Effects of DFA IV in Rats: Calcium Absorption and Metabolism of DFA IV by Intestinal Microorganisms

Katsuichi Saito, Toru Hira,* Takuya Suzuki,* Hiroshi Hara,* Atsushi Yokota, and Fusao Tomita†

Laboratory of Applied Microbiology, Department of Bioscience and Chemistry, Faculty of Agriculture, Hokkaido University, Sapporo 060-8589, Japan
*Laboratory of Nutritional Biochemistry, Department of Bioscience and Chemistry, Faculty of Agriculture, Hokkaido University, Sapporo 060-8589, Japan

Received September 24, 1998; Accepted November 27, 1998

Di-ß-fructose-2,6′:6,2′-dianhydride (DFA IV) is a disaccharide consisting of two fructose residues that can be prepared from levan by levan fructotransferase from Arthrobacter nicotinovorans GS-9, and it can be expected to have novel physiological functions from its unique structure. In this study, the effects of DFA IV on calcium absorption and the metabolism of DFA IV by intestinal microorganisms were studied in rats to examine the physiological functions of DFA IV.

The apparent calcium absorption in rats fed with DFA IV was significantly higher than that in the control rats, and it seems that calcium absorption had almost been completed at the end of the small intestine. DFA IV also increased the calcium absorption in vitro experiments, using everted jejunal and ileal sacs, and this result supports the finding obtained in the in vivo experiments. These results indicate that DFA IV may have a function for increasing the calcium absorption in the small intestine of rats. However, the effect in the large intestine could not be clearly observed because of the lack of calcium that reached there.

The results of analyses of organic acids in the cecal and colonic contents and of DFA IV in the fecal, cecal, and colonic contents showed that the metabolism of DFA IV by microorganisms in the large intestine progressed gradually, and that DFA IV was converted mainly to acetate, butyrate, and lactate.

Key words: DFA IV; calcium absorption; rats

It has been reported that oligosaccharides have various physiological functions.1-3 The effects of oligosaccharides on the calcium absorption in the small and large intestines and on the metabolism of oligosaccharides by intestinal microorganisms have been studied in the respect of the prevention of osteoporosis and improvement of the intestinal microorganism population.3-7 These reports have indicated that the small and/or large intestines were important for increasing the calcium absorption caused by oligosaccharides. It was also considered that calcium absorption in the large intestine was related with organic acids produced by intestinal microorganisms from oligosaccharides; i.e., there was a relationship between calcium absorption and the metabolism of oligosaccharides by intestinal microorganisms.

Di-ß-fructose-2,6′:6,2′-dianhydride (DFA IV) is a non-reducing and indigestible disaccharide consisting of two fructose residues, and can be expected to have novel physiological functions from its unique structure. The physiological functions of DFA IV have not been studied due to its limited availability. We have reported the effective production of DFA IV from levan by levan fructotransferase from Arthrobacter nicotinovorans GS-9.14,15 By establishing a mass-production method for DFA IV, studies on its physiological functions became possible for the first time.

We have already reported the effect of di-ß-fructose-1,2′:2,3′-dianhydride (DFA III),16 a constitutional isomer of DFA IV, on calcium absorption in rats, and it was shown that DFA III increased calcium absorption in the small and large intestines.17 In this paper, we report the effects of DFA IV on calcium absorption and the metabolism of DFA IV by intestinal microorganisms in rats.

Materials and Methods

Diets and animals. Male Sprague-Dawley rats (Japan SLC, Hamamatsu) 5 weeks old and weighing about 100 g were used in this study. The stock and test diets are shown in Table 1.

