium* levels. Analysis of purified AcP by SDS-PAGE revealed a single protein band corresponding to 44 kDa (Fig. 3-1 B). Then I compared the protease activities of Biozyme A and purified AcP. The protease activities of Biozyme A and purified AcP at pH 3.0 were 5,210 units/g and 242,000 units/g, respectively (46-fold higher).

3.3.2 Body weights, food intake, cecal contents weights, and fecal weights

Body weights and total food intake were not affected by dietary treatment (data not shown); however, the weights of the cecal contents in the group that was fed the diet containing 0.1% Amano protease were significantly higher than that of the control group ($P < 0.05$, Table 3-3). Fecal dry weights in the 0.1% Amano protease group were significantly higher than that in the control group ($P < 0.05$, Table 3-4).

3.3.3 Cecal microflora

Compared with the control group, the copy numbers of cecal *Bifidobacterium* and *Lactobacillus* were significantly elevated in the 0.0384% AcP and 0.1% Amano protease groups ($P < 0.05$) but not in the 0.0096% AcP group (Table 3-3). Compared with the control group, cecal numbers of *Clostridium leptum* were significantly reduced in the 0.1% Amano protease group ($P < 0.05$), whereas the numbers of *Akkermancia muciniphila, Clostridium coccoides* and *Enterobacteriaceae* were not affected by the dietary manipulation.

3.3.4. Fecal microflora

To confirm the results of microflora in cecal contents, fecal microflora was also examined. Compared with the control group, the numbers of fecal *Bifidobacterium* were markedly elevated in the 0.0384% AcP and 0.1% Amano protease groups ($P < 0.05$) but were unaffected in the 0.0096% AcP group (Table 3-4). Fecal numbers of *Lactobacillus*, *A. muciniphila* and *Enterobacteriaceae* were unaffected, whereas the numbers of *C. leptum* and *C. coccoides* were reduced in the 0.1% Amano protease

group ($P < 0.05$).

3.3.5 Organic acids

Rats consuming a diet containing 0.1% Amano protease had elevated levels of lactate, propionate, n-butyrate, and total organic acids in the cecum ($P < 0.05$, Table 3-5). In addition, lactate levels were significantly elevated in the 0.0384% AcP group, compared with the control group ($P < 0.05$).

Table 3-6 indicates the relation between the numbers of bacteria and the levels of organic acids in a per g of wet cecal contents. The numbers of *Bifidobacterium*, *Lactobacillus* and *Enterobacteriaceae* were positively correlated with the levels of lactate, propionate and n-butyrate ($P < 0.05$). There was an inverse association between the numbers of *A. muciniphila* and propionate levels ($P < 0.05$). The numbers of *C. leptum* were negatively correlated with lactate levels ($P < 0.05$).

Table 3-7 shows the purified amylase which equivalent to the amount in the diet containing 0.1% Amano protease did not affect the levels of *Bifidobacterium* and *Lactobacillus,* as the effects of 10-fold higher amount of purified amylase and inactivated amylase.

Table 3-8 shows that 1.5-fold higher amount of the purified alkaline protease in the diet containing 0.1% Amano protease and the inactivated alkaline protease did not affect the levels of *Bifidobacterium* and *Lactobacillus.*

3.4 Discussion

In this study, I hypothesized that the bifidogenic effect of the 0.1% Amano protease diet is because of the digestive enzymes in the preparation. To test this hypothesis, I examined the effect of supplemental AcP on microflora. The results showed the cecal and fecal *Bifidobacterium* numbers were unaffected by supplementation with 0.0096% purified AcP at the level equivalent to the AcP amount used in the 0.1% Amano protease diet. The results did not support the hypothesis that the bifidogenic effect of 0.1% Amano protease diet was because of the digestive enzymes. However, intriguingly I found that the diet containing 4-fold higher AcP content (0.0384% AcP diet) than that used in the 0.1% Amano protease diet significantly elevated the cecal *Bifidobacterium* and *Lactobacillus* numbers and the fecal *Bifidobacterium* numbers in rats. As expected from the results of increased cecal numbers of *Bifidobacteium* and *Lactobacillus*, lactate-producing bacteria, cecal lactate

levels were also elevated by the 0.0384% AcP containing diet. To the best of our knowledge, this is the first study demonstrating that AcP derived from *A. oryzae* exerts a prebiotic-like effect. *Aspergillus* protease preparations are widely used for producing many food products and are regularly consumed as a component of digestive drugs in Japan. Further, *Aspergillus*-fermented foods, such as miso, may contain *Aspergillus*-derived proteases. Therefore, it is interesting to investigate if consuming *Aspergillus*-derived AcP has beneficial effects on the colonic luminal environment in the same way as typical prebiotics, such as oligosaccharides (Yang et al. 2015).

After this study, I have failed to show any bifidogenic effect of purified alkaline protease and purified amylase in the Amano protease even if the diets were supplemented with higher amount of such enzymes than in the 0.1% Amano protease diet (Table 3-7 and 3-8). At present, it is unknown why AcP exerts a bifidogenic effect, but alkaline protease and amylase do not. I speculated that AcP is relatively stable in the acidic environment of the stomach and is able to migrate to the intestines, where it imparts bifidogenic effects. Further study is in progress to investigate the bifidogenic process.

In this study, the alterations in the numbers of cecal *Bifidobacteium* by dietary manipulation were similar to those in the feces. However, the alterations in the numbers of *Lactobacillus* in cecum were quite different from those in feces; the numbers of *Lactobacillus* in cecum were profoundly increased by the 0.0384% AcP diet and 0.1% Amano protease diet, while those in feces were not affected. The reason of the difference is unknown. However, it may be possible that *Lactobacillus* numbers are affected by transit the intestinal tract.

This study is the first to prove that consuming small quantities of active AcP derived from *A. oryzae* elevates colon *Bifidobacterium* abundance in rats. This finding may provide insights into novel applications of this protease as a functional non-nutrient food factor, e.g., a dietary supplement for colonic health. The concept of a prebiotic, as defined by Roberfloid and Valcheva *et al.* (Roberfroid 2007; Valcheva and Dieleman 2016), is “a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health.” The present study may introduce a new concept of prebiotic, and the *A. oryzae*-derived AcP may be considered as a new type of prebiotic. Furthermore, interestingly, only a very small quantity of AcP (0.0384%)

can elevate *Bifidobacterium* levels compared with 5–10% of dietary oligosaccharides and some fibers necessary to elevate it in rats (Bielecka et al. 2002; Roller et al. 2004). Further work is in progress to elucidate the underlying mechanisms by which the presence of AcP elevates colonic *Bifidobacterium* numbers. In addition, it will be interesting to investigate the response of intestinal numbers of *Bifidobacterium* to the consumption of *A. oryzae*-fermented Japanese foods, such as miso and sake flake, which contain several *Aspergillus* proteases.

