In Vitro Digestibility and Fermentation of Mannooligosaccharides from Coffee Mannan

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Digestibility of mannooligosaccharides obtained from thermal hydrolysis of spent coffee grounds was examined by in vitro digestion method. Mannooligosaccharides were resistant to human salivary α-amylase, artificial gastric juice, porcine pancreatic enzymes and rat intestinal mucous enzymes. Fermentation products of mannooligosaccharides in human large intestine were estimated by in vitro fecal incubation method. Mannooligosaccharides were fermented by human fecal bacteria and the products of fermentation were short chain fatty acids. Acetic, propionic and n-butyric acids were the main short chain fatty acids as end fermentation products. These results suggest that mannooligosaccharides are indigestible saccharides and are converted to short chain fatty acids in human large intestine. The short chain fatty acids are thought to improve the large intestinal environment. Moreover, they are absorbed and utilized by the host as an energy source.

Keywords: coffee, mannooligosaccharide, digestibility, short chain fatty acid

Intestinal bacterial florae are closely related to human health conditions, and a predominance of Bifidobacterium is particularly considered to play a beneficial role as a host (Mitsuoka, 1982). Extensive studies have shown that various indigestible oligosaccharides, e.g. fructooligosaccharides (Hidaka et al., 1986) and xylooligosaccharides (Okazaki et al., 1990), improved host health by stimulating the propagation of a limited number of bacteria such as bifidobacteria in the intestine. For instance, short chain fatty acids produced by fermenting indigestible oligosaccharides lowered pH in the intestine; consequently, Bifidobacterium sp. became dominant strains and the growth of Bacteroidaceae was inhibited (Ohtsuka et al., 1989). Low pH in the intestine also improved constipation by stimulating the intestinal inner wall and promoting intestinal peristalsis (Yajima, 1984; Okazaki et al., 1990).

It is well known that coffee beans contain hemicellulose such as mannan and arabinogalactan (Bradbury & Halliday, 1990) and that water insoluble mannan remains in spent coffee grounds. β-1,4-D-mannobiose, β-1,4-D-mannotriose, β-1,4-D-mannotetraose and β-1,4-D-mannopentaose were fractionated from a mannoooligosaccharide mixture prepared from spent coffee grounds. Each mannoooligosaccharide was investigated for its effect on the growth of an established enterobacterial strain. Each mannoooligosaccharide was used by Bifidobacterium adolescentis, Lactobacillus acidophilus and Lactobacillus gasseri but not used by bad bacteria such as Clostridium perfringens and Escherichia coli. This suggested that mannoooligosaccharides had a potential to promote the improvement of healthful human intestinal microflora as prebiotics (Asano et al., 2001). Mannooligosaccharides were required to be undigested by digestive enzymes until reaching the large intestine to act as prebiotics. However, it is not clear how mannoooligosaccharides are hydrolyzed and utilized in vivo.

We here investigated the digestibility and fermentation products of mannoooligosaccharides in vitro to predict the fate of mannoooligosaccharides in the human digestive tract.

Materials and Methods

Preparation of mannoooligosaccharides Spent coffee grounds were dispersed in water at a concentration of 14% (w/w) and milled by a colloid mill until the particle size was less than 1.0 mm. The slurry was fed to a plug flow reactor (d 10.2 cm×l 1.22 m) by high-pressure steam where it had a residence time of 8 min. The slurry was hydrolyzed in the reactor that was kept at 220°C using a 6.35 mm orifice. The reaction was terminated by spouting the slurry through the orifice of the reactor to atmospheric pressure. The hydrolyzed slurry was filtered and the filtrate was the hydrolyzed product from spent coffee grounds. The hydrolyzed product was obtained in a 29% yield from these grounds.

The hydrolyzed product was decolorized by activated carbon powder (Umehachi; Taihei Chemicals, Osaka), and desalted by cation exchange resin (SKIB; Mitsubishi Chemicals, Tokyo) and anion exchange resin (WA30; Mitsubishi Chemicals, Tokyo). One hundred grams of monosaccharides and mannoooligosaccharide mixture were obtained from 300 g of the spent coffee ground hydrolyzed products.

In vitro digestion

Materials Mannooligosaccharides were fractionated in their respective molecular weights by active carbon chromatography with stepwise gradient of water, 2.5%, 5.0% and 10.0% (v/v) ethanol. Isolated β-1,4-D-mannobiose and β-1,4-D-mannotetraose were used in the in vitro digestion test. A control experiment was carried out using maltose (Wako Pure Chemicals, Osaka).

