

## Dietary Fat and an Exogenous Emulsifier Increase the Gastrointestinal Absorption of a Major Soybean Allergen, Gly m Bd 30K, in Mice<sup>1</sup>

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**ABSTRACT** The mechanisms by which food allergens are absorbed and sensitized via the gastrointestinal tract have not been well characterized. In this study, the gastrointestinal absorption of a major soybean allergen, Gly m Bd 30K, in young and older mice, and the effects of dietary fat and exogenous emulsifier were investigated. In Expt. 1, Gly m Bd 30K [0, 500 or 2000 mg/kg body weight (BW)] was administered orally to 24-d-old mice, and blood was sampled at various time points over a 120-min period. Plasma Gly m Bd 30K was measured by sandwich ELISA and immunoblotting. Its concentration peaked at 30 min and was dose dependent. Intact Gly m Bd 30K and its 20-kDa fragments were identified in plasma after absorption. In Expt. 2, 24-d-old mice administered soy milk containing 1 mg Gly m Bd 30K showed a steady increase in plasma Gly m Bd 30K from 60 to 120 min that was significantly higher than that in 10-wk-old mice. In Expt. 3, when corn oil (5 or 30%) was coadministered with Gly m Bd 30K (2000 mg/kg BW) to 24-d-old mice, the plasma concentration increased significantly and generally reached a plateau after 30 min. The absorption after the coadministration of 30% corn oil and 3% sucrose fatty acid ester was higher than after the administration of 30% corn oil alone. Intact Gly m Bd 30K and its fragments that were <20 kDa survived digestion and were absorbed into the blood. We propose that absorption was enhanced by fat carrier-mediated transport. *J. Nutr.* 135: 1738–1744, 2005.

**KEY WORDS:** • Gly m Bd 30K • soybean allergen • gastrointestinal absorption • dietary fat • emulsifier

IgE antibody-mediated food allergies have generated much concern in developed countries, particularly in the United States and Japan. Interestingly, ~8% of children and 2% of adults have allergic reactions to food (1). Most food-allergic patients are affected after the consumption of peanuts, milk, eggs, wheat, crustaceans, soybeans, buckwheat, and rice. Soybeans are an important protein and dietary source because of their nutritional and functional benefits; they are now used in an increasing number of food products. After the FDA began allowing food products containing soybean proteins to carry a label promoting their health benefits, the consumption of many varieties of soybean products, including soybean beverages, tofu, soybean-based meat alternatives, and soybean protein powder, increased in the United States and other developed countries. This increase may lead to an increase in the incidence of soybean allergies. Ogawa et al. (2) demonstrated that ~15 soybean proteins bind to IgE antibodies in the sera of soybean-sensitive patients with atopic dermatitis. It is now clear that Gly m Bd 30K, which is recognized by IgE antibodies

in >65% of soybean-sensitive patients, is the major allergenic protein in soybeans. Gly m Bd 30K is a soybean seed vacuolar 34-kDa oil-body-associated glycoprotein, P34, which is a member of the thiol protease family of the papain superfamily, and highly homologous to the house dust mite allergen Der p 1 (3,4).

The gastrointestinal tract serves as an interface between the body and the external environment. The gastrointestinal tract includes the mouth, esophagus, stomach, and the small and large intestines, which are part of the digestive system that also includes the pancreas and liver (5). The primary function of the gastrointestinal tract is to digest and absorb dietary components from the intestinal lumen into the circulation. Another function is to act as a barrier, preventing the entrance of harmful entities including microorganisms, luminal antigens, and luminal inflammatory factors. This function is provided by immunologic mechanisms involving immunoglobulins and lymphocytes among others, and by nonimmunologic mechanisms such as selective intestinal permeability (6).