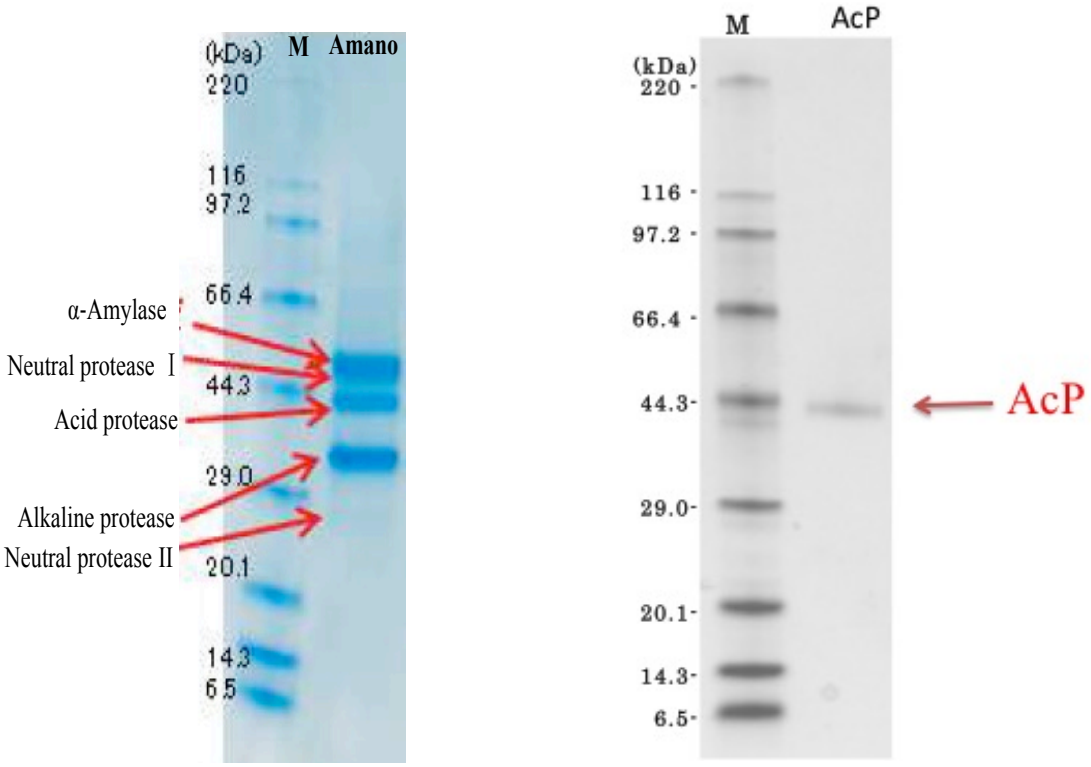
In this study, the cecal numbers of *Bifidobacterium* and *Lactobacillus* were positively correlated with cecal levels of lactate, propionate, and n-butyrate (Table 6). These organic acids are known to stimulate the growth of these intestinal beneficial bacteria (Ohashi and Ushida 2009). In addition, *Bifidobacterium* is responsible for the production of these organic acids (De Vuyst and Leroy 2011). Thus, it is reasonable to suppose that the alterations in these bacteria populations are linked to those in the organic acids in the intestine. However, the numbers or relative abundance of cecal *Bifidobacterium* appears to be too low (generally, less than several percent of total bacteria) to explain the increased production of organic acids. Other intestinal bacteria have been also reported to produce organic acids (De Vuyst and Leroy 2011). Therefore, further study is necessary to clarify the principal bacteria responsible for the alteration in cecal organic acids. On the other hand, unexpectedly, the cecal numbers of *Enterobacteriaceae* were significantly associated with the levels of lactate, propionate, and n-butyrate. Organic acids are known to inhibit the growth of *Enterobacteriaceae* (Levison 1973). However, the influence of *Enterobacteriaceae* on the production of the organic acids in the gut is unclear. Further study is warranted to investigate the relation between *Enterobacteriaceae* and the organic acids.

Some limitations of this study should be pointed out. First, this study showed the bifidogenic effect of supplemental AcP, but did not provide any information of the mechanisms. Secondary, the *Bifidobacterium*-elevating effect of the diet containing 0.1% Amano protease preparation could not be explained by AcP used in the preparation. Identification of the active factors responsible for the bifidogenic effect of the Amano protease preparation remained to be elucidated. Thirdly, because this study was performed with animals, human study is necessary to clarify the role of the AcP intake in the colonic luminal environment.

3.5 Summary

In conclusion, I demonstrated that dietary supplemental 0.0384% AcP derived from *Aspergillus* profoundly increased the cecal and fecal numbers of *Bifidobacterium* in rats fed a HF diet. This study provides the first evidence for the bifidogenic effect of dietary *Aspergillus* protease. It is of great interest to further investigate if the bifidogenic effect of dietary AcP leads to preventive effects against several disorders such as colon diseases, allergies, liver disease, insulin resistance, and brain disease (Tojo et al. 2014; Mishra et al. 2015; Arboleya et al. 2016; Rogers et al. 2016).

3.6 Figure and tables



A. SDS-PAGE of Amano protease B. SDS-PAGE of AcP

Fig. 3-1 SDS-PAGE of Amano protease and the purified acid protease (AcP). SDS-PAGE was performed with a 5-20% gradient polyacrylamide gel and the protein was stained with Coomassie brilliant blue. M; Molecular weight markers (6.5-220 kDa), AcP; the purified acid protease.