The rats were freely provided with tap water and the stock diet for one week, and were then divided into four groups (n = 7) according to body weight. Each group was given deionized water and one of four test diets for 2 weeks. The control diet had no oligosaccharide, and the other three respectively contained DFA IV (Fig. 1) produced by Arthrobacter nicotinovorans GS-9 from levan, DFA III (Nippon Beet Sugar Mfg. Co. Ltd., Obihiro), and raffinose (Nippon Beet Sugar Mfg. Co. Ltd.). These oligosaccharide were added at 30 g/kg to replace the same amount of sucrose in the control diet. CaCO3 and Cr2O3 (as an insoluble and non-absorbable marker for calculating the apparent calcium absorption) were also added at 7.5 g/kg and 0.5 g/kg to each diet. In the last week, wire-mesh anal cups18 were used to pre-

† To whom correspondence should be addressed. Fax: +81-11-706-4961; E-mail: ftomita@chem.agr.hokudai.ac.jp

Abbreviations: DFA IV, di-ß-fructose-2,6′:6,2′-dianhydride; DFA III, di-ß-fructose-1,2′:2,3′-dianhydride

NII-Electronic Library Service
vent coprophagy.

The rats were freely provided with deionized water and the stock diet for three weeks and were then starved for 24 hours before extracting intestinal segments.

The rats were individually housed in stainless-steel cages with mesh bottoms. The cages were placed in a room with a controlled temperature (22–24°C), relative humidity (40–60%), and light (lights on 8:00-20:00). The study design was approved by Hokkaido University Animal Committee, and the animals were maintained in accordance with the guidelines for the care and use of laboratory animals throughout this study.

**Animal experiment.** The body weight and food intake were measured every day. Feces collected by using wire-mesh anal cups during the last 3 days were weighed and milled after lyophilization. At the end of the experiment, the rats were anesthetized with sodium pentobarbital (Nembutal; 50 mg/ml of sodium pentobarbital; Abbott, North Chicago, IL, U.S.A.) and killed by bleeding from the abdominal aorta. The distal part of the ileum (0–10 cm proximal to the cecum), cecum, and colon were removed with their contents and then weighed, and the collected contents were stored at −80°C. The ileal, cecal, and colonic walls were washed with saline and weighed. The livers and kidneys were also removed and weighed.

**Everted sac experiment.** The rats (final body weight of 200±1 g; n = 16) were anesthetized with pentobarbital and killed by bleeding from the abdominal aorta. Four consecutive segments of 3 cm each were dissected from the upper (jejunum) and lower (ileum) half of the small intestine. The intestinal segments were each everted and ligated with surgical silk at one end. The other end of each sac was ligated immediately after the instillation of serosal fluid, and the sacs were incubated in individual tubes containing 5 ml of mucosal fluid at 37°C while shaking. The serosal fluid consisted of a 30 mM Tris- HCl buffer (pH 7.4), 125 mM NaCl, 4 mM KCl, 10 mM glucose, and 1.25 mM CaCl2. The mucosal fluid consisted of a 30 mM Tris- HCl buffer (pH 7.4), 125 mM NaCl, 4 mM KCl, 10 mM glucose, and 10 mM CaCl2. DTA IV (0, 50, 100, or 200 mm) was in the mucosal fluid. Both fluids were bubbled with a mixture of 95% O2 and 5% CO2, and warmed at 37°C for 10 min before being used.

**Analytical methods.** The analytical methods used for calculating the apparent calcium absorption in rats are the same as those reported in previous studies. To analyze the calcium and chromium concentrations in the diets, feces, and intestinal contents, the homogenized samples were wet-ashed according to the method described previously, after homogenizing the diets, feces, and intestinal contents with an appropriate amount of deionized water. The calcium concentrations of the ashed samples were measured by atomic absorption spectrophotometry (AA-6400F, Shimadzu, Kyoto, Japan) with an acetylene-air flame after appropriately diluting with 0.1 N HCl and 5 mg/ml of strontium (final concentration). The chromium concentrations of the ashed samples were measured by the same instrument after appropriate dilution with 0.1 N HCl. The concentration of strontium for analyses of the calcium concentration was determined to be 5 mg/ml by referring to the report on calcium determination in food samples by atomic absorption spectrophotometry, and it was preliminarily confirmed that this amount of strontium was enough to prevent interference by phosphate for the calcium determination.

