

Ingestion of Difructose Anhydride III Enhances Absorption and Retention of Calcium in Healthy Men

Kyoko TOMITA,^{1,†} Takuya SHIOMI,¹ Yasuhide OKUHARA,¹ Akiko TAMURA,¹ Norihiro SHIGEMATSU,¹ and Hiroshi HARA²

¹Research Institute, FANCL Co., 12-13 Kamishinano, Totsuka-ku, Yokohama 244-0806, Japan

²Division of Applied Bioscience, Graduate School of Agriculture, Hokkaido University, Kita-9, Nishi-9, Kita-ku, Sapporo 060-8589, Japan

Received August 25, 2006; Accepted December 13, 2006; Online Publication, March 7, 2007

[doi:10.1271/bbb.60465]

We examined the effects of a nondigestible disaccharide difructose anhydride III (DFAIII) on calcium absorption and retention by means of a human balance study of single-blind crossover design. Twelve healthy male subjects ingested 250 mg of shell powder as calcium carbonate (corresponding to 100 mg of calcium) with or without 1.0 g DFAIII three times a day for 13 d. In the last 4 d as a balance period, all urine and feces were collected and evaluated for calcium excretion. The apparent calcium absorption (mg/d) and rate of absorption (%) were higher, and those of retention were much higher, in the DFAIII group than in the control group. Furthermore, serum osteocalcin increased after the experimental period in the DFAIII group but not in the control group. These results indicate that DFAIII ingestion enhances intestinal calcium absorption, which might be beneficial for bone metabolism.

Key words: difructose anhydride III; calcium absorption; balance study; bone metabolism; human

Calcium is an essential nutrient, and adequate calcium intake is recommended for the development of high peak bone mass and the prevention of osteoporosis.¹⁾ To improve nutritional status as to calcium, increases in intake are important, but intestinal calcium absorption is affected by various factors, such as the source of supplied calcium^{2,3)} and co-ingested food components.⁴⁾ The ability of calcium absorption is also influenced by age.⁵⁾ Therefore, it is necessary to consider the absorptive efficacy of ingested calcium together with adequate intake.

Several nondigestible carbohydrates, such as fructooligosaccharides, have been reported to promote calcium absorption in *in vivo* studies.^{6–10)} Difructose anhydride III (DFAIII; di-D-fructofuranose-1,2':2,3'-dianhydride)

has also been shown to enhance calcium absorption in both the small and the large intestine of rats.^{11–18)} DFAIII is a naturally occurring nondigestible disaccharide in the root of *Lycoris radiate*,¹⁹⁾ and is also found in caramels^{20,21)} and roasted chicory.²²⁾ This disaccharide has recently been manufactured from inulin with *Arthrobacter* sp. H65-7 fructosyltransferase^{23,24)} and is used as a food material. The mechanisms of promotion of calcium absorption with DFAIII have been identified as follows: (i) intact DFAIII stimulates paracellular calcium absorption in the small intestine, and (ii) DFAIII increases large intestine calcium absorption *via* short-chain fatty acid (SCFA) production.

The aim of the present study was to examine the effects of DFAIII ingestion on calcium absorption in human subjects. In a previous study, we observed temporal increases in urinary calcium excretion after an oral load of calcium with DFAIII, which suggests stimulation of DFAIII on intestinal calcium absorption in humans.²⁵⁾ However, this method was based on a positive correlation between intestinal calcium absorption and urinary calcium excretion in the short term, and results were affected by renal clearance of calcium. In the present study, we adopted a balance study in human subjects to examine the effects of DFAIII ingestion on calcium absorption.

Material and Methods

Test food and control food. DFAIII (>97%) was obtained from Nippon Beet Sugar (Tokyo). The test foods were composed of 1.0 g of DFAIII and 250 mg of scallop shell powder as calcium carbonate (corresponding to 100 mg of calcium). Control foods were composed of 250 mg of folded scallop shell powder only.

