Preparation of Bamboo Hemicellulose Hydrolysate Possessing Anti-oxidative Properties and Their Effects on Mice Plasma Cholesterol

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Bamboo hemicellulose hydrolysate (BHH) was extracted from bamboo by high temperature and enzymatic treatment. The ratio of total sugars in BHH was 75.7% on a dry matter basis (sugar components: 46.4% glucose, 12.7% arabinose, 40.9% xylose). These were mainly comprised of low molecular weight oligosaccharides containing ferulic acid. BHH exhibited strong anti-oxidative activities. In high fat (HF) diet-fed mice, 5% BHH supplementation significantly ameliorated increases in plasma cholesterol levels compared to the control mice fed HF diet alone. Also, while the fecal pH of control mice increased, that of test mice (5% BHH supplementation) did not increase to similar levels. Greater amounts of propionic acid were detected in the feces of HF diet-fed mice fed HF supplemented with BHH than one fed HF diet alone.

Keywords: bamboo hemicellulose hydrolysates, ferulic acid, anti-oxidant, cholesterol, short-chain fatty acids (SCFA)

Introduction

Moso bamboo (Phyllostachys pubescens), a gramineous plant, is one of the most abundant sources of biomass material in Asian countries. Particularly in Japan, bamboo is one of the most popular biomass-energy plants due to its abundant holocellulose content (almost 65 – 70%) and lower lignin content (< 20%) and faster growth compared to wood. However, converting mature bamboo materials into bio-ethanol remains challenging due to the high resource costs of energy conversion. Thus, in addition to using bamboo as a bio-ethanol material, new applications for bamboo are currently being developed (Peng and She, 2014).

Hemicellulose comprises almost 30% of the dry matter in so-called soft-biomass such as bamboo or nepiagrass, and is one of the most abundant components of the bamboo plant along with cellulose. Hemicellulose consists of a main chain of beta-1,4 xylan that branches into alpha-1,3-arabinofranose or 1,4-glucopyranosyl glucan, which in turn branch into alpha-1,6 -D-xylopyranose. These hemicellulose components are known to be health-supporting materials (Broekaert et al., 2011). For example, to control high blood pressure or decrease the LDH cholesterol concentration in serum (Kodama et al., 1996; Lopez et al., 1999), hemicellulose prepared from cereals such as rice or wheat bran was applied as a functional food. Shiiba et al. developed an efficient method of preparing hemicellulose oligosaccharides from wheat bran using a high-temperature treatment followed by an enzymatic reaction using cell-wall lytic enzymes, including cellulase and beta-xylanase (Shiiba et al., 1992; Shiiba et al., 1993). Additionally, it was reported that their hydrolysed oligosaccharides possessed a bifidobacterium or lactobacilli activation factor (Morishita et al., 1993). The anti-oxidative activity of feruloylated oligosaccharides prepared from cereal arabinoxylan has also been reported (Katapodis et al., 2003; Veenashri and Muralikrishna, 2011; Wang et al., 2011). These results suggest that bamboo hemicellulose materials also possess prebiotic effects and could possibly be applied as a functional food. Actually, immature bamboo has long been used as a healthy food material called as “Takenoko”, in Japan.

To apply bamboo hemicellulose as a functional food, cosmetic
or medical material, it is necessary to characterize the physiological properties and kinetic functions of bamboo hemicellulose hydrolysate (BHH). First, methods for the extraction and preparation of BHH were investigated. Second, to determine the physiological properties of BHH, an investigation of the relationship between the components and the structure and chemical properties of BHH was performed. Then, a high fat diet supplemented with BHH was fed to mice to investigate the effects of BHH on plasma cholesterol.

Materials and Methods

Moso bamboo and enzyme materials Milled moso bamboo tips (passed through 2 mm × 2 mm mesh) were generated from mature bamboo stems originally obtained from a field located in the most southern Japanese prefecture of Kagoshima.

Cell wall lytic enzymes containing cellulase and beta-xylanase, commercially called “cellulase Onozuka RS”, were purchased from Yakult Co. (Tokyo). These enzymes are derived from the fungus Trichoderma viride.