Gastrointestinal development is a highly organized process, both topologically and temporally. The changing dietary inputs during ontogeny (amniotic fluid, maternal milk, weaning, postweaning diet) impose different demands on the intestine and influence its morphology, enzyme capacity, and transport (7). Malnutrition or starvation during the suckling and wean-

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ing periods is associated with marked changes in mucosal barrier functions and also modulates the intestinal transport of nutrients and ions. The permeability of macromolecules is enhanced (8,9) and the concentrations of mucosal IgA and secretory components markedly decrease (10). Studies of intestines of adults revealed that the regulation of intestinal transport depends not only on the intrinsic properties of enterocytes, but also on local hormones, growth factors, neurotransmitters, and complex interactions between enteric nerves in the submucosa and immune cells in the lamina propria (11).

Dietary proteins are digested primarily within the gastrointestinal tract by the action of secreted enzymes or peptidases in the enterocyte microvillous membrane. However, a small amount of food allergens escape this enzymatic breakdown and are available for intestinal absorption (12–14). Several studies showed the permeability of proteins and macromolecules in vitro using the Caco-2 human colon adenocarcinoma cell line (15–18). Koda et al. (19) reported that higher concentrations of interferon- $\gamma$  enhance the uptake and transport of ovalbumin in Caco-2 cells. In addition,  $\beta$ -lactoglobulin and ovalbumin were absorbed into the circulation after an oral allergen challenge in ovalbumin-sensitized rats in vivo (20,21). However, the mechanisms by which a soybean allergen is absorbed and sensitized via the gastrointestinal tract have not been well characterized in vivo.

Among dietary factors, fats have been proposed as a risk factor for diseases such as diabetes, hyperlipidemia, hypertension, and cancer. The consumption of dietary fats in processed food products is increasing, particularly in the developed countries. It is still unclear how various coexisting dietary factors, such as dietary fats, affect the intestinal absorption of Gly m Bd 30K and the mechanisms underlying allergic reactions to Gly m Bd 30K.

In this study we investigated the gastrointestinal absorption of Gly m Bd 30K after oral administration to mice. We studied the effects of dietary fats and an exogenous emulsifier on absorption, and measured the plasma Gly m Bd 30K concentration by sandwich ELISA and immunoblotting using specific antibodies.

## MATERIALS AND METHODS

**Purification of Gly m Bd 30K.** The major soybean allergen, Gly m Bd 30K, was purified from the oil body pad of soybeans as described by Kalinski et al. (22). Briefly, the oil body pad was isolated from soybean seeds that were soaked in water for 24 h by homogenization in 0.1 mol/L Tris-HCl and 2 mmol/L mercaptoethanol, pH 8.6, using a Polytron homogenizer. The homogenate was filtered through 3 layers of gauze and then centrifuged at  $15,000 \times g$  for 20 min. The oil body pad was resuspended in the homogenization buffer and recentrifuged. Afterwards, the oil body pad was resuspended in a solution containing 0.1 mmol/L NaCl, 0.1 mol/L Tris-HCl, and 2 mmol/L mercaptoethanol, pH 8.6, and centrifuged as described above. The oil body pad was resuspended and centrifuged in the same buffer again. Then, the oil body pad was resuspended in 1 mol/L  $\text{Na}_2\text{CO}_3$  and allowed to stand for 3 h at 4°C. The supernatant was recovered by centrifugation as described above. Total protein in the supernatant was precipitated with 70% saturated ammonium sulfate. The precipitate was collected and dissolved in PBS, followed by dialysis against the same buffer for 20 h at 4°C. The extract was stored at  $-28^\circ\text{C}$  until use.

**Preparation of soy milk.** Soybeans were soaked in water overnight and homogenized in distilled water (1:2 wt:wt) using a Polytron homogenizer. The homogenate was filtered through 3 layers of gauze to remove insoluble materials, and the filtrate was used as the soy milk.

**Animals, diets, and experimental design.** Male ddY mice (24 d old, weighing 10–12 g, and 10 wk old, weighing 35–40 g) were

TABLE 1

Compositions of solutions containing Gly m Bd 30K (2000 mg/kg BW) administered to food-deprived 24-d-old mice (Expt. 3)

| Group | Supplemented constituent                                  |
|-------|-----------------------------------------------------------|
| 1     | None                                                      |
| 2     | 5% (v:v) corn oil                                         |
| 3     | 30% (v:v) corn oil                                        |
| 4     | 3% (wt:v) sucrose fatty acid ester                        |
| 5     | 30% (v:v) corn oil and 3% (wt/v) sucrose fatty acid ester |

purchased from Japan SLC. They were housed in a room with a 12-h light:dark cycle. The temperature and humidity were maintained at 23°C and 48–62%, respectively. All mice were fed a commercial diet<sup>3</sup> (CRF-1; Oriental Yeast). The experimental design was in accordance with the guidelines for animal experimentation and was approved by the Animal Experimental Committee of Kyoto University.