Subjects. We recruited volunteers. Twenty healthy

[†] To whom correspondence should be addressed. Fax: +81-45-820-3509; E-mail: tomita@fancl.co.jp

Abbreviations: DFAIII, difructose anhydride III; SCFA, short chain fatty acid; Ca, calcium; PTH, parathyroid hormone; Cre, creatinine

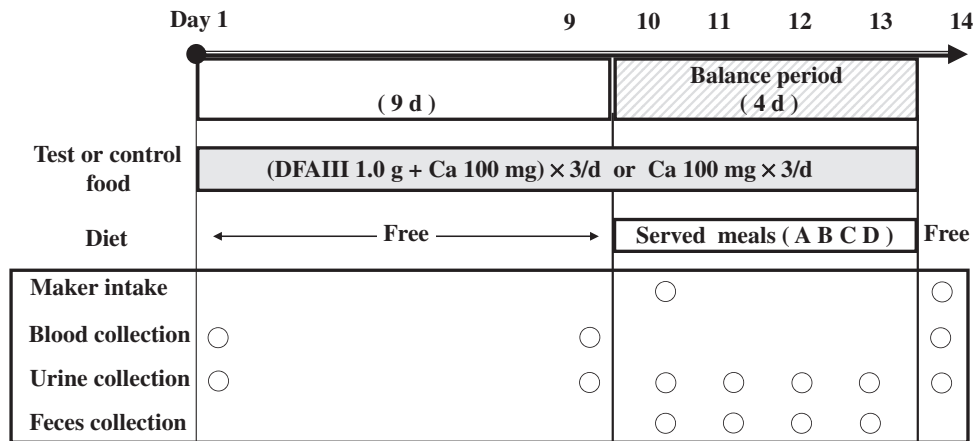


Fig. 1. Experimental Schedule.

Subjects ingested calcium or calcium and DFAIII for 13 d. After the first 9 d, the balance study was performed.

males agreed to participate in this study. The protocol complied with the Helsinki Declaration and received approval from the ethics committees of the Fancl Corporation. We informed all participants of the purpose and plan of this study and of their right to resign at any time of their own free will, and of our duty to guard their personal privacy against outsiders. All subjects satisfied the following conditions: (i) No history of daily intake of calcium-enriched foods or health foods to stimulate calcium absorption. (ii) No intake of vitamin D, vitamin K, or other drugs to treat or prevent osteoporosis, and no agents mediating bone metabolism. (iii) No history of bone-related disorders, diabetes, or other hepatic or renal function disorders. We finally selected 14 males by fecal condition and their volume.

Study design. The study was designed in a single-blind, crossover manner. The 14 subjects were divided into two groups. They ingested the test food or the control food three times a day before each meal for 13 d. The whole feces and urine were collected for the last 4 d (day 10–day 13) as a balance period. The subjects repeated the 13-d ingestion period with another test food after an 8-day washout period. The experimental schedule is illustrated in Fig. 1.

On the first day of the experiment (day 1), the subjects underwent a physical examination and health interviews with a physician, and blood and urine samples were collected for the initial value. The subjects were instructed to record their overall physical condition, stool frequency and condition, and the presence and severity of specific abdominal or any other symptoms every day on a specified log sheet. They did not have any dietary restriction during the first 9 d of the experimental period, without taking test food. The nutrient contents of their diets were analyzed for 3 d of this period by the previously reported method,²⁶⁾ taking photographs of their meals with a camera equipped with a personal digital assistant (PDA). The data were sent to dietitians *via* an attached mobile phone card for

calculation of the nutrients using computer software (Wellnavi; Matsushita Electric Works, Osaka, Japan).

From day 9, 1 d before the start of a balance period, to day 13, the subjects were kept in the hospital under a unified protocol with restrictions on movement out of the hospital, and abstinence from both alcohol and excessive exercise. They were served meals to control nutrition intake during the balance period. Four different meal sets were prepared for each experimental day. Each subject had breakfast at 9:00, lunch at 12:00, and dinner at 19:00. The menu contents and the nutrition compositions of the meals are shown in Table 1.

On day 10, subjects collected their urine before breakfast and ingested 0.5 g of coloring marker (carmine, Sigma, St. Louis, MO) with 50 ml of water to determine the start line of fecal sampling, then ingested the test food or control food with 100 ml of water (natural mineral water, Suntoryfoods, Tokyo). The subjects were allowed to drink this freely (recording the amount by themselves), and 24-h total urine and all fecal samples were collected for 4 d. The 4 d of collected urine for each subject was pooled, weighed, and stored at -20°C until analysis. Feces were collected in plastic bottles, freeze-dried, and stored at -20°C until analysis. Blood samples were also stored at -20°C . On day 14 (the first day of the washout period), the subjects again ingested 0.5 mg of carmine to determine the end line of feces sampling and underwent a physical examination and health interviews, and blood samples were collected.