Preparation of hemicellulose hydrolysate from bamboo Bamboo hemicellulose hydrolysate (BHH) was prepared according to the modified method of Shiiba et al. (1992). Bamboo tips (100 g) were dried after being washed in a large volume of water, and then re-suspended in 100 mL of distilled water and autoclaved (121°C, 2.0 atm) for 20 min to sterilize and break apart the cell wall structure. After cooling, the suspension was incubated with 0.1% (w/w) of cellulose Onozuka RS at 50°C for 60 min. After the enzymatic reaction, the suspension was centrifuged at 3000 rpm (352 G) for 10 min. The supernatant was lyophilized and the obtained dry matter of crude hemicellulose oligosaccharides was stored at −20°C until use. Of the BHH components, the protein content was determined by Lowry’s method (Lowry et al., 1951) and compared with a standard curve of bovine serum albumin, and the carbohydrate constituents were analyzed by the following methods.

Determination of molecular weight distribution of hemicellulose hydrolysate from bamboo To determine the molecular weight distribution, the BHH solution (0.5%, w/w) was analyzed by an HPLC system (HT-1, Hitachi Ltd., Tokyo) equipped with a gel filtration column (TSKGEL G-3000 PWXL; 7.8 mm ID × 30 cm L, TOSOH Co., Tokyo) and RI detector under the following conditions: 50°C, super pure water elution at a flow rate of 1 mL/min, and UV detection at 320 nm. The concentration of total sugars was measured using the phenol-H₂SO₄ method (OD 490 nm) using glucose as the standard sugar (Dubois et al., 1956).

Sugar component analysis The sugar components in the crude hemicellulose or oligosaccharides were analyzed by HPLC after hydrolysis with 2 mol/L of TFA (trifluoroacetic acid) at 120°C in N₂ atmosphere for 1 h. An HPLC system (HT-1) equipped with an InertSustain NH2 column (4.6 mm ID × 250 mm L, GL Science Co., Tokyo) was used at 50°C with acetonitrile-water (80:20) elution at a flow rate of 1 mL/min to determine the sugar constituents. Sugar solution mixtures with specific concentrations of glucose, arabinose and xylose were used as the standard sugars. These procedures were performed according to the method reported previously (Shiiba et al., 1992; Shiiba et al., 1993).

Measurement of ferulic acid content The ferulic acid in the hemicellulose hydrolysate or oligosaccharides was analyzed by HPLC after hydrolysis with 2 mol/L of TFA at 120°C for 1 h. An HPLC system (HT-1) equipped with a LiChroCART 250-4 column (4.6 mm ID × 250 mm L, Merck KGaA, Darmstadt, Germany) was used at 50°C with formic acid-acetonitrile-water (10:13:70) elution at a flow rate of 1 mL/min and UV detection at 320 nm. Specific amounts of pure ferulic acid (Wako Pure Chemical Industries Ltd., Tokyo) were used as the standard (Katapodis et al., 2003).

Measurement of anti-oxidative activity The total anti-oxidative capacity of BHH was spectrophotometrically determined by a method using DPPH (2,2-diphenyl-1-pircylhydrazyl) (Tokyo Chemical Industry Co., Ltd., Tokyo) (Williams et al., 1995). The anti-oxidative activity was determined by the decrease in the spectrophotometric absorbance (517 nm) of the DPPH solution. Ascorbic acid (AA) was selected as the standard anti-oxidant. The anti-oxidative activities were converted into ascorbic acid concentration (µmol AA/mg).

Animals and diet intervention Two cycles of diet experiments were performed using two groups of two mice each (ICR; CLEA Japan, Inc). The young group mice were 5 weeks old, and the aged group mice were 54 weeks old, at the beginning of the experiment. Two male mice per cycle of the experiment were housed in a controlled environmental cage (12-hour daylight cycle) with free access to food and water. After two weeks of acclimatization to a standard diet (0 time), the mice were divided into two groups (n = 4/group): the control group (n = 4), which was fed a HF (high fat) diet for 4 weeks, and the test group (n = 4), which was fed a HF diet supplemented with 5% BHH. After 4 weeks of HF diet feeding, both the test and control mice were fed the same initial standard diet.