Table 3-1. Composition of experiment diets

Ingredient	Control (%, w/w)	0.0096% AcP[2] (%, w/w)	0.0384% AcP[3] (%, w/w)	0.1% Amano protease[4] (%, w/w)
Beef tallow	30.00	30.00	30.00	30.00
Casein[1]	20.00	20.00	20.00	20.00
L-Cystine	0.30	0.30	0.30	0.30
Vitamin mixture (AIN-93G)	1.00	1.00	1.00	1.00
Mineral mixture (AIN-93G)	3.50	3.50	3.50	3.50
Cellulose	5.00	5.00	5.00	5.00
Sucrose	20.00	20.00	20.00	20.00
Corn starch	20.20	20.19	20.16	19.87
AcP	-	0.0096	0.0384	-
Amano protease	-	-	-	0.33

[1]Casein: net protein content, 87% (w/w)

[2]0.0096% AcP: Equivalent to the amount of AcP in the diet containing 0.1% Amano protease
[3]0.0384% AcP: Four-fold higher amount than the amount of AcP in the diet containing 0.1% Amano protease

[4]Amano Protease: This powder contains 70% (w/w) dextrin as a thickening agent

Table 3-2. Target bacteria group, primers sequence and product size for quantitative PCR

Target bacteria group	Sequence (5' to 3')	Product size (bp)
Total bacteria	F: GRGTTYGATYMTGGCTCAG R: ACTGCTGCCTCCCGTAGGAGT	300
Bifidobacterium spp.	F: CGCGTCYGGTGTGAAAG R: CCCCACATCCAGCATCCA	244
Lactobacillus spp.	F: AGCAGTAGGGAATCTTCCA R: CACCGCTACACATGGAG	341
Clostridium coccoides	F: GACGCCGCGTGAAGGA R: AGCCCCAGCCTTTCACATC	1000
Clostridium leptum	F: CCTTCCGTGCCGSAGTTA R: GAATTAAACCACATACTCCACTGCTT	100
Akkermansia muciniphila	F: CAGCACGTGAAGGTGGGGAC R: CCTTGCGGTTGGCTTCAGAT	327
Enterobacteriaceae	F: CATTGACGTTACCCGCAGAAGAAGC R: CTCTACGAGACTCAAGCTTGC	195

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

Table 3-3. Effects of dietary addition of AcP and Amano protease on the copy numbers of microflora in cecal contents of rats

	Control	0.0096% AcP	0.038% AcP	0.1%Amano protease
Number of animals	8	6	6	6
Cecal contents (g)	1.61 ± 0.11^{a}	1.35 ± 0.22^{a}	1.76 ± 0.12^{a}	3.61 ± 0.27^{b}
(numbers/g wet cecal contents)				
Total bacteria ($\times 10^{10}$)	6.42±1.38	3.40±0.72	6.02±1.48	2.78±0.22
Bifidobacterium spp. ($\times 10^{8}$)	0.17 ± 0.13^{a}	1.93 ± 0.34^{a}	10.33 ± 2.43^{b}	12.17 ± 2.58^{b}
Lactobacillus spp. ($\times 10^{9}$)	5.4 ± 1.2^{a}	$7.3\pm2.0^{a,b}$	$31.7\pm12.3^{b,c}$	35.8 ± 7.7^{c}
Akkermancia muciniphila ($\times 10^{9}$)	9.3±2.5	3.9±0.8	13.7±4.5	5.1±4.7
Clostridium leptum subgroup ($\times 10^{9}$)	39.5 ± 11.04^{a}	$22.95\pm5.39^{a,b}$	$22.73\pm11.94^{a,b}$	0.66 ± 0.34^{b}
Clostridium coccoides group ($\times 10^{9}$)	9.42±3.92	5.66±2.15	6.54±1.82	1.78±0.50
Enterobacteriaciae ($\times 10^{9}$)	2.90±0.45	1.80±0.53	3.37±0.78	12.30±7.22

Data: means ± standard error. [abc] Significantly different by Tukey-Kramer HSD test ($P < 0.05$), n = 6-8

Table 3-4. Effects of dietary addition of AcP and Amano protease on the copy numbers of microflora in feces of rats

	Control	0.0096% AcP	0.038% AcP	0.1%Amano protease
Fecal dry wt (g)	1.85 ± 0.07[a]	2.11 ± 0.10[a]	2.19 ± 0.07[a]	2.76 ± 0.15[b]
(numbers/g dry feces)				
Total bacteria ($\times 10^{10}$)	6.47±1.30	9.80±2.64	8.55±1.86	3.73±1.00
Bifidobacterium spp. ($\times 10^{8}$)	1.0±0.6[a]	6.4±1.3[a]	43.8±10.1[b]	60.7±15.7[b]
Lactobacillus spp. ($\times 10^{9}$)	6.3±1.3	10.1±1.4	14.1±4.8	5.4±2.1
Akkermancia muciniphila ($\times 10^{9}$)	1.31±0.35	1.60±0.66	1.16±0.55	0.64±0.38
Clostridium leptum ($\times 10^{9}$)	3.30±0.81[a]	2.51±1.01[a,b]	1.78±0.44[a,b]	0.07±0.01[b]
Clostridium coccoides ($\times 10^{9}$)	18.3±4.0[a]	18.7±5.4[a]	14.3±2.8[a,b]	1.3±0.4[b]
Enterobacteriaciae ($\times 10^{9}$)	0.45±0.13	0.45±0.15	0.47±0.16	0.55±0.13

Data: means ± standard error. [ab] Significantly different by Tukey-Kramer HSD test ($P < 0.05$), n = 6-8

Table 3-5. Effects of dietary addition of AcP and Amano protease on cecal short-chain fatty acids in rats

(μmol/g wet cecal contents)	Control	0.0096% AcP	0.038% AcP	0.1%Amano protease
Succinate	$28.6 \pm 8.5^{a,b}$	8.4 ± 2.1^{a}	$14.1 \pm 3.7^{a,b}$	40.8 ± 9.1^{b}
Lactate	7.8 ± 2.4^{a}	4.1 ± 0.9^{a}	27.2 ± 9.5^{b}	49.0 ± 9.5^{c}
Acetate	38.6 ± 4.0	44.7 ± 5.3	50.5 ± 4.6	33.3 ± 5.1
Propionate	15.2 ± 0.7^{a}	10.4 ± 1.5^{a}	$16.3 \pm 3.2^{a,b}$	26.6 ± 4.5^{b}
n-Butyrate	15.4 ± 2.2^{a}	17.3 ± 5.5^{a}	20.7 ± 3.0^{a}	46.3 ± 5.3^{b}
Total organic acids	106 ± 10^{a}	84 ± 11^{a}	124 ± 15^{a}	189 ± 14^{b}

Data: means ± standard error. ^{abc} Significantly different by Tukey-Kramer HSD test ($P < 0.05$), n = 6-8.