Analytical methods. Duplicated meals were prepared and homogenized. The meals were dried at 110°C for 48 h, wet-ashed with nitric acid, and diluted adequately with hydrochloric acid solution. The calcium contents of the meals were analyzed by the inductively coupled plasma atomic emission spectrometry (ICP-AES) method. The fecal samples were pretreated the same way as for the meals, and the concentrations of calcium were measured by atomic absorption spectrometry (Hitachi 170-50A, Hitachi, Tokyo). Blood and urinary calcium

Table 1. Four Sets of Served Meals

	A	B	C	D
Breakfast	Boiled rice Miso soup Pork saute Namul (vegetable marinated with sesame oil) Fry boiled down in soy sauce	Bread Jam Tuna salad Fruit	Boiled rice Miso soup Grilled fish Boiled vegetables Steamed fish paste Roasted laver	Boiled rice Miso soup Fermented soybeans Pickles Boiled dried-radish strips
Lunch	Festive red rice Fried shrimp Spaghetti with neapolitan sauce Tartar sauce Sauteed vegetables Salad Pickles	Boiled rice Sliced beef boiled with vegetables Sauteed onion and spaghetti Food boiled down in soy sauce Cooked fried-bean curd and konnyaku Fruit	Boiled rice Pork cutlet with curry Macaroni salad Fruit Pickles	Boiled rice Meuniere fish Pickles Food boiled down in soy sauce Potato salad Fruit
Dinner	Boiled rice Grilled fish Grated radish Boiled spinach Tomato Simmered meat and potatoes White radish sprouts Fruit	Boiled rice Fish boiled with soy Pickles Boiled spinach Cooked spanish potato Food boiled down in soy sauce Slight soup Boiled hijiki (brown algae)	Chicken bowl Miso soup Vinegared cucumber Sauteed french bean Pickles Jelly	Boiled rice with fixing Roasted laver Tofu and ground meat dressed with miso Fried vegetable Egg roll Pickles Fruit
Total energy and nutrient contents of each meal set				
	A	B	C	D
Energy (kcal)	1,512.0	1,796.7	1,776.2	1,654.4
Protein (g)	66.6	79.7	54.8	58.3
Calcium (mg)	466.2	406.4	317.1	330.0
Magnesium (mg)	220.5	217.4	184.3	267.0
Zinc (mg)	9.8	7.2	7.1	6.2
Dietary fiber (g)	14.4	15.3	14.9	16.9

concentrations were measured by the OCPC method (Clinimate CA, Daiichi Pure Chemicals, Tokyo). Serum osteocalcin and plasma intact parathyroid hormone (PTH) levels were measured by immunoradiometric assay (BGP IRMA Mitsubishi, Mitsubishi Kagaku Iatron, Tokyo; Allegro Intact PTH kit, Nichols Institute Diagnostics, San Clemente, CA). Calcitonin and 1,25-dihydroxyvitamin D₃ were analyzed by radioimmunoassay (Calcitonin RIA Mitsubishi, Mitsubishi Kagaku Iatron, Tokyo; 1,25-dihydroxyvitamin D₃ RIA kit, TFB, Tokyo). Urinary deoxypyridinoline was measured by the CLIA method (ChemilumiACS-DPD, Sumitomo Pharmaceuticals, Osaka, Japan) and creatinine was measured by the Jaffe method (Creatinine-HR, Wako Pure Chemical Industries, Osaka, Japan). Deoxypyridinoline levels were adjusted for creatinine excretion, and were presented as nmol per mmol creatinine.

Calculation. Apparent calcium absorption and rate of absorption and calcium retention over the 4 d of the balance period were calculated as follows;

Apparent calcium absorption (wg/d) = daily calcium intake – daily calcium fecal excretion.

Absorption rate (%) = 100 × {(daily calcium intake – daily calcium fecal excretion)/daily calcium intake}.

Calcium retention (mg/d) = daily calcium intake – daily calcium fecal excretion – daily calcium urinary excretion.

Retention rate (%) = 100 × {(daily calcium intake – daily calcium fecal excretion – daily calcium urinary excretion)/daily calcium intake}.

Statistical analysis. Values of measured parameters are presented as the mean ± SEM. Comparisons between the experimental products were performed by paired-*t*-test (Statview version 5.0, SAS Institute, Cary, NC). Differences were considered significant at *P* < 0.05.

Results

Two subjects dropped out during the experiment due to common cold and diarrhea, confirmed not to have been caused by DF-III ingestion by the subjects' records on the specified log sheet. Consequently, 12 subjects were finally analyzed in this study. The characteristics of the 12 subjects are shown in Table 2. The daily nutrient intakes of the subjects during 3 d are shown in Table 3. The average total calcium intake was 358 mg.