Digestion by human salivary α-amylase β-1, 4-mannobiose, β-1,4-mannotetraose and maltose were dissolved in 50 mM sodium maleate buffer (pH 6.0) at a concentration of 2.0%. Human salivary α-amylase (Sigma Chemical, St. Louis, MO) was dis-
solved in deionized water at an amylase activity of 20 units/ml. One unit will liberate 1.0 mg of maltose from starch in 3 min at pH 6.0 at 37˚C. Digestion was started by adding 0.5 ml of α-amylase to 1 ml of each substrate solution in a test tube and incubated at 37˚C for 4 h. Then the test tube was dipped into a boiling water bath for a few minutes to inactivate α-amylase (Kato et al., 1991). Oligosaccharides remaining after digestion were measured by high-performance liquid chromatography (HPLC). The results were shown as the average of three experiments.

Digestion by artificial gastric juice β-1,4-Mannobiose, β-1,4-mannotetraose and maltose were dissolved in water at a concentration of 4.0%. Artificial gastric juice was prepared according to Okazaki et al. (1991). Porcine pepsin used in this gastric juice was purchased from Sigma Chemical. Digestion was started by adding 0.5 ml of each substrate solution to 1 ml of artificial gastric juice in a test tube and incubated at 37˚C for 4 h. Then the test tube was dipped into a boiling water bath for a few minutes to inactivate pepsin. Oligosaccharides remaining after digestion were measured by HPLC and the results were shown as the average of three experiments.

Digestion by porcine pancreatic enzymes Porcine pancreatin (Wako Pure Chemicals) was dissolved in 50 mM potassium phosphate buffer (pH 8.0) at an amylase activity of 70 units/ml. One unit will liberate 1.0 mg of glucose from starch in 1 min at pH 5.0 at 37˚C. β-1,4-Mannobiose, β-1,4-mannotetraose and maltose were dissolved in the phosphate buffer as a concentration of 4.0%. Digestion was started by adding 0.5 ml of porcine pancreatin solution to 1 ml of each substrate solution in a test tube and incubated at 37˚C for 4 h; the test tube was then dipped into a boiling water bath for a few minutes to inactivate the pancreatin enzymes. Oligosaccharides remaining after digestion were measured by HPLC and the results were shown as the average of three experiments.

Digestion by rat intestinal mucous enzymes Rat intestinal acetone powder (Sigma Chemical) was used as mucous enzymes. The powder was dissolved in 50 mM of sodium maleate acetone powder (Sigma Chemical) was used as mucous enzymes. Oligosaccharides remaining after digestion were measured by HPLC and the results were shown as the average of three experiments.

Analysis Mannooligosaccharides were measured by HPLC. After deactivation of digestive enzymes, the incubation solutions were diluted 100 fold with water and centrifuged at 5200×g for 10 min. The supernatant was used for HPLC analysis after filtering through a 0.45 μm membrane filter (Advantec). HPLC was performed with a Shimadzu LC-10 device and a refractive index detector (Erma ERC-7512, Erma, Tokyo). A Merck-packed PolyspherOA KC column was used for anion exchange chromatography with 0.012 N H₂SO₄ elution. The flow rate was maintained at 0.4 ml/min; the sensitivity of detection was 1 μg/ml.

Results and Discussion Digestibility of β-1,4-mannobiose and β-1,4-mannotetraose as mannooligosaccharides was carried out in vitro digestion experiments. Initially we examined decomposition of oligosaccharides by saliva using human salivary α-amylase (Fig. 1). β-1,4-Mannobiose and β-1,4-mannotetraose were not decomposed during 4 h incubation, but maltose was decreased by about 40% after 4 h. Next we examined decomposition of oligosaccharides by artificial gastric juice (Fig. 2), and found that β-1,4-mannobiose, β-1,4-mannotetraose and maltose showed resistance to this juice. We then examined decomposition of oligosaccharides by a pancreatic enzymes using porcine pancreatin (Fig. 3). There was little decomposition of β-1,4-mannobiose, β-1,4-mannotetraose by porcine pancreatin, but maltose was decreased by about 10% after 4 h. Finally, our examination of the decomposition of oligosaccharides by intestinal mucous enzymes using rat intestinal acetone powder (Fig. 4) showed that β-1,4-mannobiose and β-1,4-mannotetraose were not decomposed during 4 h incubation. About 90% of maltose was hydrolyzed by rat intestinal mucous enzymes after 2 h incubation and about 99% of maltose was...
hydrolyzed after 4 h. In summary, β-1,4-mannobiose and β-1,4-mannotetraose did not undergo hydrolysis by human salivary α-amylase, artificial gastric juice, porcine pancreatic enzymes or rat intestinal mucous enzymes. These results suggested that manno-oligosaccharides were indigestible oligosaccharides. Therefore, it was expected that manno-oligosaccharides reached the large intestine following the digestive tract without digestion, and intestinal bacteria used them.