Table 3-6. Correlation coefficient (r) between numbers of microflora and organic acids per g of cecum contents

	Succinate	Lactate	Acetate	Propionate	n-Butyrate
Bifidobacterium spp.	-0.04	0.56**	-0.03	0.43*	0.58**
Lactobacillus spp.	-0.09	0.59**	0.02	0.48*	0.55**
Akkermancia muciniphila	0.07	-0.28	0.26	-0.42*	-0.27
Clostridium leptum	-0.22	-0.44*	-0.12	-0.30	-0.31
Clostridium coccoides	-0.04	-0.24	0.37	-0.25	-0.23
Enterobacteriaciae	-0.10	0.63**	-0.20	0.69**	0.48*

*P < 0.05, **P < 0.01

Table 3-7. Effects of dietary addition of amylase and Amano protease on the abundance of microflora in cecal contents of rats

	Control	0.1% Amano protease	Amylase[1] (×1)	Amylase[2] (×10)	Inactivated Amylase[2] (×10)
Bifidobacterium spp.	0.036±0.016	3.235±1.324*	0.011±0.003	0.015±0.005	0.004±0.001
Lactobacillus spp.	2.01±0.69	24.28±8.25*	0.97±0.22	2.40±0.38	1.10±0.31

Data: means ± standard error. *: Significantly different from the control group by Dunnett's multiple-range test ($P < 0.05$), n = 6

[1]Amylase (×1): Equivalent to the amount of amylase in the diet containing 0.1% Amano protease

[2]Amylase (×10): Ten-fold higher amount than the amount of amylase in the diet containing 0.1% Amano protease

Table 3-8. Effects of dietary addition of amylase and Amano protease on the abundance of microflora in cecal contents of rats

	Control	Alkaline protease[1] (×1.5)	Inactivated Alkaline protease[1] (×1.5)
Bifidobacterium spp.	0.173±0.062	0.099±0.012	0.106±0.079
Lactobacillus spp.	3.373±0.983	2.088±1.025	1.594±0.551

Data: means ± standard error, n = 6
[1]Alkaline protease (×1.5): 1.5-fold higher amount than the amount of alkaline protease in the diet containing 0.1% Amano protease

Chapter 4

Effects of dietary supplemental *Aspergillus* protease preparation on gut-protective amino acids and related metabolites in the cecum of rats

4.1 Brief introduction

Amino acids (AA) are confirmed as one of the main building blocks of protein synthesis and also play critical roles in other functions, such as cell signaling, gene expression, intracellular protein turnover, reproduction, oxidative stress, and immunity regulation (Wu 2009). As the major fuels for the small intestinal mucosa, AA are also important substrates for syntheses of intestinal proteins, nitric oxide, polyamines, and other products with enormous biological importance. Some kinds of AA may contribute to the intestinal homeostasis by their important roles in connection with apoptosis and proliferation of intestinal epithelial cells, expression in tight junction proteins, alleviation of inflammation and oxidative stress by inhibiting nuclear factor kappa-B (NF-κB) signaling pathway and activating nuclear erythroid-related factor 2 (Nrf2) signaling pathway (Song et al. 2016; Zhou et al. 2018). For example, threonine (Thr), one of essential AA, is a primary ingredient for intestinal IgA and mucins synthesis. Thus, the deficiency of Thr induced inflammation and influenced the immune responses through the NF-κB pathway (Zhang et al. 2017). In reverse, dietary Thr supplementation could promote intestinal health via regulating intestinal mucins and IgA expression, and microbial population of laying hens (Dong et al. 2017). The main AA and their metabolites that play important roles in keeping the intestinal homeostasis by several signaling pathways are shown in the Table 4-1.

In the chapter 2, I found that the addition of 0.2% Amano protease preparation markedly elevated cecal *Bifidobacterium* levels (Yang et al. 2015). The protease preparation contains several digestive enzymes, including acid protease (AcP), alkaline protease, and amylase. Subsequently, in the chapter 3, I demonstrated that dietary supplemental AcP derived from *Aspergillus* profoundly increased the cecal and fecal numbers of *Bifidobacterium* in rats. Such bifidogenic effect was not observed in the rats fed inactivated AcP and inactivated Amano protease preparation. In view of these facts, I hypothesized that a part of digestive enzymes in the protease preparation may

successfully pass through the tough condition of stomach and small intestine, and then influence the protein and AA metabolisms in the large intestine. Accordingly, in this chapter, I investigated the free AA profile alteration in the cecum contents of rats fed 0.1% Amano protease supplemented diet.

4.2 Methods and materials

4.2.1 Animals and diets

A total of 16 male Sprague–Dawley rats (3 weeks old) were purchased from the Hiroshima Laboratory Animal Center (Hiroshima, Japan) and maintained according to the "Guide for the Care and Use of Laboratory Animals" established by Hiroshima University. This study was approved by the Ethics Committee of Hiroshima University (Ethical approval No. C15-12). The rats were individually housed in an air-conditioned room at 23–24°C under a 12-h light/dark cycle (lights on from 08:00–20:00). Following acclimatization with a non-purified commercial rodent diet (MF, Oriental Yeast, Tokyo) for 7 days, the rats (mean body weight: 151 g) were randomly assigned to one of the two groups (n = 8 rats per group).

The composition of experimental diets was based on the 25% casein diet, a high-protein diet, indicated in Table 4-2. The groups of rats were randomly assigned to one of the two diets: a control diet (25% casein diet) or 0.1% Amano protease supplemented experimental diets.

During the feeding period, all rats were allowed free access to assigned diets and water. The weight of diets consumed were recorded each day during the feeding study. The weight of spilled diet was recorded daily and appropriately incorporated in calculations of food intake. At the end of feeding period, rats were euthanized by decapitation following anaesthesia (13:00–15:00) with inhalation exposure of isoflurane for about 20–30 sec in total in the desiccator to reduce the suffering. The cecum was immediately excised, and its contents were completely removed, weighed, and stored at −80°C until subsequent analysis of AA. Immediately after collecting the contents, a portion was used for DNA extraction.

4.2.2 Measurements

The analysis of microflora was performed according to the method described in the chapter 3. To determine the protein concentrations and free AA in the cecal contents, 100 mg contents were homogenized well in 4 volumes of distilled water. The

homogenate was centrifuge at 12,000 × g for 10 min at 4°C, then the supernatant was collected in duplicate for each sample. One portion was directly subject to analysis the protein concentrations with use of DC™ Protein Assay (Bio-Rad, Life Science) according the protocol. Another portion of supernatant was deproteinized with the same volume of 3% sulfosalicylic acid, then centrifuge at 12,000 × g for 10 min at 4°C. The collected supernatant was filtered and subjected to Amino Acid analyser (JLC-500/V2, JEOL, Tokyo, Japan).