Table 2. Characteristics of the Subjects

Age (years)	23.0 ± 0.5
Height (cm)	169.6 ± 1.9
Weight (kg)	62.3 ± 2.8
BMI	21.5 ± 0.6

Values are presented as means ± SEM (n = 12).

Table 3. Subjects' Daily Nutrient Intake per D (mean of 3 d)

Energy (kcal)	1611 ± 57
Moisture (g)	786 ± 24
Protein (g)	50 ± 2
Lipids (g)	50 ± 2
Carbohydrates (g)	224 ± 10
Sodium (mg)	3048 ± 193
Potassium (mg)	1359 ± 40
Calcium (mg)	358 ± 28
Magnesium (mg)	147 ± 3
Phosphorus (mg)	674 ± 33
Ferrous (mg)	4.9 ± 0.2
Zinc (mg)	6.1 ± 0.3
Fiber (g)	8.7 ± 0.2

Calcium intake during the balance period was similar between the groups. Fecal excretion of calcium was 462 ± 26 mg/d in the DFAlII group, and 525 ± 29 mg/d in the control group. Therefore, the apparent calcium absorption (mg/d) and rate of absorption (%) were 43.8% and 39.1% higher respectively in the DFAlII group than in the control group. Urinary excretion of calcium was very similar between the control group and

the DFAlII group. Consequently, the calcium retention (mg/d) and rate of retention (%) were much higher in the DFAlII group (Table 4).

Concentrations of plasma intact parathyroid hormone (PTH), calcitonin 1,25-dihydroxyvitamin D₃, and calcium showed no differences between the two groups (Table 5). In comparisons between before and after the experimental period in each group, blood calcium increased and intact PTH decreased after ingestion of the test and the control food ($P < 0.05$). The concentration of 1,25-dihydroxyvitamin D₃ decreased only after the ingestion of food containing DFAlII ($P < 0.01$). Serum osteocalcin, a bone formation marker, increased in the DFAlII group ($P < 0.05$), but not in the control group (Fig. 2). The concentrations of urinary deoxypyridinoline, a bone resorption marker, and creatinine did not change throughout the experimental period (Table 6).

Discussion

The aim of this study was to examine the effects of DFAlII on calcium absorption and retention using the balance method. We have reported that DFAlII promoted urinary calcium excretion in a human study.²⁵⁾ This method offered only a suggestion, because urinary calcium clearance is not consistent with different renal functions and bone metabolism. The present study with a carefully designed balance method indicates that apparent calcium absorption and retention are increased by DFAlII in healthy men.

Table 4. Absorption and Retention of Calcium during the 4-D Balance Period

Group	Intake (Ca mg/d)	Feces (Ca mg/d)	Urine (Ca mg/d)	Apparent absorption ^{a)} (mg/d)	Retention ^{b)} (mg/d)	Absorption rate ^{c)} (%)	Retention rate ^{d)} (%)
Control	677 ± 14	525 ± 29	138 ± 14	153 ± 22	15 ± 23	23 ± 4	2 ± 4
DFAlII	683 ± 16	462 ± 26	133 ± 14	220 ± 21*	87 ± 24*	32 ± 3*	13 ± 4*

Values are presented as means ± SEM (n = 12).

Significant differences between ingestion groups: * $P < 0.05$.

a) Apparent absorption = intake - feces

b) Retention = intake - feces - urine

c) Apparent absorption rate = {(intake - feces)/intake} × 100

d) Retention rate = {(intake - feces - urine)/intake} × 100

Table 5. Changes in Blood Parameters Related to Calcium Metabolism

	Control		DFAlII	
	Day 1	Day 14	Day 1	Day 14
Calcium (mg/dl)	9.48 ± 0.13	9.70 ± 0.07*	9.37 ± 0.12	9.68 ± 0.14*
1-25-di-hydroxyvitamin D ₃ (pg/ml)	49.17 ± 3.06	49.25 ± 6.74	53.00 ± 3.26	42.67 ± 2.51**
PTH-INTACT (pg/ml)	42.08 ± 3.29	29.42 ± 2.55*	42.83 ± 2.44	28.00 ± 3.29*
Calcitonin (pg/ml)	27.92 ± 3.72	28.67 ± 3.06	25.86 ± 2.84	27.32 ± 2.88

Values are presented as means ± SEM (n = 12).

Significant differences vs. the value at day 1 in each group: * $P < 0.05$, ** $P < 0.01$.

