Dietary daidzein induces accumulation of *S*-equol in enterohepatic circulation to far higher levels than that of daidzein in female rats with and without ovariectomy

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(Received 6 February 2019; and accepted 8 March 2019)

ABSTRACT

We previously found that daidzein decreased food intake in female rats. To understand the mechanism of anorectic action of dietary daidzein, it is necessary to determine distributions of daidzein and *S*-equol, a metabolite of intestinal bacterial conversion from daidzein, in the body. In the present study, we measured the concentrations of daidzein and *S*-equol in serum and bile in sham-operated and ovariectomized female rats fed a diet containing 150 mg/kg daidzein for 7 days. Dietary daidzein increased serum and bile concentrations of *S*-equol to far higher levels than those of daidzein. *S*-equol concentration was more than several hundred fold-higher in bile than in serum, regardless of ovariectomy. Moreover, to investigate whether accumulation of *S*-equol is facilitated by efficient enterohepatic circulation during continuous intake of daidzein and *S*-equol, female rats were fed diet containing daidzein or *S*-equol (both 150 mg/kg), or control diet for 1, 2, 3, or 5 days. Dietary daidzein significantly increased serum and bile concentrations of *S*-equol in a time-dependent manner, but not those of daidzein. These results indicated that substantial proportion of dietary daidzein was converted to *S*-equol, which underwent efficient enterohepatic circulation and predominantly accumulated there.

Obesity results in numerous associated metabolic diseases, such as cardiovascular disease, type 2 diabetes and fatty liver disease. Appetite control is an effective approach for obesity and its comorbidities. Appetite-mediated neuropeptides and hormones are promising targets for obesity treatment (3). Some studies have indicated that food ingredients exert anorectic effects by targeting multiple appetite-mediated neuropeptides and hormones (17). However, additional studies need to be completed to understand mechanisms involved.

Isoflavones are a subclass of the more ubiquitous flavonoids. Daidzein and genistein are the predominant isoflavones in the soy. Most dietary isoflavones are present in their glycosidic forms, whereas in fermented soy products, the aglycones dominate (18). In our previous study, dietary isoflavone aglycone-rich fermented soybean extract (FSBE) and daily intubation of FSBE decrease food intake in both ovariectomized (OVX) and non-OVX female rats, but not in male rats, suggesting that the bitter taste of soy isoflavone was not the cause of reduced food intake in female rats, and that the observed reduction in intake was a postabsorptive effect (8, 9). We found that dietary daidzein, but not dietary genistein,

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has the anorectic effect, and changes gene expression of appetite-mediated neuropeptides in hypothalamus, an important appetite-control center that integrates peripheral hormone signals and interacts with other brain region (2, 6). These results indicate that daidzein is a potential candidate to treat obesity due to their ability to regulate food intake and energy homeostasis.

Intestinal bacteria convert daidzein to the *S*-enantiomer of equol or to *O*-desmethylanagolensin (*O*-DMA) in both humans and animals (4, 16). Rachoń *et al.* reported that a 400 mg/kg diet of dietary equol decreases food intake and weight gain (13). Dietary daidzein increases serum concentration of *S*-equol to far higher levels than that of daidzein in female rats in our previous studies (6, 9). *S*-equol may be responsible for decreases in food intake and serum cholesterol concentration in FSBE- and daidzein-fed female rats. *S*-equol is a selective agonist of estrogen receptor β (16), which is involved in the anorectic action of estrogen (12). On the other hand, the anorectic effect of a subcutaneous injection of 17β -estradiol is more pronounced in male and OVX female rats than in intact female rats, probably because of low endogenous estrogen level (9). It is possible that the decrease in food intake in daidzein-fed female rats was not attributable to a purely estrogenic function.

To understand the mechanism of anorectic action of *S*-equol, it is necessary to determine its distributions in the body. Although little is known about the distribution of *S*-equol in the body, the second and third peaks of plasma concentration of racemic equol after oral administration may indicate that it undergoes enterohepatic circulation (11). Enterohepatic circulation is often associated with multiple peaks and a longer apparent half-life in a plasma concentration-time profile (14). In the present study, to investigate the distribution of daidzein and *S*-equol and whether *S*-equol undergoes efficient enterohepatic circulation, we directly measured the concentrations of daidzein and *S*-equol in serum and bile, as well as their urinary excretion, in sham-operated (sham) and OVX female rats fed a diet containing 150 mg/kg daidzein for 7 days (Experiment 1). The results indicated that a substantial proportion of dietary daidzein is converted to *S*-equol, which undergoes efficient enterohepatic circulation regardless of ovariectomy. We further investigated whether continuous daidzein or *S*-equol intake for 5 days induces accumulation of *S*-equol in enterohepatic circulation in intact female rats (Experiment 2).