4.2.3 Statistical analysis

All values are expressed as means with their standard error (SE). Statistical analysis was evaluated by Student's t-test. Some data were analyzed using Sperman rank correlation analysis. For all tests, $P < 0.05$ was considered statistically significant.

4. 3 Results

4.3.1 Characteristics of the groups

The final body weights and food intake in the control and experiment groups were not different (Table 4-3). The wet weight of cecal content in 0.1% Amano protease was much greater than in the control group (2.02-fold, $P < 0.01$).

4.3.2 Cecal microflora

When expressed as bacterial numbers per gram of cecal contents, the level of total bacteria was no significantly different in the experiment group compared to that in the control group. The level of *Bifidobacterium* per gram of cecal contents in the experiment group significantly increased compared to that in the control group (5.52-fold, $P < 0.05$). There were no changes in the level of *Lactobacillus* between the two groups when expressed as bacterial numbers per gram of cecal contents. The proportion of *Bifidobacterium* was significantly higher in the Amano protease group compared to the control group. The proportion of *Lactobacillus* has a tendency to increase in Amano protease group.

4.3.3 Cecal free amino acids and related metabolites

Amano protease was shown to have a strong effect on the concentrations of free amino acids (AA) and related metabolites (Table 4-4 and Table 4-5). For the essential AA, Amano protease supplementation significantly increased the concentrations of valine (Val), Thr, and histidine (His). For the non-essential AA, Amano protease

supplementation significantly increased the concentrations of alanine (Ala), glycine (Gly), Asparagine (Asp), and proline (Pro). However, the concentration of arginine (Arg) was markedly decreased by Amano protease supplementation (-83%, $P < 0.05$). The concentrations of Lys were slightly decreased by Amano protease (-28%, $P < 0.05$). Free cysteine (Cys) and phenylalanine (Phe) were undetectable in the control group, but detectable in the Amano protease group. For the AA related metabolites, Amano protease supplementation significantly increased the concentrations of taurine (Tau), γ-aminobutyric acid (GABA), ornithine (Orn), and phosphoserine (P-Ser). The concentrations of total free amino acids were greater in Amano protease group than that in the control group. In addition, total protein concentration has a tendency to be high in the cecum of Amano protease group. Therefore, the ratio of total amino acids to total proteins was significant higher in Amano protease group than that in the control group.

4.4 Discussion

As similar to my previous studies, consumption of Amano protease significantly elevated the level of cecal *Bifidobacterium*. Furthermore, by analysing the profile of free AA in cecal contents, this study showed that Amano protease supplementation markedly increased the concentrations of several kinds of free AA and related metabolites. In addition, the ratio of total free AA and total proteins were significantly increased by Amano protease supplementation. These alterations might be at least partially due to the higher protein digestion by Amano protease. However, the AA pattern appears to be quite different from the AA composition of casein (Zhaorigetu et al. 2007). Possibly, the AA profile in the cecum might be affected by the fermentation by intestinal microflora.

In this study, supplementation of Amano protease significantly increased 9 kinds of AA in cecal contents, including Val, Cys, Thr, Ala, Gly, Phe, Asp, His, and Pro. There is growing evidence for the beneficial effects of these AA on gut health. For example, a recent study suggested Cys exerts protective effects in the intestinal barrier that involves anti-inflammation and antioxidation by suppressing the NF-κB pathway and activating the Nrf2 signaling pathway (Song et al. 2016). Dietary Thr supplementation promotes intestinal health via regulating intestinal mucins and IgA expression, and microbial population of laying hens (Dong et al. 2017). Ala was demonstrated to stimulate the antioxidant defense proteins such as heme oxygenase-1 and ferritin, and

exert cytoprotective effects in human endothelial cells (Grosser et al. 2004). Gly improves chemical-induced diarrhea and intestinal mucosal barrier, and prevented the increases of IL-1β and TNF-α production (Tsune et al. 2003; Li et al. 2016). Phe combined with chromium has a protective effect against IBD induced by indomethacin in rats, which might be attributed to antioxidant and anti-inflammatory characteristics of Phe (Nagarjun et al. 2017). Asp improves intestinal integrity, and attenuates intestinal injury may by inhibiting TLR4 and NOD signaling (Chen et al. 2016; Wang et al. 2017). His supplementation alleviates colitis of murine by suppressing the generation of proinflammatory cytokines by inhibiting the activation of NF-*κ*B (Andou et al. 2009). Pro supplementation plays important roles in regulating the proliferation and differentiation of intestinal epithelial cells, increasing superoxide dismutase (SOD) activities, and expressions of tight junction proteins (Wu et al. 2011; Kang et al. 2014). These increased free AA by Amano protease supplementation may directly contribute the gut health via their different ameliorative effects.

On the other hand, cecum Arg levels were markedly decreased by dietary Amano protease. Arg is also suggested to exhibit protective functions against colon damage (Farghaly and Thabit 2014; Liu et al. 2017), however, the adverse effects of Arg supplementation on the gut were also reported (Grimble 2007). Thus, it is not clear if the decreased Arg by Amano protease supplementation is favorable or harmful for gut health.

In addition, 4 kinds of AA related metabolites, including Tau, GABA, Orn, and P-ser, were elevated by Amano protease supplementation. Among them, Tau and GABA are well-known for their favorable effects on gut health. Tau plays a wide range of critical roles in both human and animal physiology, including functions in antioxidation, osmoregulation, bile acid conjugation, regulation of blood pressure, maintenance of retinal and cardiac function, regulation of neuroendocrine activity, and prevention and treatment of fatty liver disease (Ruiz-Terán and Owens 1996). A recent study indicated Tau could regulate the gut microbiota by inhibiting the growth of harmful bacteria, accelerate the production of SCFA and reduce lipopolysaccharide (LPS) concentration (Yu et al. 2016). Meanwhile, there is no study indicating the dietary modulation of intestinal free Tau levels. To the best of my knowledge, this study provides the first evidence for the increase in intestinal free Tau by dietary manipulation. GABA is the main inhibitory neurotransmitter of central nervous system and is significantly involved