To calculate the calcium solubility in the intestinal contents, homogenized samples were centrifuged (17,000 × g for 20 min at 4°C), and the concentration of calcium dissolved in each supernatant was measured by atomic absorption spectrophotometry as already described. These concentrations were taken to be the same as those in the liquid part of the intestinal contents.

The calcium concentration of the serosal fluid in the

---

**Table 1. Composition of the Stock and Test Diets (g/kg)**

<table>
<thead>
<tr>
<th></th>
<th>Stock diet</th>
<th>Test diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein</td>
<td>250</td>
<td>250</td>
</tr>
<tr>
<td>Sucrose</td>
<td>645</td>
<td>568.5</td>
</tr>
<tr>
<td>Maize oil</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Mineral mixture</td>
<td>40</td>
<td>28.5**</td>
</tr>
<tr>
<td>Vitamin mixture</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Granulated vitamin E</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Oligosaccharide</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>CaCO3</td>
<td>7.5</td>
<td></td>
</tr>
<tr>
<td>Cellulose</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Cr2O3</td>
<td>0.5</td>
<td></td>
</tr>
</tbody>
</table>

---

* The minerals in the stock diet were based on the AIN-76 mixture. This provided (mg/kg diet): Ca 4491, P 2997, K 3746, Mg 375, Fe 100, I 0.32, Mn 10.0, Zn 34.7, Cu 6.0, Na 6279, Cl 6542, Se 0.2, Mo 1.0, Cr 0.5, B 0.5, V 0.25, Sn 1.05, As 1.0, SI 20.0, Ni 1.0, F 2.72, and Co 0.2.

** The minerals in the test diet were based on the AIN-76 mixture, except for Ca.

† Casein (Alacid; New Zealand Dairy Board, Willowin, New Zealand)

‡ Retinyl palmitate (7.66 μmol/kg of diet) and ergocalciferol (0.0504 μmol/kg of diet) were added to the maize oil.

§ The vitamin mixture was prepared in accordance with the AIN-76 mixture, except that menadione and L-ascorbic acid were added at concentrations of 5.81 and 284 μmol/kg of diet.

† Vitamin E granules (Juveta; Eisai Co., Ltd., Tokyo, Japan) supplied 423 μmol of dl-α-tocopheryl acetate/kg of diet.

# The same amount of sucrose was added to the control diet instead of oligosaccharide.

† This provided 3000 mg of Ca per kilogram of the test diet.

† Crystalline cellulose (Aviceel; Asahi Chemical Industry Co. Ltd., Tokyo, Japan).

---

Fig. 1. Structure of di-α-Fructose-2,6′:6,2′-Dianhydride (DFA IV).
everted sacs was assayed with a kit (Calcium C-test, Wako Pure Chemical Industries, Osaka, Japan).

The amounts of organic acids in the cecal and colonic contents were measured as described previously.\textsuperscript{17} A TLC analysis of saccharides was done by method B as described previously,\textsuperscript{20} after homogenizing the diets, feces, and intestinal contents with an appropriate amount of deionized water.

All implements in this study, except for the metal ware, were washed in 4 N HNO\textsubscript{3} and rinsed in deionized water to avoid any calcium contamination.

**Calculations and statistics.** The apparent calcium absorption of the entire intestine and part of digestive tract (%) is given by 100 × [(Ca/Cr of diet - Ca/Cr of feces, contents of distal ileum, cecum, or colon)/(Ca/Cr of diet)]

The solubility of calcium (%) is given by 100 × [(calcium dissolved in liquid part of contents of distal ileum, cecum, or colon)/(total calcium of contents of distal ileum, cecum, or colon)].

Each value is shown as the mean with SE. The results were analyzed by one-way ANOVA, and the significance of differences between groups was evaluated by Duncan’s multiple-range test. Differences with \( p < 0.05 \) were taken to be statistically significant, and values not sharing a common superscript are significantly different.