MATERIALS AND METHODS

Daidzein and *S*-equol. Daidzein and racemic equol were purchased from LC laboratories (MA, USA). *S*-equol was obtained from racemic equol by HPLC-UV under the following conditions: a chiral column (10 $\times$ 250 mm, CHIRALCEL OJ, particle size 10 μ m; Daicel Co., Ltd., Osaka, Japan), mobile phase consisting of n-hexane/ethanol/acetic acid = 60 : 40 : 0.1 (v/v), column temperature of 25°C, flow rate of 5.0 mL/min, and UV-visible detector at 280 nm (SPD-10AV; Shimadzu Co., Kyoto, Japan). *S*-equol was confirmed to be of >98% enantioselective purity by HPLC-UV under above-mentioned conditions.

Animal and diets. Female Sprague-Dawley rats aged 6 weeks were purchased from Japan SLC (Hamamatsu, Japan). The experimental protocol was approved by the Laboratory Animal Care Committee of Ehime University, and the rats were maintained in accordance with the Guidance for the Care and Use of Laboratory Animals of Ehime University. All animals were housed individually in a room with controlled 12-h light-dark cycle (dark phase: 15 : 00–3 : 00) and constant temperature (23 $\pm$ 1°C). Each morning during experimental period, body weight and amount of diet consumed by weight were recorded, and diet was replenished. The basal composition of the experimental control diet was (in g/kg): casein (New Zealand Dairy Board, Wellington, New Zealand), 200; cellulose (Danisco Japan, Inc, Tokyo, Japan), 50; soybean oil (J-oil Mills, Inc, Tokyo, Japan), 70; AIN-93 mineral mixture, 35; AIN-93 vitamin mixture, 10; L-cystine (Nacalai Tesque, Kyoto, Japan), 3; sucrose (Nippon Beet Sugar Manufacturing, Tokyo, Japan), 100; and α -corn starch (Nihon Shokuhin Kako, Tokyo, Japan), 532. Additions of daidzein and *S*-equol were performed at the expense of α -corn starch.

Bilateral ovariectomy (Experiment 1) and blood and bile sampling were performed under combined anesthesia. The combined anesthesia was prepared with 0.0375 mg/kg body weight (b.w.) medetomidine (Domitor; Nippon Zenyaku Kogyo, Fukushima, Japan), 2.0 mg/kg b.w. midazolam (Sandoz K.K., Tokyo, Japan), and 2.5 mg/kg b.w. butorphanol (Vetorphale; Meiji Seika Pharma Co., Ltd., Tokyo, Japan). The anesthetics were administered to rats by intraperitoneal injection. At day 7 (Experiment 1), and days 1, 2, 3, and 5 (Experiment 2) after dietary supplementation with daidzein or *S*-equol, mid-line laparotomy was performed on the rats and the bile duct was then cannulated with a PE-10 polyethylene tube

(Clay Adams, Parsippany, NJ, USA) connected to a silicon tube (SH No.00; Fuji Systems Corporation, Tokyo). The bile was then collected over 30 min while maintaining body temperature of the rats at 37°C. The blood was then collected from the abdominal aorta. Next, blood samples were kept at room temperature for a minimum of 30 min. After that, serum was separated by centrifugation at $1,500 \times g$ for 10 min at 4°C. Bile and serum samples were stored at -50°C until analysis. Urine was collected from individual rats for 24 h using trays fixed lying under their cages at days 1, 2, 3, and 5 of the experimental period for Experiment 2, and at day 7 for Experiment 1. The trays contained 50 mL of 120 mg/L sodium azide. Urine samples were filtered, freeze-dried, weighed, and stored in a desiccator until analysis. In Experiment 2, feces were collected from individual rats for 24 h at days 1, 2, 3, and 5, and then freeze-dried, weighed, and stored in a desiccator until analysis.