Indigestible oligosaccharides escape digestion and are then broken down by intestinal anaerobic bacteria. This microbial breakdown of carbohydrates is an anaerobic process that yields short chain fatty acids as its main end products together with the
gases hydrogen, methane and carbon dioxide. Concentrations of acetic, propionic, iso-butyric, butyric, iso-valeric and valeric acids in regions of the human large intestine were measured to seek evidence of the occurrence of microbial breakdown of carbohydrate in the human colon (Cummins et al., 1987). The *in vitro* human fecal incubation experiments were performed using fresh human feces obtained from seven healthy persons to predict fermentation end products of mannooligosaccharides in the large intestine. Because acetate, propionate, iso-butyrate, butyrate, iso-valerate and valerate were produced from carbohydrate by intestinal anaerobic bacteria, these short chain fatty acids in fecal cultures were determined by HPLC. β-1,4-Mannobiose or mannooligosaccharide mixtures were used for the substrates of fecal incubation and fructooligosaccharides were used as positive control. Fructooligosaccharides are known to be broken down to short chain fatty acids by intestinal bacteria (Hidaka et al., 1986; Gibson & Fuller, 1998; Hosoya et al., 1988). At first, carbohydrate contents in the fecal cultures were measured after 0 h, 4 h, 8 h, 15 h and 24 h to determine the time oligosaccharides disappeared (Fig. 5). In the cultures with a β-1,4-mannobiose or mannooligosaccharide mixture the carbohydrate disappeared after a 15 h incubation period. Both the β-1,4-mannobiose and mannooligosaccharide mixtures seemed to be broken down by fecal flora after 15 h under this experimental condition. On the other hand, carbohydrate was not detected in the fecal culture with fructooligosaccharides after 8 h of incubation. The time course of mannooligosaccharide disappearance in the culture was different from that of fructooligosaccharides. Fructooligosaccharides were broken down faster than mannooligosaccharides by fecal flora. The difference of the time course seemed to be due to differences in species and quantity of bacteria in the feces which utilized each oligosaccharide (Hidaka et al., 1986; Asano et al., 2001).

Determination of short chain fatty acids as the fermentation end products were carried out at the time when oligosaccharides disappeared. Detailed composition of these short chain fatty acids is given in the Table 1. Acetic, propionic and n-butyric acids were main fatty acids in the fecal fermentation with mannooligosaccharides or fructooligosaccharides. Compositions of short chain fatty acids showed some scatter between the volunteers, and the variance in individual composition was apparently due to the difference of intestinal microflora. The variation in individual quantity of total short chain fatty acids was minimal, however. In addition, the quantity of short chain fatty acid that was produced from 1.0 g of each oligosaccharide showed almost the same value.

The short chain fatty acids acetate, propionate and butyrate produced in the large intestine by intestinal bacteria lower pH in the large intestine. It is known that low pH in the intestine suppresses the production of putrid substances (Mitsuoka, 1982), and promotes mineral absorption (Rémésy et al., 1993; Chonan & Watanuki, 1995). Propionate and butyrate also improved constipation by stimulating the intestinal inner wall and promoting intestinal peristalsis (Yajima, 1984; Okazaki et al., 1990). Therefore, the fermentation products of mannooligosaccharides prepared from coffee are expected to show these physiological functions.