**Results and Discussion**

**Effect of DFA IV on the apparent calcium absorption**

Body weight, food intake, and dry feces weight are shown in Table 2. These values and the weights of the kidney and liver (data not shown) were not significantly different among the groups, and it is considered that there were no differences in growth among the groups of rats.

The apparent calcium absorption is shown in Fig. 2. The values from the feces, as a percentage of the total calcium intake, were significantly higher in the rats fed on DFA IV (89.8\% ± 1.8), DFA III (91.4\% ± 2.4), and raffinose (89.2\% ± 2.4) than in the rats fed on the control diet (74.8\% ± 1.9), there being no significant differences among the groups fed on oligosaccharides.

The apparent calcium absorption at the end of the small intestine was also significantly higher in the three experimental groups than the control group, the values being especially high in the DFA IV and DFA III groups. These results indicate that DFA IV had the function of increasing calcium absorption in the small intestine.

While the apparent calcium absorption in the raffinose group increased gradually through the entire intestine, the apparent calcium absorption in the DFA IV, DFA III, and control groups didn’t increase through the large intestine. There are two possible reasons why this occurred. One is that DFA IV and DFA III weren’t metabolized by microorganisms in the cecum and colon, and calcium absorption didn’t increase any more like that in the control group. Another reason is that the amount of calcium that reached the large intestine was too small to consider its increased absorption in this part of the intestines, because of the strong effect of DFA IV and DFA III on calcium absorption in the small intestine. As shown in Table 3, DFA IV and DFA III were converted to organic acids in the large intestine and were fermented by intestinal microorganisms. Thus, it is presumed that calcium absorption in the DFA IV and DFA III groups was almost completed at the end of the small intestine, and that the effect in the large intestine could not be clearly observed because of the lack of calcium reaching there.

**Effect of DFA IV on the calcium absorption by using everted jejunal and ileal sacs**

In order to confirm the effects of DFA IV in the small

![Fig. 2. Apparent Calcium Absorption by the Entire Intestine and Parts of the Digestive Tract in Rats Fed with DFA IV and other Oligosaccharides.](image)

Each value is the mean of seven rats with SE shown as a vertical bar. Values not sharing a common superscript are significantly different at \( p < 0.05 \).

**Table 2.** Body Weight, Food intake, Dried Fecal Weight, and Weight of Cecal and Colonic Contents

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>DFA IV</th>
<th>DFA III</th>
<th>Raffinose</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight gain (g/14 days)</td>
<td>69.4 ± 6.1</td>
<td>69.9 ± 2.4</td>
<td>69.1 ± 1.8</td>
<td>69.5 ± 2.8</td>
<td>0.9991</td>
</tr>
<tr>
<td>Food intake (g/14 days)</td>
<td>214.9 ± 5.6</td>
<td>215.8 ± 6.7</td>
<td>215.0 ± 4.4</td>
<td>216.9 ± 8.0</td>
<td>0.9950</td>
</tr>
<tr>
<td>Fecal weight (g/3 days)</td>
<td>2.61 ± 0.14</td>
<td>3.13 ± 0.17</td>
<td>3.05 ± 0.24</td>
<td>3.00 ± 0.25</td>
<td>0.3036</td>
</tr>
<tr>
<td>Cecal content (g)</td>
<td>1.54 ± 0.20\textsuperscript{a}</td>
<td>2.07 ± 0.20\textsuperscript{b}</td>
<td>2.67 ± 0.26\textsuperscript{b}</td>
<td>3.20 ± 0.21\textsuperscript{a}</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Colonic content (g)</td>
<td>0.44 ± 0.08</td>
<td>0.62 ± 0.06</td>
<td>0.86 ± 0.19</td>
<td>0.49 ± 0.09</td>
<td>0.0722</td>
</tr>
</tbody>
</table>

DFA IV, di-\( \delta \)-fructose-2,6'-6,2'-dianhydride; DFA III, di-\( \delta \)-fructose-1,2'-2,3'-dianhydride.