Experiment 1—The rats were fed a commercial solid diet (Rodent Diet EQ; Japan SLC) during acclimatization period and recovery period. After 7 days of acclimatization period, the rats underwent bilateral ovariectomy or sham operation. After a week of recovery, the sham and OVX rats were divided into 2 groups according to mean weight, and each group was provided free access to either control diet or diet containing 150 mg/kg daidzein for 7 days ($n = 5-6$). At the end of experiment, we failed to collect blood sample from one daidzein-fed OVX rat.

Experiment 2—The rats were fed control diet during acclimatization and recovery period. After 7 days of acclimatization period, the rats were divided into 3 groups according to mean weight, and each group was provided free access to either control diet or diet containing 150 mg/kg daidzein or *S*-equol for 1, 2, 3, or 5 days ($n = 6$). We failed to collect bile samples from one rat of the daidzein group and three of the *S*-equol group, blood sample from one of the *S*-equol group and urine sample from one of the daidzein group at day 1, blood sample from one of the *S*-equol group at day 2, and bile sample from one of the daidzein group at day 3.

Isoflavone level measurement

Sample preparation—Isoflavone concentrations in serum, bile, and urine were determined using the method of Janning *et al.* (7). Frozen samples of rat serum (100 μ L) and bile (5 μ L bile + 95 μ L MilliQ water) were mixed with 100 μ L of hydrolysis buffer

[0.1 mol/L sodium acetate pH 5, 0.1% (w/v) ascorbic acid, 0.01% (w/v) EDTA], 8 μ L of glucuronidase, and 4 μ L of sulfatase. Freeze-dried urine samples (10 mg) were mixed with 100 μ L of ammonium acetate buffer (10 mmol/L, pH 5) to make a stock solution. Next, 10 μ L of stock solution was mixed with 100 μ L of hydrolysis buffer, 8 μ L of glucuronidase, and 4 μ L of sulfatase. Reaction mixtures of serum, bile, and urine samples were allowed to hydrolyze to glucuronide and sulfate metabolites at 37°C for at least 15 h. Subsequently, 10 μ L of an internal standard (formononetin, 5 μ g/mL in dimethyl sulfoxide), 120 μ L of water, 75 μ L of ammonium acetate buffer (1 mmol/L, pH 7), and 85 μ L of triethylammonium sulfate buffer (3 mol/L, pH 7) were added. The mixtures were then heated to 60°C for 10 min to facilitate dissociation of isoflavones from proteins and then centrifuged. Deproteinized samples were passed through 0.5-g Sep-Pak C-18 cartridges (Nihon Waters, Tokyo, Japan) that had been previously washed with 5 mL of ammonium acetate buffer (10 mmol/L, pH 5) and 5 mL of water at room temperature. The absorbed isoflavones were eluted with 1.5 mL of methanol. The methanol effluent was evaporated to dryness under a gentle stream of nitrogen at 45°C, dissolved in 100 μ L of methanol/1% aqueous acetic acid (40 : 60, v/v).

De-conjugation and solid phase extraction were not conducted on fecal samples. Before methanol extraction, 10 μ L of an internal standard (formononetin, 5 μ g/mL in dimethyl sulfoxide) was added to 3 mg of freeze-dried feces powder. Ice-cold methanol (1000 μ L) was added and the mixture was vortexed vigorously. The samples were then sonicated for 30 min and centrifuged at $2000 \times g$ at 4°C for 10 min, and the supernatant was recovered in evaporation tubes. The above steps from adding 1000 μ L of ice-cold methanol were repeated three times. Finally, the collected methanol effluent (3000 μ L) was evaporated to dryness under a gentle stream of nitrogen at 45°C, and dissolved in 100 μ L of methanol/1% aqueous acetic acid (40 : 60, v/v).

HPLC Methods—Serum, bile, fecal, and urine daidzein and *S*-equol concentrations were analyzed by HPLC as detailed previously (7). A 5- μ L aliquot of the sample was applied to a reverse-phase HPLC column (2.0 $\times$ 150 mm, CAPCELL PAK C18, particle size 5 μ m; Shiseido, Tokyo, Japan). The mobile phase was potassium phosphate buffer containing 40% of a mixture of methanol and acetonitrile (3 : 2, v/v) at 40°C, and the flow rate was 0.2 mL/min. Daidzein and *S*-equol were detected using an electrochemical detector (electrochemical detector 3005;