Short chain fatty acid are also absorbed and subsequently utilized by host as a substrate of energy metabolism. Indigestible oligosaccharides seem to contribute to host as energy resources after broken down to short chain fatty acids in large intestine. Hosoya et al. (1988) investigated utilization of fructooligosaccharides by an anaerobic incubation of [U-14C]fructooligosaccharides with human feces *in vitro*. They attempted to estimate energy of fructooligosaccharides from quantity of short chain fatty acid that generated by fecal incubation. Okada et al. (1990) also estimated energy of pullulan, microbial polysaccharide, from amounts of short chain fatty acid that were end products of *in vitro* human fecal incubation. It is interesting to grasp how short chain fatty acids produced from mannooligosaccharides are metabolized as energy resources in the human body. Therefore, we attempted to estimate energy of mannooligosaccharides from quantity of short chain fatty acids produced by *in vitro* human fecal incubation in the same manner. Short chain fatty acids produced from 1.0 g of oligosaccharide were shown in Table 1. Availability of short chain fatty acids in the human body has not been elucidated except acetic acid. We assumed that availability of all short chain fatty acids were equal to that of acetic acid. Availability of acetic acid is 69% (Kagawa, 1995). Estimation of energy was calculated using the following equation:

$$\text{Energy of oligosaccharides} \approx \sum \{(\text{quantity of each short chain fatty acid (g)}) \times (\text{availability of short chain fatty acids (69%)}) \times (\text{energy of short chain fatty acids (kJ/g)})\}.$$ 

### Table 1. Short chain fatty acids (SCFA) produced in the *in vitro* fecal incubation.

<table>
<thead>
<tr>
<th>Carbohydrate in the culture (μg/ml)</th>
<th>β-1,4-Mannobiose mixture (mg)</th>
<th>Mannooligosaccharide mixture (mg)</th>
<th>Fructooligosaccharide mixture (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td>361.0 ± 69.9</td>
<td>248.7 ± 69.9</td>
<td>339.8 ± 54.1</td>
</tr>
<tr>
<td>Propionate</td>
<td>100.5 ± 34.5</td>
<td>118.7 ± 30.1</td>
<td>108.5 ± 43.4</td>
</tr>
<tr>
<td>n-Butyrate</td>
<td>145.3 ± 33.3</td>
<td>136.1 ± 42.4</td>
<td>191.1 ± 31.1</td>
</tr>
<tr>
<td>iso-Butyrate</td>
<td>12.3 ± 10.4</td>
<td>11.5 ± 12.7</td>
<td>21.5 ± 10.6</td>
</tr>
<tr>
<td>Valerate</td>
<td>15.1 ± 22.7</td>
<td>16.3 ± 20.3</td>
<td>9.6 ± 14.1</td>
</tr>
<tr>
<td>iso-Valerate</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.4 ± 0.8</td>
</tr>
<tr>
<td>Total SCFA</td>
<td>634.2 ± 52.7</td>
<td>631.2 ± 60.9</td>
<td>670.4 ± 93.2</td>
</tr>
</tbody>
</table>

SCFA were measured after 15 h in the β-1,4-mannobiose and mannooligosaccharide mixture and after 8 h in the fructooligosaccharide. Figures represent the amounts of generated short chain fatty acids (mg) from one gram of each oligosaccharide. Values are means±standard deviations of seven volunteers.
The energy value of each short chain fatty acid, quoted from International Critical Tables, is as follows: acetic acid; 14.6 kJ/g, propionic acid; 20.8 kJ/g, butyric acid; 24.9 kJ/g, iso-butyric acid; 24.8 kJ/g, iso-valeric acid; 27.8 kJ/g. Estimated energy value of mannooligosaccharides and fructooligosaccharides was 7.9 kJ/g (1.9 kcal/g) and 8.7 kJ/g (2.1 kcal/g), respectively. These values are estimated under the assumption that the availabilities of all short chain fatty acids is equal to that of acetic acid. Determination of the availability of each short chain fatty acid in the human body is required to more precisely estimate the energy of mannooligosaccharides by this method.

In conclusion, mannooligosaccharides comprising one to six molecules of mannose were resistant to the digestive enzymes salivary α-amylase, gastric juice, pancreatin and intestinal mucous enzymes. Mannooligosaccharides were also fermented by human fecal bacteria and the products of fermentation were short chain fatty acids. It was assumed that mannooligosaccharides escaped digestion and were broken down to short chain fatty acids by intestinal anaerobes in the large intestine. Therefore, mannooligosaccharides can be expected to improve the intestinal environment. To study these physiological functions of mannooligosaccharides in vivo, experiments with animals and human are in progress.

References