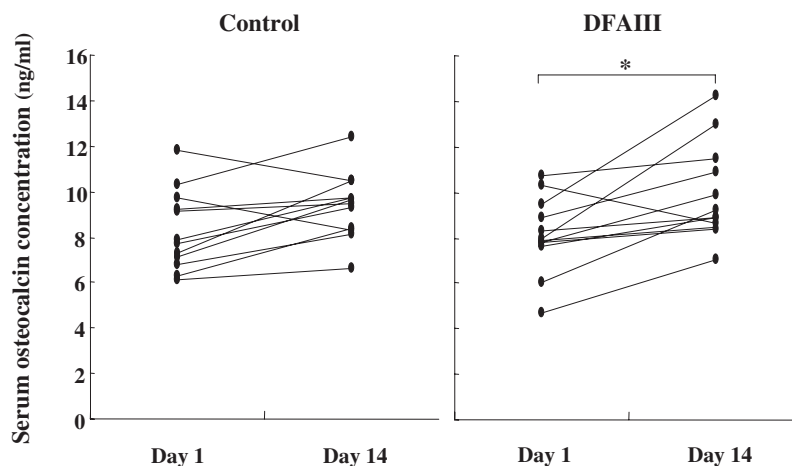


Fig. 2. Changes in Serum Osteocalcin Levels between Day 1 (pre-ingestion) and Day 14 (post-ingestion).

Values are presented as means \pm SEM ($n = 12$). Significant differences vs. the value at day 1 (pre-ingestion) in each group: $*P < 0.05$.

Table 6. Changes in Urinary Creatinine and Deoxypridinoline Levels during the Experimental Period

	Day 1	Day 10–13 (balance period)
Creatinine (mg/dl)		
Control	1.14 \pm 0.23	1.13 \pm 0.06
DFAlII	0.81 \pm 0.12	1.34 \pm 0.10
Deoxypridinoline (nmol/mmol Cre)		
Control	6.06 \pm 0.36	5.20 \pm 0.12
DFAlII	7.05 \pm 0.50	5.37 \pm 0.16

Values are presented as means \pm SEM ($n = 12$).

It was found that the daily nutrition intake of the subjects was less than complete. The calorie and protein intake of the subjects were relatively low compared with that in their age group of Japanese males. The subjects in this study consisted of students and part-time workers, and their lifestyle was thought to be irregular. In particular, the calcium intake of all subjects over 3 d was much lower than the average intake in Japanese males of ages 18–29, which is estimated to be 468 mg/d.²⁷⁾ It can be predicted that the subjects in this study show low calcium intake in their daily life. Apparent calcium absorption in the balance period was increased 67 mg by DFAlII, but urinary calcium excretion was not affected. This led to increases in calcium retention in the DFAlII group. In this nutritional status, increased calcium supplied by promoting absorption by DFAlII can be efficiently retained and utilized in the body.

The mechanism by which DFAlII promotes calcium absorption is studied by fermentation in the large intestine, as with other nondigestible oligosaccharides. It has been reported that repeated ingestion of DFAlII changes intestinal microflora in humans.^{28–30)} Also, DFAlII promotes calcium absorption by acting directly on the paracellular transport pathway in the small intestinal epithelium.^{11–14)} Enhancement of calcium

absorption by DFAlII has been reported in *in vivo* rat studies.¹⁴⁾ DFAlII increased calcium absorption in both the small and the large intestine, and the increase was greater than that of fructooligosaccharides, which promote calcium absorption only in the large intestine. Fructooligosaccharides and galactooligosaccharides have been found to improve absorption of calcium and magnesium, and the effects have been examined also in human subjects.^{31–36)} However, some results of a stable isotope study failed to show improvement in calcium absorption due to fructooligosaccharides.³⁷⁾ In the present study, we did not compare the effects of DFAlII with other nondigestible oligosaccharides. Further studies should clarify whether DFAlII has an advantage over other nondigestible saccharides in promoting calcium absorption.

Serum osteocalcin, a bone formation marker, increased after ingestion of DFAlII-containing food. This finding suggests that DFAlII might promote bone formation *via* increased calcium absorption. Osteocalcin in the control group also tended to increase at the end of the experimental period. Ingestion of 300 mg calcium in control food might also have affected calcium status and bone metabolism, because the subjects' daily calcium intakes were low. But we found much higher calcium retention in the DFAlII group. Since more than 99% of body calcium exists in bone, changes in the retention level of calcium can reflect the bone calcium level. The level of urinary deoxypridinoline, a bone resorption marker, tended to be lower after ingestion (day 10–13) of both control and DFAlII foods than the level at day 1. These results suggest that increased calcium absorption by DFAlII preferentially promotes bone formation, more than it suppresses bone resorption. It has been found that feeding DFAlII to ovariectomized rats improves their bone strength and femoral mineral concentrations.^{17,18)} Long-term observation of changes in bone mass are required to evaluate the effects of DFAlII ingestion on bone metabolism in humans too.