Fig. 1 Daily changes in food intake in sham (circle) and OVX (triangle) female rats fed control diet (open mark) or diet containing 150 mg/kg daidzein (closed mark). Each value represents the mean $\pm$ standard error. Asterisks show significant difference relative to the control group in sham rats, and number signs show significant difference relative to the control group in OVX rats, determined by three-way repeated measures ANOVA followed by Student's *t*-test with Bonferroni corrections. * and #, $P < 0.05$. OVX C, ovariectomized rats fed control diet; OVX D, ovariectomized rats fed daidzein diet; sham C, sham-operated rats fed control diet; sham D, sham-operated rats fed daidzein diet. Day, effect of time; Diet, effect of dietary daidzein; NS, not significant; Ope, effect of operation.

Shiseido, Tokyo, Japan) under the following conditions: working electrode, glassy carbon; applied voltage, 800 mV. Formononetin was detected simultaneously using a UV-Visible detector (SPD-10AV; Shimadzu Corporation, Kyoto, Japan) at 254 nm.

Statistical analysis. All data are presented as the mean $\pm$ standard error. In Experiment 1, data were analyzed by three-way repeated measures ANOVA followed by Student's *t*-test with Bonferroni corrections for the time course of food intake. For other data, statistical analysis was performed with the Student's *t*-test. In Experiment 2, data were analyzed by two-way repeated measures ANOVA followed by Student's *t*-test with Bonferroni corrections for the time course of food intake. For other data, statistical analysis was performed with one-way ANOVA followed by Student's *t*-test with Bonferroni corrections for daidzein concentrations and excretions, and two-way ANOVA followed by Student's *t*-test with Bonferroni corrections for *S*-equol concentrations and excretions. All statistical analyses were performed using the IBM SPSS Statistics software package (SPSS Japan Inc., IBM, Tokyo, Japan). Statistical significance was defined as $P < 0.05$.

RESULTS

In Experiment 1, daily food intake in rats fed diet containing 150 mg/kg daidzein was significantly

lower than that in rats fed control diet at days 5–7 in the OVX group, and at day 7 in the sham group (Fig. 1), suggesting that continuous intake of daidzein for at least several days was required to exert its anorectic effect, regardless of ovariectomy. Daidzein and *S*-equol were not detected in serum, bile, and urine from the rats fed control diet, and daidzein was not detected in the serum in all cases, including cases of daidzein administration. Bile concentration of *S*-equol was approximately 50-fold higher than that of daidzein, and more than 1000-fold higher than serum *S*-equol concentration in both sham and OVX rats fed daidzein diet (Fig. 2A, Fig. 2B), suggesting that substantial proportion of dietary daidzein was converted to *S*-equol, which underwent efficient enterohepatic circulation, and only a small portion of *S*-equol escaped enterohepatic circulation and reached systemic circulation. Ovariectomy did not affect these concentrations. Urinary *S*-equol excretion tended to be higher in OVX rats than in sham rats, but the difference was not significant ($P = 0.059$) (Fig. 2C).

In Experiment 2, daily food intake in intact female rats fed diet containing 150 mg/kg *S*-equol was significantly lower than that in those fed control diet at day 3 (Fig. 3), similar to observations in Experiment 1. Daidzein in serum, bile, urine, and feces from rats fed control or *S*-equol diet and *S*-equol in serum, bile, urine, and feces from rats fed control diet were not detected. Dietary daidzein significantly

Fig. 2 Distribution of daidzein and *S*-equol in sham (open bar) and OVX (closed bar) rats fed diet containing 150 mg/kg daidzein for 7 days. Each value represents the mean $\pm$ standard error. There was no significant difference between sham and OVX, according to results of statistical analysis with Student's *t*-test. Sham, sham-operated rats; OVX, ovariectomized rats.