Each value is the mean ± SE for seven rats. Values not sharing a common superscript in the same row are significantly different at \( p < 0.05 \).
intestine, the calcium absorption was evaluated by using everted sacs of the jejunum and ileum, the result being shown in Fig. 3. The amount of calcium in the serosal fluid increased linearly with increasing incubation time in this condition. In both sacs, the calcium absorption was two to three times higher when DFA IV was added to the mucosal fluid than with the sacs incubated without DFA IV, regardless of the DFA IV concentration.

It has been shown that a sugar alcohol, maltitol, increased calcium absorption only in the ileum in experiments with everted sacs, and it was considered that maltitol modulated the tight junction of the small intestine. The tight junction regulates the passive paracellular diffusion of calcium in the intestinal mucosa. It is presumed that DFA III also affected the tight junction and increased the calcium absorption through passive paracellular diffusion in the small intestine, judging from the increase in calcium absorption when using a high calcium concentration in the mucosal fluid (10 mM). DFA IV in this study also increased calcium absorption with the jejunal and ileal sacs when using 10 mM of calcium in the mucosal fluid, like the case of DFA III. Thus, DFA IV is also presumed to have increased calcium absorption in the small intestine by modulating the tight junction. Details of the mechanism of action of DFA IV and DFA III in the small intestine are being investigated.

![Diagram]

**Fig. 3.** Effect of DFA IV on the Calcium Absorption of Everted Sacs Prepared from the Jejunum and Ileum.

Symbols indicate the concentrations of DFA IV in the mucosal fluid. Each value is the mean of eight rats with SE shown as a vertical bar. Values not sharing a common superscript are significantly different at *p*<0.05.

### Table 3. Amounts of Organic Acids Produced in the Cecum and Colon

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>DFA IV</th>
<th>DFA III</th>
<th>Raffinose</th>
<th><em>P</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cecum</strong> (μmol/ccum content)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetate</td>
<td>54.4±6.9*</td>
<td>96.1±13.6°</td>
<td>95.5±19.7°</td>
<td>161.2±13.1°</td>
<td>0.0002</td>
</tr>
<tr>
<td>Propionate</td>
<td>23.5±5.3°</td>
<td>15.1±2.8°</td>
<td>21.2±5.8°</td>
<td>37.2±4.1°</td>
<td>0.0177</td>
</tr>
<tr>
<td>Butyrate</td>
<td>31.6±3.5°</td>
<td>52.7±8.0°</td>
<td>72.2±13.7°</td>
<td>131.2±19.0°</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Valerate</td>
<td>17.4±3.4°</td>
<td>34.3±7.3°</td>
<td>48.2±18.6°</td>
<td>137.1±23.3°</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Succinate</td>
<td>24.7±6.8°</td>
<td>26.6±3.5°</td>
<td>22.7±4.0°</td>
<td>123.8±21.9°</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Lactate</td>
<td>19.3±5.3°</td>
<td>53.0±7.5°</td>
<td>61.2±12.8°</td>
<td>52.4±6.6°</td>
<td>0.0077</td>
</tr>
<tr>
<td>Total</td>
<td>170.9±17.3°</td>
<td>277.8±31.5°</td>
<td>320.1±62.8°</td>
<td>643.1±53.2°</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>Colon</strong> (μmol/colon content)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetate</td>
<td>19.6±4.5°</td>
<td>49.9±7.1°</td>
<td>47.6±7.6°</td>
<td>25.3±9.0°</td>
<td>0.0113</td>
</tr>
<tr>
<td>Propionate</td>
<td>8.1±1.2°</td>
<td>17.6±10.2</td>
<td>14.6±8.9</td>
<td>3.0±2.3</td>
<td>0.2584</td>
</tr>
<tr>
<td>Butyrate</td>
<td>75.0±13.8</td>
<td>137.3±25.6</td>
<td>100.4±14.9</td>
<td>90.7±11.0</td>
<td>0.0086</td>
</tr>
<tr>
<td>Valerate</td>
<td>17.5±5.1</td>
<td>26.6±6.9</td>
<td>26.4±7.5</td>
<td>24.9±5.7</td>
<td>0.7262</td>
</tr>
<tr>
<td>Succinate</td>
<td>17.8±4.6</td>
<td>31.4±3.0</td>
<td>18.4±2.4</td>
<td>18.6±5.3</td>
<td>0.0626</td>
</tr>
<tr>
<td>Lactate</td>
<td>7.4±2.7</td>
<td>18.0±10.2</td>
<td>17.7±7.1</td>
<td>33.1±13.2</td>
<td>0.2872</td>
</tr>
<tr>
<td>Total</td>
<td>138.4±27.4</td>
<td>280.8±38.3</td>
<td>225.2±29.1</td>
<td>195.6±22.1</td>
<td>0.0199</td>
</tr>
</tbody>
</table>