The components of both the standard and HF diets were...
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The standard diet (CLEA Rodent Diet CE-2) was composed of 8.9% moisture, 24.9% crude protein, 4.6% crude fat, 4.1% crude fiber, 6.6% crude ash and 51.0% nitrogen free extract (NFE), with 344.9 Kcal/100 g of total energy. The high fat diet (CLEA High Fat Diet 32, HFD32) was composed of 6.2% moisture, 25.5% crude protein, 32.0% crude fat, 2.9% crude fiber, 4.0% crude ash and 29.4% nitrogen free extract (NFE), with 507.6 Kcal/100 g of total energy. From the start of the experiment, the body weight of mice was measured weekly, a small blood sample was obtained from the tail vein, and fresh fecal samples were collected from each cage within 16 hours every 2 weeks. All blood and fecal samples (at 0, 2, 4, 6 weeks) were stored at −70°C before being used for the next experiment. Thus, the series of samples (at 2, 4 weeks) of the blood and fecal were obtained from mice fed high fat diet supplemented with/without 5% of BHH, the other samples (at 0 week and 6 weeks) were obtained form mice fed standard diet only.

All experiments in the current study were performed in triplicate. All experimental procedures were performed in accordance with institutional guidelines for animal research, and the study was approved by the animal ethics committee of Tokyo Denki University.

Plasma and faecal cholesterol measurement The total cholesterol from blood and faecal samples was measured using a Cholesteryl Ester Quantitation Kit (Bio Vision Inc. Milpitas, USA) coupling enzymatic reaction and spectrophotometric detection of reaction end-products. Because mice are HDL-dominant animal, the ratio of LDL/HDL had not been determined.

pH measurement of the faeces A shaking mixture was prepared with some of the faeces and 10 times the amount of distilled water. The pH values of the faeces solution samples were measured using a pH meter (DKK-TOA Co., Tokyo). To avoid experimental error, faecal pH was measured from ten fecal pieces (n = 10).

Organic acids measurements of the faeces The organic acids were extracted from fecal samples according to the modified method of Villalba et al. (2012). Fecal samples (0.1 g) were suspended in 1 mL of 0.5% orthophosphoric acid and frozen at −20°C for 16 h. After thawing under running water, the suspension was vigorously homogenized with 1 mL of ethyl acetate using a vortex mixer for 2 min. The homogenized sample was centrifuged at 18000 G for 10 min. A 5-μL aliquot of the ethyl acetate solution was injected into a gas chromatography system (GC-1413, Shimadzu Co., Kyoto) equipped with an Inert Cap FFAP capillary column (0.25 mm ID × 30 m L; GL Science Co.) and a flame ionization detector (FID). The column temperature was raised from 60°C to 240°C at 10°C/min. The injection temperature was 240°C. Identification was based on the amounts of acetic, propionic and butyric acids in the ethyl acetate solution. All experiments were performed in triplicate.

Statistical analysis The data were expressed as means ± standard error (SE) and analyzed by Tukey-Kramer’s multiple comparison post hoc test. The analysis was carried out using SSRI (version 1.03 for Windows, SSRI Co., Ltd., Tokyo). Differences were considered to be significant at P < 0.05.

Results and Discussion

Characterization of bamboo hemicellulose hydrolysate The prepared lyophilized hemicellulose hydrolysate from bamboo (BHH) was weighed, and its yield from the original bamboo was determined. A total of 2834 mg of BHH from 100 g of original bamboo was obtained. The ratio of total sugars in BHH on a dry matter basis was 75.7%, and the sugar components were 46.4% glucose, 12.7% arabinose and 40.9% xylose. Some ferulic acid (9.8 mg/g of BHH) was also present. The protein content measured by Lowry’s method was 20.2%. The anti-oxidative activity was 2.01 µmol AA/mg of BHH. This activity was greater than reported for bamboo leaf extract (1.51 µmol AA/mg; Hu et al., 2000) and corresponded to almost 35.4% pure ascorbic acid (5.6 µmol AA/mg).

The molecular distribution of BHH constituents is shown in Fig. 1. The elution profile generated using the RI detector showed that BHH contained a main component (peak a in Fig. 1 A) with a peak at 7.06 min and minor components (peak b in Fig. 1 A) with a broad peak at around 8.38 min. The molecular weight of each component was estimated using a calibration line (L) to obtain a value of almost 700 for the main component and about 600 for the minor components. On the other hand, the profile by UV detection at OD 320 nm suggested that BHH contained at least two compounds combined with ferulic acid, main (peak a' in Fig. 1 B) and minor compounds (peak c in Fig. 1 B), respectively. Comparing the two profiles (A and B), the compound at peak a would be almost identified with the compound at peak a' because of similar elution profiles with the same elution time. However, only a limited amount of compound at peak c with a peak time of 8.82 min, with an estimated molecular weight of about 560, was detected because an RI peak corresponding to peak c was not observed.