Blood calcium significantly increased and plasma intact PTH decreased during both the control and the DFAIII test periods. The concentration of blood calcium and that of PTH are closely related. When blood calcium is low, PTH secretion is stimulated and bone resorption is enhanced. The daily calcium intake of the subjects was low at the beginning of the experiment, but increased to about 680 mg/day in the balance period (day 10–day 13). An improvement in nutritional status as to calcium in both groups might induce an increase in the blood calcium concentration and thereby, suppression of PTH secretion. In contrast, 1,25-dihydroxyvitamin D₃ decreased only in the DFAIII group subjects. The reason for this difference between the change in PTH and that in 1,25-dihydroxyvitamin D₃ is not known, but changes in PTH are perhaps much more sensitive to small changes in calcium absorption than those in 1,25-dihydroxyvitamin D₃. Higher levels of 1,25-dihydroxyvitamin D₃ are known to have adverse effects on lipid metabolism.³⁸⁾

In this study, we chose males as subjects so that the results would not be affected by menstruation-related changes in hormonal balance. Uenishi *et al.* have reported on the calcium requirement estimated by a balance study in young and elderly Japanese adults and mentioned gender difference in calcium balance according to body weight, sex hormones, and physical activity.^{39,40)} Studies using female subjects are on the agenda to be examined in the future.

The results of the present study indicate that DFAIII enhances calcium absorption and retention in human subjects. They also suggest that co-ingestion of calcium and DFAIII might make a contribution to efficacious bone formation.