in regulating many physiological and psychological processes. The administration of GABA could alleviate hypertension, neurological disorders, and regulate sensation of pain and anxiety, and lipid levels in serum (Dhakal et al. 2012). Some microbial species belong to lactic acid bacteria, such as *Bifidobacterium* and *Lactobacillus* have the potential to synthesize GABA (Barrett et al. 2012). GABA-producing gut microbiota were demonstrated to modulate visceral sensitivity in the intestine (Pokusaeva et al. 2017). In this study, the concentration of GABA was not detectable in the control group, but detected in Amano protease group. Thus, it is easy to speculate that Amano protease supplementation may increase the level of GABA-producing gut microbiota. Orn is an essential component of the urea cycle and the ammonia-detoxifying system in the liver. In addition, several human studies have indicated some beneficial effects of Orn by attenuation of fatigue evoked by exercise or alcohol consumption (Sugino et al. 2008; Kokubo et al. 2013), stimulation of growth hormone release (Demura et al. 2010), and improvements in sleep disturbance (Horiuchi et al. 2013). However, there is no study have been published for the beneficial effect of Orn on colon health.

Recent *in vitro* study indicated cysteine specifically stimulates the growth of *Bifidobacterium bifidum* (Ferrario et al. 2015). In this study, Cys was detected in the Amano protease group, but not in the control group. In addition, cecum levels of Thr, His and total AA significantly correlated with the numbers of *Bifidobacterium* (data was not shown). Thus, it seems possible that the alterations in these AA might partially relate to the increase in *Bifidobacterium* by Amano protease. However, the information appears to be not enough for the explanation of the remarkable bifidogenic effect of Amano protease. Thus, further study is necessary to investigate the underlying mechanisms of the bifidogenic effect.

In this study, there were significant correlation among several AA and related metabolites. This is not surprising results because several lines of studies indicated these metabolites are converted to each other metabolites by microflora in the large intestine of human and rodents (Dai et al. 2011). Cys is utilized for the biosynthesis of Tau in prokaryotes (Agnello et al. 2007). Possibly, the increased Cys levels in the Amano protease group may relate to the increased Tau levels. Tau also exists as forms of taurine conjugations with several bile acids in intestine. Thus, the levels of taurine conjugated metabolites remain to be studied. Our study implies the possibility that intestinal fermentation is associated with higher Tau in the cecum. This raises a question

if intake of typical prebiotics such as fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS) is also able to increase intestinal Tau levels, which in turn leads to a favorable impact on the colon health.

4.5 Summary

This study indicated consumption of Amano protease significantly increased cecum numbers of *Bifidobacterium* per gram contents without affecting those of *Lactobacillus*. Remarkably, Amano protease intake have an impact on intestine by modulating cecum free AA and related metabolites. The functions of these free AA metabolites in intestine are considered to be associated with gut health by improving intestinal barrier and integrity, suppressing oxidative stress and inflammation (Fig 4-1). Therefore, through increasing cecal *Bifidobacterium* and modulating free AA metabolites, Amano protease may exert favourable effect on gut health. However, it is necessary to elucidate the relationship between the alteration of gut bacteria and the modified free AA metabolites in the future.

4.6 Tables and figure

Table 4-1. The functions and signalling pathway of amino acids and related metabolites in intestinal homeostasis

Amino acids	Subjects	Key functions	Signaling pathways	Reference(s)
Leucine	Mice/ Piglets	Enhances mucin production and intestinal integrity; suppresses intestinal inflammation	mTOR, GCN2, NF-κB, MAPK	(Mao et al. 2015; Ravindran et al. 2016)
Isoleucine	Human intestinal epithelial cells	Increases anti-microbial peptides β-defensins	GPCRs, ERK	(Youkou et al. 2012)
Methionine	Piglets	Improves intestinal integrity, cysteine and glutathione	NF-κB	(Chen et al. 2014)
Cysteine	Piglets	Reduces intestinal permeability and cell apoptosis; inhibits TNF-α, IL-6, IL-12p40, and IL-1β; enhances *Bifidobacterium* growth	mTOR, NF-κB, Nrf2	(Kim et al. 2009; Ferrario et al. 2015)
Arginine	Mice/ Piglets	Improves clinical parameters and body weight loss; reduces colonic permeability and proinflammatory cytokine	iNOS	(Coburn et al. 2012)

Threonine	Rats/ Pigs	Increases IgG, IgA, and mucin production; decreases IL-6	mTOR, NF-κB, MAPK	(Faure et al. 2006; Wang et al. 2006)
Alanine	Human endothelial cells	Stimulates the antioxidant defense proteins heme oxygenase-1 and ferritin; exerts cytoprotective effects	Unclear	(Grosser et al. 2004)
Glycine	Rats/ Piglets	Diarrhea improvement, prevention of the increases of IL-1β and TNF-α, improvement of intestinal mucosal barrier	NF-κB	(Tsune et al. 2003; Li et al. 2016)
Serine	Mice/ Intestinal porcine epithelial cell	Increases barrier function; reduces inflammation, oxidative stress, and permeability	AMPK	(Zhou et al. 2017)
Asparagine/ Aspartate	Piglets	Improves intestinal integrity; inhibits proinflammatory cytokines	NF-κB, MAPK	(Chen et al. 2016; Wang et al. 2017)
Glutamate	Piglets/ Porcine epithelial cells	Improves intestinal mucosa morphology and tight junction proteins; reduces oxidative stress	Unclear	(Wu et al. 2012; Jiao et al. 2015)
Phenylalanine	Rats	Exerts anti-inflammatory, inhibits TNF-α, IL-6, and oxidative stress; modulates gut hormone	CaSR	(Alamshah et al. 2017; Nagarjun et al. 2017)
Histidine	Mice	Reduces histologic damage, IL-6, and TNF-α production; inhibits NF-κB	NF-κB	(Andou et al. 2009)

Proline	Piglets	Increases SOD activity; improves small-intestinal morphology	mTOR	(Wu et al. 2011; Kang et al. 2014)
Tryptophan	Piglets	Ameliorates clinical symptoms; reduces gut permeability and cell apoptosis; inhibits TNF-α, IL-6, IL-12p40, INF-γ, IL-1β, and ICAM-1	mTOR, CaSR, MAPK	(Kim et al. 2010)
Taurine	Mice	Suppresses TNF-α and IL-1β; inhibits harmful bacteria, reduces LPS, and increases SCFAs	NF-κB	(Wang et al. 2013; Yu et al. 2016)
GABA		Modulates the GI motility and inflammation	ENS	(Auteri et al. 2015)