DFA IV, di-o-fructose-2,6:6,2'-dianhydride; DFA III, di-o-fructose-1,2'-2,3'-dianhydride.

Each value is the mean±SE for seven rats. Values not sharing a common superscript in the same row are significantly different at *p*<0.05.
that these organic acids produced from DFA IV and DFA III might have enhanced calcium absorption in the large intestine, even though no effects on calcium absorption in the large intestine could be observed as already described. The results indicate that DFA IV and DFA III were utilized by different kinds of microorganisms and/or in different manners from raffinose.

**Relationships among organic acids, intestinal wall weight, pH, and calcium solubility in the small and large intestines**

It has been reported that organic acids produced from oligosaccharides caused an enlargement of the large intestine and increased calcium solubility after the low pH value of the contents, and that calcium absorption was increased in the cecum as a result of these effects. As shown in Fig. 5, the relationships among the organic acids, intestinal wall weight, pH, and calcium solubility are considered to agree well with these reports. In the case of the raffinose group, it is considered that the calcium absorption that was shown occurred mainly from such secondary effects after the rapid fermentation of raffinose in the cecum. In the groups of rats fed with DFA IV or DFA III in comparison with the raffinose group, organic acid production and decreasing pH progressed gradually through the intestines, and there was tendency for the wall weight of the colon and calcium solubility in the colonic content to be high.

These results and the organic acid production in the large intestine indicate that DFA IV and DFA III had effects on the enhancement of calcium absorption in the large intestine, even though the effects on calcium absorption in the large intestine were not clearly observed due to the lack of calcium that reached there.

**Metabolisms of oligosaccharides by intestinal microorganisms**

The remaining oligosaccharides in the diet, intestinal contents, and feces were analyzed by TLC (Fig. 6). DFA IV and DFA III were detected in all contents, including the feces, while raffinose was detected only in the ileal content. This result agrees with the productivity of organic acids. It was considered that DFA IV and DFA III were gradually metabolized by microorganisms.

TLC analyses detected two kinds of saccharides corresponding to fructose and levansaccharides in the ileal and colonic contents of rats fed with DFA IV, while no other oligosaccharides without DFA III and fructose were detected with any content of DFA III. It is also presumed that DFA IV was degraded to fructose through levansaccharides, whereas DFA III was taken directly into the cell by microorganisms without the formation of inulinose.

Improving the population and property of microorganisms in the intestines is one of the important physiological functions of oligosaccharides. As already indicated, DFA IV was utilized in a characteristic way by the intestinal microorganisms of rats, and it is expected that
DFA IV had different effects from those of other oligosaccharides. Studies on the effects of DFA IV and of DFA III on intestinal microorganisms are in progress.

Acknowledgment
The authors are grateful to Nippon Beet Sugar Mfg. Co. Ltd for kindly supplying DFA III and raffinose.

References