References

- 1) National Institutes of Health, Osteoporosis prevention, diagnosis, and therapy. NIH Consensus. Statement, **17**, 1–45 (2000).
- 2) Heaney, R. P., Recker, R. R., and Weaver, C. M., Absorbability of calcium sources: the limited role of solubility. *Calcif. Tissue Int.*, **46**, 300–304 (1990).
- 3) Uenishi, K., Ezawa, I., Kajimoto, M., and Tsuchiya, F., Calcium absorption from milk, fish (pond smelt, sardine) and vegetables (komatsuna-green, Jew's marrow, saltwort) in Japanese young women. *J. Jpn. Soc. Nutr. Food Sci.* (in Japanese), **51**, 259–266 (1998).
- 4) Heaney, R. P., Weaver, C. M., and Fitzsimmons, M. L., Soybean phytate content: effect on calcium absorption. *Am. J. Clin. Nutr.*, **53**, 745–747 (1991).
- 5) Bullamore, J. R., Wilkinson, R., Gallagher, J. C., Nordin, B. E., and Marshall, D. H., Effects of age on calcium absorption. *Lancet*, **2**, 535–537 (1970).
- 6) Hara, H., Suzuki, T., Kasai, T., Aoyama, Y., and Ohta, A., Ingestion of guar gum hydrolysate, a soluble fiber, increases calcium absorption in totally gastrectomized rats. *J. Nutr.*, **129**, 39–45 (1999).
- 7) Shiga, K., Hara, H., Takahashi, T., Aoyama, Y., Furuta, H., and Maeda, H., Ingestion of water-soluble soybean fiber improves gastrectomy-induced calcium malabsorption and osteopenia in rats. *Nutrition*, **18**, 636–642 (2002).
- 8) Mitamura, R., Hara, H., Aoyama, Y., Takahashi, T., and Furuta, H., Ingestion of water-soluble soybean fiber prevents osteopenia and hypercholesterolemia induced by ovariectomy in rats. *J. Agric. Food Chem.*, **51**, 1085–1089 (2003).
- 9) Roberfroid, M. B., Concepts in functional foods: the case of inulin and oligofructose. *J. Nutr.*, **129**, 1398S–1401S (1999).
- 10) Ohta, A., Ohtsuki, M., Baba, S., Adachi, T., Sakata, T., and Sakaguchi, E., Calcium and magnesium absorption from the colon and rectum are increased in rats fed fructooligosaccharides. *J. Nutr.*, **125**, 2417–2424 (1995).
- 11) Mineo, H., Hara, H., Kikuchi, H., Sakurai, H., and Tomita, F., Various indigestible saccharides enhance net calcium transport from the epithelium of the small and large intestine of rats *in vitro*. *J. Nutr.*, **131**, 3243–3246 (2001).
- 12) Mineo, H., Hara, H., Shigematsu, N., Okuhara, Y., and Tomita, F., Melibiose, difructose anhydride III and difructose anhydride IV enhance net calcium absorption in rat small and large intestinal epithelium by increasing the passage of tight junctions *in vitro*. *J. Nutr.*, **132**, 3394–3399 (2002).
- 13) Mineo, H., Amano, M., Chiji, H., Shigematsu, N., Tomita, F., and Hara, H., Indigestible disaccharides open tight junctions and enhance net calcium, magnesium, and zinc absorption in isolated rat small and large intestinal epithelium. *Dig. Dis. Sci.*, **49**, 122–132 (2004).
- 14) Suzuki, T., and Hara, H., Effects of difructose anhydride III on calcium absorption in small and large intestines of rats. *Biosci. Biotechnol. Biochem.*, **62**, 837–841 (1998).
- 15) Mineo, H., Amano, M., Chiji, H., Shigematsu, N., Tomita, F., and Hara, H., Absorptive activity of calcium in the isolated cecal epithelium adaptively increased by 2 week's feeding of difructose anhydride III in rats. *Biosci. Biotechnol. Biochem.*, **67**, 1847–1851 (2003).
- 16) Shiga, K., Hara, H., Okano, G., Ito, M., Minami, A., and Tomita, F., Ingestion of difructose anhydride III and voluntary running exercise independently increase femoral and tibial bone mineral density and bone strength with increasing calcium absorption in rats. *J. Nutr.*, **133**, 4207–4211 (2003).
- 17) Mitamura, R., Hara, H., Aoyama, Y., and Chiji, H., Supplemental feeding of difructose anhydride III restores calcium absorption impaired by ovariectomy in rats. *J. Nutr.*, **132**, 3387–3393 (2002).
- 18) Mitamura, R., and Hara, H., Prolonged feeding of difructose anhydride III increases strength and mineral concentrations of the femur in ovariectomized rats. *Br. J. Nutr.*, **94**, 268–274 (2005).
- 19) Li, H. Y., Hagiwara, H., Shu, W., Yokoyama, C., and Harada, N., Isolation and NMR studies of di-D-fructose anhydride III from *lycoris radiata* Herbert by supercritical extraction with carbon dioxide. *Carbohydr. Res.*, **299**, 301–305 (1997).
- 20) Manley-Harris, M., and Richards, G. N., Di-D-fructose dianhydrides and related oligomers from thermal treatments of inulin and sucrose. *Carbohydr. Res.*, **287**, 183–202 (1996).