These results suggested that the BHH extracted from bamboo using high temperature and enzymatic treatment mainly consisted of oligosaccharides with a molecular weight of about 700, which could correspond to an oligosaccharide in bamboo such as O-(4-O-trans-feruloyl-α-D-xylopyranosyl)-(1→6)-D-glucopyranose (Ishii and Hiroi, 1990).

Sugar contents and anti-oxidative activities of bamboo hemicellulose hydrolysate The oligosaccharides in the BHH were fractionated by Sephadex LH-20 column chromatography. A total of 15 fractions were collected. Table 1 lists the total sugar contents and anti-oxidative activities in the fractions. The results indicated that BHH contained some types of oligosaccharides that exhibited anti-oxidative activities. The main oligosaccharides (fractions No. 1-4) eluted by water exhibited weaker anti-oxidative activity than

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the oligosaccharides (fraction No. 7-12) eluted by aqua-methanol or methanol, which showed stronger anti-oxidative activity than pure ascorbic acid. Katapodis et al. (2003) reported that glycoside derivatives of ferulic acid exhibited stronger anti-oxidative activities than ferulic acid itself because they possess increased hydrogen donor ability. However, additional structural studies to verify the detailed structure of these oligosaccharides are necessary.

Changes in plasma cholesterol level and body weight of mice fed HF diet with/without 5% BHH supplementation. For the young mice, 5 weeks old at the start of the experiment (shown in Fig. 2A), a higher plasma cholesterol level was induced by the HF diet compared to the standard diet. Mice fed the standard diet for 2 weeks (shown as 0 time in Fig. 2A) showed a plasma cholesterol level of 4.0 – 4.5 µg/μL, which was almost the same cholesterol level observed before the HF feeding test. While control mice fed a HF diet for 2 – 4 weeks showed an increased plasma cholesterol level of 5.5 – 6.8 µg/μL, mice fed HF diet supplemented with 5% of BHH maintained a plasma cholesterol level of 4.5 – 5.5 µg/μL. Moreover, after cessation of the HF diet (shown as 6 weeks in Fig. 2A), the plasma cholesterol level in the test mice decreased to the same value as initially (0 time), while that of control mice showed a higher level.

In the aged mice, 54 weeks old at the start of the experiment (shown in Fig. 2B), and similar to the young mice, a higher plasma cholesterol level was induced by the HF diet. While both groups of aged mice showed increased plasma cholesterol levels when fed a HF diet, BHH supplementation inhibited the increase in plasma cholesterol level. While the 4 weeks of HF diet feeding induced an increase in the total plasma cholesterol from about 3 to 10 – 12.5 µg/μL, supplementation with 5% BHH resulted in an increase from about 5 to 7 – 9 µg/μL. Even after cessation of the HF diet (shown as 6 weeks in Fig. 2B), the plasma cholesterol level of both groups did not reverse to the initial level (0 time).

Both the young and aged mice showed significantly increased body weight of almost the same amounts in the control and test mice, respectively (Fig. 3A, B). Though the HF diet apparently induced almost the same weight gain for both the young and aged mice irrespective of BHH supplementation, the plasma cholesterol level differed between groups fed HF diets with and without BHH supplementation. Similar results were previously reported for wheat arabinoxylan, a type of hemicellulose (Lopez et al., 1999; Neyrinck et al., 2011).

It was previously reported that in aged mice fed a HF diet, supplementation with long-chain wheat arabinoxylan polysaccharides, likely gramineous as well as bamboo hemicellulloses, inhibited increases in plasma cholesterol levels, while those fed HF diet alone showed significant increases in plasma cholesterol levels (Lopez et al., 1999; Lu et al., 2004; Hughes et al., 2007; Neyrinck and Delzenne, 2010). I added it into the References.

Notably, BHH supplementation significantly restricted increases in plasma cholesterol levels even though these bamboo hemicellulose hydrolysates are not long-chain polysaccharides such as wheat arabinoxylan, which is highly viscous and counteracts the increase in the plasma cholesterol levels caused by a HF diet.