IL: interleukin; NF-κB: nuclear factor-kappa-B; TNF-α: tumor necrosis factor; GCN2: general controlled nonrepressed kinase; CaSR: calcium-sensing receptor; iNOS: inducible nitric oxide synthase; IFN-γ: interferon-γ; mTOR: mechanistic target of rapamycin; MAPK: mitogen-activated protein kinase; AMPK: AMP-activated protein kinase; GPCRs: G protein coupled receptors; Nrf2: transcription factor; NF-E2-related factor 2; LPS: lipopolysaccharides; GABA: gamma aminobutyric acids; ENS: enteric nervous system

Table 4-2. Composition of experimental diets

Ingredient	Control (%, w/w)	Amano protease (%, w/w)
Beef tallow	30.00	30.00
Casein[1]	25.00	25.00
Vitamin mixture[2]	1.00	1.00
Mineral mixture[2]	3.50	3.50
Cellulose	5.00	5.00
Sucrose	20.00	20.00
Corn starch	15.50	15.17
Amano protease[3]		0.33

[1]Casein: net protein content, 87% (w/w).

[2]American Institute for Nutrition (AIN-93G).

[3]Amano protease: This powder contains 70% (w/w) dextrin.

Table 4-3. Effect of dietary Amano protease on cecum microflora

	Control	0.1% Amano protease
Initial body wt (g)	151±2	151±1
Gains in body wt (g/14 days)	119±3	105±6
Final body wt (g)	270±4	256±6
Food intake (g/14 days)	245±4	225±2
Cecum contents wt (g)	1.69±0.13	3.41±0.30*
(numbers/g wet cecal contents)		
Total bacteria ($\times 10^{10}$)	7.97±1.35	4.45±2.43
Bifidobacterium ($\times 10^{6}$)	0.31±0.18	1.71±0.39*
Lactobacillus ($x10^{8}$)	0.87±0.28	1.10±0.46
(% of total bacteria)		
Bifidobacterium (%)	0.006±0.004	0.286±0.066*
Lactobacillus (%)	0.314±0.189	1.653±0.708

Means ± SE (n =7~8). *Significantly different from control at $P < 0.05$ by Student's t-test.

Table 4-4. Effect of dietary Amano protease on cecum free amino acids

Amino acids	Control	0.1% Amano protease
Val (μmol/g contents)	0.75±0.08	1.41±0.20*
Leu (μmol/g contents)	0.86±0.08	1.28±0.18
Ile (μmol/g contents)	0.62±0.06	0.76±0.11
Met (μmol/g contents)	0.20±0.05	0.27±0.04
Cys (nmol/g contents)	ND	0.08±0.02
Arg (μmol/g contents)	0.12±0.02	0.02±0.01*
Lys (μmol/g contents)	0.89±0.08	0.64±0.09
Thr (μmol/g contents)	0.58±0.06	1.25±0.19*
Ala (μmol/g contents)	1.34±0.14	3.60±0.55**
Gly (μmol/g contents)	0.86±0.15	2.29±0.31**
Ser (μmol/g contents)	0.58±0.05	0.48±0.04
Asp (μmol/g contents)	0.04±0.01	0.14±0.02**
Glu (μmol/g contents)	3.26±0.23	3.23±0.47
Phe (μmol/g contents)	ND	0.11±0.03
Tyr (μmol/g contents)	0.43±0.04	0.36±0.06
His (μmol/g contents)	0.12±0.01	0.26±0.03**
Pro (μmol/g contents)	0.40±0.07	1.47±0.29**
Total free amino acids (μmol/g contents)	11.1±1.0	17.7±1.8**
Total protein (mg/g contents)	108±4	126±9
Total free amino acids/total protein (μg/mg)	13.2±1.2	17.9±0.9**

Means ± SE (n = 8). *, **Significantly different from control at $P < 0.05$ and $P < 0.01$, respectively, by Student's t-test. ND: Not detectable (< 0.001 μmol/g contents).

Table 4-5. Effect of dietary Amano protease on cecum amino acid relating metabolites

Amino acids relating metabolites	Control	0.1% Amano protease
Tau (μmol/g contents)	0.45±0.14	2.95±0.64*
GABA (μmol/g contents)	ND	0.93±0.13
Orn (μmol/g contents)	0.13±0.02	0.33±0.03*
P-Ser (μmol/g contents)	0.24±0.03	1.44±0.31*
NH_3 (μmol/g contents)	5.70±0.70	4.56±0.54

Means ± SE (n =8). *Significantly different from control at $P < 0.05$ by Student's t-test. ND: Not detectable (< 0.001 μmol/g contents).