- 21) Ratsimba, V., Garcia-Fernandez, J. M., Defaye, J., Nigay, H., and Voilley, A., Qualitative and quantitative evaluation of mono- and disaccharides in D-fructose, D-glucose and sucrose caramels by gas-liquid chromatography-mass spectrometry. *J. Chromatogr. A*, **844**, 283–293 (1999).
- 22) Defaye, J., and Garcia-Fernandez, J. M., The oligosaccharide components of caramels. *Zuckerrind.*, **120**, 700–704 (1995).
- 23) Yokota, A., Hirayama, S., Enomoto, K., Miura, Y., Takao, S., and Tomita, F., Production of inulin fructotransferase (depolymerizing) by *Arthrobacter* sp. H65-7 and preparation of DFA III from inulin by the enzyme. *J. Ferment. Bioeng.*, **72**, 258–261 (1991).
- 24) Yokota, A., Enomoto, K., and Tomita, F., Purification and properties of an inulin fructotransferase (depolymerizing) from *Arthrobacter* sp. H65-7. *J. Ferment. Bioeng.*, **72**, 262–265 (1991).
- 25) Shigematsu, N., Okuhara, Y., Shiomi, T., Tomita, F., and Hara, H., Effect of difructose anhydride III on calcium absorption in humans. *Biosci. Biotechnol. Biochem.*, **68**, 1011–1016 (2004).
- 26) Wang, D. H., Kogashiwa, M., Ohta, S., and Kira, S., Validity and reliability of a dietary assessment method: the application of a digital camera with a mobile phone card attachment. *J. Nutr. Sci. Vitaminol.* (Tokyo), **48**, 498–504 (2002).
- 27) “The National Health and Nutrition Survey in Japan (in Japanese),” Ministry of Health, Labour and Welfare of Japan, Daiichi Shuppan (2003).
- 28) Tamura, A., Shiomi, T., Tamaki, N., Shigematsu, N., Tomita, F., and Hara, H., Comparative effect of repeated ingestion of difructose anhydride III and palatinose on the induction of gastrointestinal symptoms in humans. *Biosci. Biotechnol. Biochem.*, **68**, 1882–1887 (2004).
- 29) Minamida, K., Sujaya, I. N., Tamura, A., Shigematsu, N., Sone, T., Yokota, A., Asano, K., Benno, Y., and Tomita, F., The effects of di-D-fructofuranose-1,2':2,3'-dianhydride (DFA III) administration on human intestinal microbiota. *J. Biosci. Bioeng.*, **98**, 244–250 (2004).
- 30) Minamida, K., Asakawa, C., Sujaya, I. N., Kaneko, M., Abe, A., Sone, T., Hara, H., Asano, K., and Tomita, F., Effects of long-term ingestion of difructose anhydride III (DFA III) on intestinal bacteria and bile acid metabolism in humans. *J. Biosci. Bioeng.*, **101**, 149–156 (2006).
- 31) Griffin, I. J., Davila, P. M., and Abrams, S. A., Non-digestible oligosaccharides and calcium absorption in girls with adequate calcium intakes. *Br. J. Nutr.*, **87**, S187–S191 (2002).
- 32) Coudray, C., Bellanger, J., Castiglia-Delavaud, C., Remesy, C., Vermorel, M., and Rayssiguier, Y., Effect of soluble or partly soluble dietary fibres supplementation on absorption and balance of calcium, magnesium, iron and zinc in healthy young men. *Eur. J. Clin. Nutr.*, **51**, 375–380 (1997).
- 33) van den Heuvel, E. G., Schoterman, M. H., and Muijs, T., Transgalactooligosaccharides stimulate calcium absorption in postmenopausal women. *J. Nutr.*, **130**, 2938–2942 (2000).
- 34) Morohashi, T., Sano, T., Ohta, A., and Yamada, S., True calcium absorption in the intestine is enhanced by fructooligosaccharide feeding in rats. *J. Nutr.*, **128**, 1815–1818 (1998).
- 35) van den Heuvel, E. G., Muys, T., van Dokkum, W., and Schaafsma, G., Oligofructose stimulates calcium absorption in adolescents. *Am. J. Clin. Nutr.*, **69**, 544–548 (1999).
- 36) Tahiri, M., Tressol, J. C., Arnaud, J., Bornet, F., Bouteloup-Demange, C., Feillet-Coudray, C., Ducros, V., Pepin, D., Brouns, F., Rayssiguier, A. M., and Coudray, C., Five-week intake of short-chain fructooligosaccharides increases intestinal absorption and status of magnesium in postmenopausal women. *J. Bone Miner. Res.*, **16**, 2152–2160 (2001).
- 37) Tahiri, M., Tressol, J. C., Arnaud, J., Bornet, F. R., Bouteloup-Demange, C., Feillet-Coudray, C., Brandolini, M., Ducros, V., Pepin, D., Brouns, F., Roussel, A. M., Rayssiguier, Y., and Coudray, C., Effect of short-chain fructooligosaccharides on intestinal calcium absorption and calcium status in postmenopausal women: a stable-isotope study. *Am. J. Clin. Nutr.*, **77**, 449–457 (2003).
- 38) Shi, H., Norman, A. W., Okamura, W. H., Sen, A., and Zemel, M. B., 1 α ,25-Dihydroxyvitamin D3 modulates human adipocyte metabolism *via* nongenomic action. *FASEB J.*, **15**, 2751–2753 (2001).
- 39) Uenishi, K., Ishida, H., Kamei, A., Shiraki, M., Ezawa, I., Goto, S., Fukuoka, H., Hosoi, T., and Orimo, H., Calcium requirement estimated by balance study in elderly Japanese people. *Osteoporos. Int.*, **12**, 858–863 (2001), Erratum in: *Osteoporos. Int.*, **13**, 184 (2002).
- 40) Nishimuta, M., Kodama, N., Morikuni, E., Yoshioka, Y. H., Takeyama, H., Yamada, H., Kitajima, H., and Suzuki, K., Balances of calcium, magnesium and phosphorus in Japanese young adults. *J. Nutr. Sci. Vitaminol.*, **50**, 19–25 (2004).