Although the anti-oxidative activity of these compounds is
evident, it is fully established that in vitro anti-oxidative activity using DPPH has no direct relationship with the in vivo physiological activity. Further studies are needed to clarify the mechanism by which plasma cholesterol levels are ameliorated by BHH.

Comparison of fecal pH, colour and organic acid contents between mice fed HF diet with/without 5% BHH supplementation

The relationship between the bacterial flora and pH was investigated. At the start of the experiment, the feces of the young mice fed the standard diet were brown in color with a pH of 7.1 – 7.5 (Fig. 4A). While the HF diet changed the fecal color to light brown or white-brown with a pH of 8.0 – 8.5. BHH supplementation resulted in black or dark-brown feces with a pH of 7.8 – 8.1. For the aged mice, at the start of the experiment, the feces were brown in color with a pH of 7.5 – 7.9 (Fig. 4B), which was higher than that of the young mice. After being fed the HF diet, the fecal color changed to light brown or white-brown color and the pH increased to 8.3 – 8.8. However, with 5% BHH supplementation, the fecal color became black or dark-brown, and the pH changed slightly to 8.0 – 8.4. These changes in pH and color in both experiments using young and aged mice were almost the same as those observed in previous reports (Vardakou et al., 2007; Damen et al., 2011) of wheat arabinoxylans. The report noted that pH decreases upon feeding arabinoxylans were caused by the production of short-chain fatty acids, such as acetate, propionate or butyrate, and the promotion of specific bacterial growth.

The amounts of fecal organic acids in aged mice fed the HF diet with/without 5% BHH supplementation over a period of 4 weeks were compared. Acetic acid, propionic acid and butyric acid were detected in the feces of both the control and test mice. The amounts of acetic acid, propionic acid and butyric acid in the feces of mice fed the HF diet were 307 ± 12.5 µg/g, 378 ± 20.5 µg/g and 151 ± 22.5 µg/g fecal weight (mean ± SD), respectively. The amounts of acetic acid, propionic acid and butyric acid in the feces of mice fed the HF diet supplemented with 5% BHH were 315 ± 39.1 µg/g, 811 ± 55.5 µg/g and 389 ± 31.9 µg/g fecal weight (mean ± SD), respectively. Compared to the control, the test group contained a greater amount of fecal short-chain fatty acids (SCFA).
Notably, the fecal amount of propionic acid in the test mice (811 ± 55.5 µg/g) was almost two times greater than that of the control mice (378 ± 20.5 µg/g), and both propionic acid and butyric acid values differed significantly between the control and test groups (P < 0.01). It was suggested that the excess SCFA in the test feces might have caused a greater decrease in the pH compared to the control.

This suggests that BHH supplementation causes the activation of intestinal microorganisms, resulting in the production of organic acids such as acetic acid or propionic acid, as reported previously (Neyrinck et al., 2011). In particular, higher amounts of fecal propionic acid were found in the test compared to the control groups. Propionic acid, which is absorbed through the intestinal wall, primarily acts as a precursor for gluconeogenesis in that it helps reduce hepatic cholesterol synthesis (Cheng and Lai, 2000).

**Comparison of fecal cholesterol contents in mice fed HF diet with/without 5% BHH supplementation** The fecal cholesterol contents of aged mice fed HF diet alone (control) and with 5% BHH supplementation (test) were determined. Fecal cholesterol contents of test mice at 4 weeks were 542.1 ± 29.1 µg/g (mean ± SD). However, the fecal cholesterol content in the control mice was barely detected.

These results suggest that BHH can promote the production of SCFA and the excretion of cholesterol, however, further studies are necessary.

**Conclusion**

Bamboo hemicellulose hydrolysate (BHH) prepared by high temperature and enzymatic treatment contained oligosaccharides as the main component. BHH was characterized as a material containing ferulic acid with high anti-oxidative properties. BHH supplementation in mice fed a HF diet resulted in the amelioration of plasma cholesterol levels. Further, while the fecal pH in control mice fed HF diet alone increased, that in mice fed a HF diet supplemented with BHH did not increase to the same level. For fecal short-chain fatty acid (SCFA) determinations, higher amounts of total SCFA, especially propionic acid, were detected in the aged mice fed a HF supplemented with BHH compared to mice fed HF diet alone. It was suggested that the BHH diet maintained a lower pH in the intestinal environment through the increased production of SCFA.

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