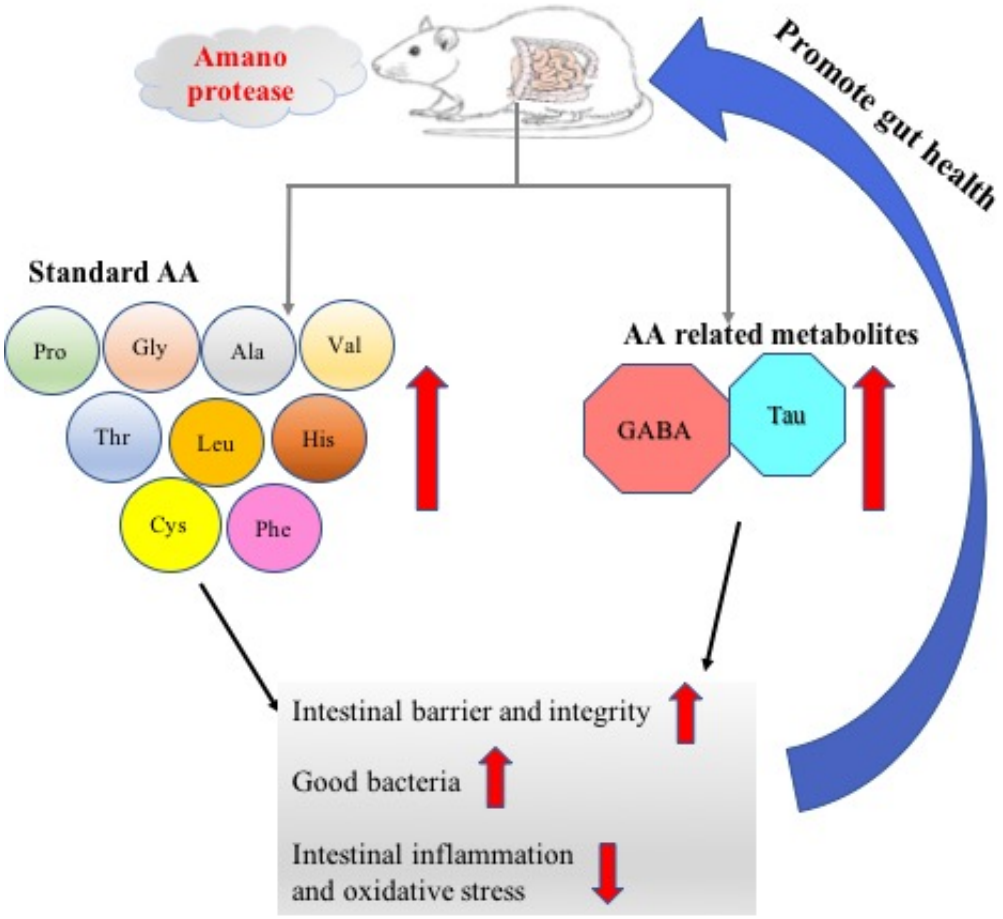


Fig 4-1. The tentative beneficial effects of Amano protease on the GI tract health by amino acids metabolism. AA, amino acids

Chapter 5

Conclusions

Aspergillus is a fungal genus contains more than 200 species found in various ecological niches. Some species of *Aspergillus*, especially *A. oryzae*, are extensively used in the fermented food production and other industries. The catalytic abilities of their secreted enzymes may help to increase biological and functional characters of some foodstuffs. In order to know whether the dietary of these enzymes have potential beneficial effects on mammals, here, I investigated the effects of *Aspergillus*-derived protease preparations on the colonic luminal environment in rats.

In the chapter 2, through the screening experiments, I found consumption of two kinds of *Aspergillus*-derived protease preparations could exert some extent favorable effects on colonic environment in rats. Amano protease supplementation markedly elevated the levels of cecal *Bifidobacterium* and *Lactobacillus*, which are commonly seen as beneficial for health. Furthermore, the concentrations of cecal n-butyrate, propionate and lactate, playing important roles in gut health, were increased by Amano protease and Orientase supplementation. In addition, dietary Amano protease elevated fecal IgA and mucins, which are indicators of intestinal immune and barrier functions. Taken together, these results suggest 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 Amano protease preparation contains several digestive enzymes, including acid protease, alkaline protease, amylase, etc. In the chapter 3, I hypothesized that the digestive enzymes in the protease preparation are responsible for the bifidogenic effect. Then, I tested this hypothesis by investigating whether cecal *Bifidobacterium* levels are elevated by the addition of the purified AcP at the dose equivalent to the level found in the 0.1% Amano protease diet, which is considered the effective dose for generating a bifidogenic effect. The results showed the cecal and fecal *Bifidobacterium* numbers were unaffected by supplementation with 0.0096% purified AcP at the level equivalent to the AcP amount used in the 0.1% Amano protease diet. However, intriguingly I found that the diet containing 4-fold higher AcP content (0.0384% AcP diet) than that used in

the 0.1% Amano protease diet significantly elevated the cecal *Bifidobacterium* and *Lactobacillus* numbers and the fecal *Bifidobacterium* numbers in rats. As expected from the results of increased cecal numbers of *Bifidobacterium* and *Lactobacillus*, lactate-producing bacteria, cecal lactate levels were also elevated by the 0.0384 % AcP containing diet.

These results are the first to prove that consuming small quantities of active AcP derived from *A. oryzae* exerts a prebiotic-like effect in rats. This finding may provide insights into novel applications of this protease as a functional non-nutrient food factor, e.g., a dietary supplement for colonic health. The study perhaps to challenge the current concept of prebiotic, and the *A. oryzae*-derived AcP may be considered as a new type of prebiotic. Furthermore, interestingly, only a very small quantity of AcP (0.0384%) can elevate *Bifidobacterium* levels compared with 5–10% of dietary oligosaccharides and some fibers necessary to elevate it in rats (Bielecka et al. 2002; Roller et al. 2004). After this study, I have failed to show any bifidogenic effect of purified alkaline protease and purified amylase, and their combinations in the Amano protease preparation even if the diets were supplemented with higher amount of such enzymes than in the 0.1% Amano protease diet.

Furthermore, accumulating evidence indicates several kinds of AA and their metabolites play critical roles in the intestinal homeostasis. In the chapter 4, I hypothesized that some parts of digestive enzymes in the Amano protease preparation may successfully pass through the tough condition of stomach, and then influence the protein and AA metabolism in the cecum. Accordingly, I investigated the free AA profile changes in the cecum contents of rats fed 0.1% Amano protease supplemented diet. Remarkably, Amano protease intake had a remarkable impact on cecal free AA profile by increasing 12 kinds of metabolites, including Val, Cys, Thr, Ala, Gly, Phe, His, Pro, Tau, GABA, Orn, and P-ser. Most of them exert positive and protective functions to intestinal health by improving intestinal barrier and integrity, suppressing oxidative stress and inflammation. Therefore, the results suggest supplemental Amano protease increases cecal several gut-protective AA and the related metabolites beneficial for colon health. On the other hand, cecum Arg levels were markedly decreased by dietary Amano protease. Arg is also suggested to exhibit protective functions against colon damage (Farghaly and Thabit 2014; Liu et al. 2017), however, the adverse effects of Arg supplementation on the gut were also reported (Grimble

2007). Thus, due to the controversial functions, it is not clear if the decreased Arg by Amano protease supplementation is favorable or harmful for gut health.

In conclusion, these studies suggest dietary *Aspergillus*-derived protease preparations exert prebiotic-like effects in rats by increasing the levels of *Bifidobacterium* and *Lactobacillus,* several gut-protective AA and the related metabolites, and fecal IgA and mucins. However, some limitations of these studies should be pointed out. First, these studies showed the prebiotic-like effect of supplemental Amano protease, but did not provide the specific mechanisms. Secondary, the *Bifidobacterium*-elevating effect of the diet containing 0.1% Amano protease preparation could not be explained by AcP used in the preparation. Therefore, identification of the active factors responsible for the bifidogenic effect of the Amano protease preparation remained to be elucidated. Thirdly, it is necessary to clarify the relationship between the gut bacteria alterations and the AA metabolites changes after the Amano protease intake in the colonic luminal environment.

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