Fig. 3 Daily changes in food intake in intact female rats fed control diet (open square) or diet containing 150 mg/kg daidzein (closed circle) or 150 mg/kg *S*-equol (closed square). Each value represents the mean $\pm$ standard error. Asterisks show significant difference relative to the control group, determined by two-way repeated measures ANOVA followed by Student's *t*-test with Bonferroni corrections. *, *P* < 0.05. C, control group; D, daidzein group; NS, not significant; S-EQL, *S*-equol group. Day, effect of time; Diet, effect of dietary daidzein or *S*-equol.

increased serum and bile concentrations of *S*-equol in a time-dependent manner, to far higher levels than those of daidzein (Fig. 4A–D). Thus, daily changes in daidzein concentrations in serum and bile were negligible (Fig. 4B, D). *S*-equol concentration was several hundred-fold higher in the bile than in the serum of rats fed daidzein diet (Fig. 4A and C). Dietary *S*-equol also significantly increased serum and bile *S*-equol concentrations in a time-dependent manner (Fig. 4A and C). These results indicate that continuous daidzein intake induces accumulation of *S*-equol, but not daidzein, in the body, which was not attributed to the adaptation of the intestinal bacte-

ria involved in the conversion of daidzein to *S*-equol. Serum and bile *S*-equol concentrations in rats fed *S*-equol diet were significantly higher than those in rats fed daidzein diet (Fig. 4A and C).

Urinary excretions may indicate the amounts of daidzein and *S*-equol that enter systemic circulation, while fecal excretions may indicate the amounts of daidzein and *S*-equol that undergo enterohepatic and enteric circulations. Dietary daidzein significantly increased urinary *S*-equol, as well as fecal daidzein and *S*-equol excretions in a time-dependent manner, whereas urinary daidzein did not significantly change from day 1 to 5 (Fig. 4E–H). Dietary *S*-equol also

Fig. 4 Daily changes in daidzein (right panel) and S-equol (left panel) concentrations in serum (A, B), bile (C, D), urine (E, F), and feces (G, H) in intact female rats fed diet containing 150 mg/kg daidzein (closed circle) or 150 mg/kg S-equol (closed square). Each value represents the mean $\pm$ standard error. Asterisks show significant difference relative to day 1, and number signs show significant difference relative to the daidzein group. Statistical analyses were performed with one-way ANOVA followed by Student's *t*-test with Bonferroni corrections for daidzein concentrations and excretions, and two-way ANOVA followed by Student's *t*-test with Bonferroni corrections for S-equol concentrations and excretions. * and #, $P < 0.05$; ** and ##, $P < 0.01$; *** and ###, $P < 0.001$. D, daidzein group; S-EQL, S-equol group. Day, effect of time; Diet, effect of dietary daidzein or S-equol.

significantly increased urinary and fecal *S*-equol excretions in a time-dependent manner (Fig. 4E and G). Urinary and fecal *S*-equol excretions of rats fed *S*-equol diet were significantly higher than those of rats fed daidzein diet (Fig. 4E and G). These results indicate that accumulation of *S*-equol in enterohepatic circulation may be accompanied with daily increases in serum *S*-equol concentration, and urinary and fecal excretions of *S*-equol.

DISCUSSION

Dietary daidzein increased serum and bile concentrations of *S*-equol to far higher levels than those of daidzein itself, and *S*-equol concentration was several hundred-fold higher in the bile than in the serum of both non-OVX (intact and sham) and OVX rats (Fig. 2 and Fig. 4), suggesting that substantial proportion of dietary daidzein was converted to *S*-equol, which underwent efficient enterohepatic circulation, and only a small portion of *S*-equol escaped enterohepatic circulation and reached systemic circulation. Enterohepatic circulation may influence drug concentrations in the body by a delayed elimination (14). Continuous intake of daidzein and continuous intake of *S*-equol induced daily increases in serum and bile *S*-equol concentrations for 5 days (Fig. 4), suggesting that these daily increases in *S*-equol concentrations in the body were not attributed to the adaptation of the intestinal bacteria involved in the conversion of daidzein to *S*-equol. Accumulation of *S*-equol in the body may be facilitated by efficient enterohepatic circulation. In addition, dietary daidzein decreased food intake of OVX rats on days 5–7 and that of sham rats on day 7 (Fig. 1), suggesting that continuous intake of daidzein for at least several days is required for its anorectic effect to occur. That was also the case for dietary *S*-equol; continuous intake for 3 days was required (Fig. 3). Accumulation of *S*-equol in enterohepatic circulation may lead to an increase in its abundance in the small intestinal lumen. The small intestine releases hormones regulating satiety. It is speculated as one possibility that accumulation of *S*-equol in enterohepatic circulation may induce its anorectic effect via the release of satiety hormones such as cholecystokinin, glucagon-like peptide 1 and peptide YY.

In Experiment 2, we did not observe significant decreases in food intake in rats fed daidzein diet from day 1 to 4, and bile *S*-equol concentration in these rats were lower than that in rats that had decreased food intake due to *S*-equol intake (Fig. 3 and Fig. 4). It is possible that the accumulated level of

S-equol in enterohepatic circulation was not sufficient to exert its anorectic effect in rats fed daidzein diet for 4 days, although there were few data that can be used to assess how much *S*-equol accumulated in enterohepatic circulation is required to exert its anorectic effect. A longer time course, at least for 7 days, is needed to assess the relations between accumulated level of *S*-equol in enterohepatic circulation and its anorectic effect.

Dietary daidzein significantly increased urine level of *S*-equol in a time-dependent manner, but not that of daidzein itself (Fig. 4). Urinary excretion may indicate the amount of drug that enters systemic circulation. A daily increase in urinary *S*-equol excretion may accompany its accumulation in enterohepatic circulation, whereas urinary daidzein excretion did not change daily because it may not accumulate during continuous intake. On the other hand, urinary excretion of *S*-equol was lower than that of daidzein, although serum and bile concentrations of *S*-equol were several-fold higher than those of daidzein in rats fed daidzein diet for 1 day (Fig. 4). This suggested that excretion of *S*-equol from enterohepatic circulation to urine via systemic circulation may be lower relative to that of daidzein. Previously, it has been suggested that rapid excretion of daidzein may be related to its weak estrogenicity *in vivo*, compared to that of other estrogenic chemicals with comparatively low affinity to the estrogen receptor (1). Conversely, the slower urinary excretion of *S*-equol partly explains the higher abundance of it in serum and bile relative to those of daidzein, which could be important for effects of *S*-equol. Ovariectomy tended to increase urinary *S*-equol excretion (Fig. 2), suggesting that estrogen deficiency may facilitate urinary *S*-equol excretion. Sepehr *et al.* claimed that the sex of rats has significant effects on isoflavone bioavailability (15). Our previous study also showed that serum *S*-equol concentration in non-OVX rats fed diet containing daidzein tends to be higher than that in OVX rats (5, 9), which could be explained by high urinary *S*-equol excretion due to ovariectomy. However, we did not observe any decreases in serum and bile *S*-equol concentrations due to ovariectomy in the present study (Fig. 2). Further studies will be needed to determine whether estrogen deficiency affects *S*-equol concentration in systemic and enterohepatic circulations and its urinary excretion.

Dietary daidzein and *S*-equol significantly increased fecal *S*-equol excretion in a time-dependent manner (Fig. 4). In rats fed *S*-equol diet, daily urinary and fecal *S*-equol excretions gradually approxi-

mated its daily intake (approximately 6.5 nmol/day) over 5 days, speculating that the accumulation rate of *S*-equol gradually decreased (Fig. 4). Dietary daidzein also significantly increased fecal daidzein excretion in a time-dependent manner (Fig. 4). A previous study has suggested that daidzein could undergo enteric recycling (10, 19). Daidzein could also accumulate within the gastrointestinal tract through enteric recycling during its continuous intake. However, we do not further discuss the accumulation of daidzein in the gastrointestinal tract and do not assess the balance between intake of daidzein and excretions of daidzein and its metabolites in the present study, because we did not measure the levels of *O*-DMA and its metabolites (4). Further studies will be needed to determine why fecal daidzein excretion was increased by continuous intake of daidzein.

In conclusion, we found that continuous intake of daidzein induced accumulation of *S*-equol in enterohepatic circulation to far higher levels than that of daidzein itself, and only a small portion of *S*-equol escaped enterohepatic circulation and reached systemic circulation. In addition, continuous intake of daidzein for at least several days was required to exert its anorectic effect. It is possible that accumulation of *S*-equol in enterohepatic circulation was needed to exert its anorectic effect.

Acknowledgement

This work was supported in part by the Ministry of Education, Culture, Sports, Science, and Technology, Japan, under the Monbukagakusho scholarship, by JSPS KAKENHI Grant Number 16K07738, and by a grant from Fuji Foundation for Protein Research. This work was supported by the Ministry of Education, Culture, Sports, Science, and Technology, Japan, under the Monbukagakusho scholarship, and by Rotary Yoneyama Memorial Foundation scholarship.

CONFLICT OF INTEREST

This study was funded by FANCL Co. Ltd. Junji Ishikawa is a current employee of FANCL Co. Ltd.

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