**Expt. 1.** The aim of the first study was to determine the dose dependence of the gastrointestinal absorption of Gly m Bd 30K. The 24-d-old mice were food-deprived with free access to water 24 h before the experiment. Using a 20-gauge needle equipped with a curved bulb-ended gavage tube, they were orally administered 1 mL of PBS containing Gly m Bd 30K [500 or 2000 mg/kg body weight (BW)<sup>4</sup>] or PBS alone as the control condition ( $n = 5/\text{group}$ ). Using a capillary tube, blood (0.15 mL) was drawn from the ophthalmic venous plexus from anesthetized mice at 0, 30, 60, 90, and 120 min, and placed into heparinized containers. Blood was immediately centrifuged at  $3000 \times g$  for 5 min at 4°C and plasma was stored at  $-20^\circ\text{C}$  until analysis.

**Expt. 2.** The aim of the second study was to evaluate the gastrointestinal absorption of soy milk Gly m Bd 30K in young and older mice. Mice (24 d and 10 wk old;  $n = 5/\text{group}$ ) were food-deprived with free access to water 24 h before the experiment and then orally administered 1 mL of soy milk as the natural source of Gly m Bd 30K. The concentration of Gly m Bd 30K in soy milk, determined using sandwich ELISA, was  $\sim 1$  g/L. After administration, blood was sampled and processed as in Expt. 1.

**Expt. 3.** The aim of the third study was to demonstrate the effects of corn oil and/or sucrose fatty acid ester on the gastrointestinal absorption of Gly m Bd 30K. Food-deprived 24-d-old mice were divided into 5 groups ( $n = 5/\text{group}$ ). Each group was administered 1 mL of PBS containing Gly m Bd 30K (2000 mg/kg BW) and the supplemented constituents listed in Table 1. The control was PBS containing Gly m Bd 30K alone (2000 mg/kg BW). Corn oil was obtained from Nacalai Tesque. Sucrose fatty acid ester was kindly supplied by Mitsubishi Kagaku Food. Gly m Bd 30K was mixed for 1 min with each constituent and sonicated for 1 min using a Branson Sonifier 450. After administration, blood was sampled and processed as in Expt. 1.

### Generation of rabbit polyclonal antibody against Gly m Bd 30K.

A rabbit polyclonal antibody against Gly m Bd 30K was generated using purified Gly m Bd 30K with Freund's complete adjuvant. The postimmune serum was affinity-purified with purified Gly m Bd 30K. For the quantification of Gly m Bd 30K in plasma using sandwich ELISA, the affinity purified rabbit polyclonal antibody was biotinylated using an EZ-Link Sulfo-NHS-LC-biotinylation kit (Pierce).

**ELISA.** ELISA plates (96 wells, Asahi Technoglass) were incubated with 0.01  $\mu\text{g/L}$  rabbit polyclonal antibody against Gly m Bd 30K overnight at 4°C and blocked with Zepto-Block (ZeptoMetrix)

<sup>3</sup> The commercial diet was composed of defatted soybean, fish meal, wheat, soybean oil, corn oil, salt, vitamins, and minerals. The nutritional values of the commercial diet were as follows (g/100 g): soluble nonnitrogen compounds, 54.5; protein, 22.4; water, 7.8; ash, 6.6; fat, 5.7, and fiber 3.1.

<sup>4</sup> Abbreviations used: BW, body weight; HRP, horseradish peroxidase; TMB, tetramethylbenzidine.

for 2 h at room temperature. The wells were washed with PBS, pH 7.4, containing 0.1% (v/v) Tween 20 (PBST). Gly m Bd 30K used as the standard was mixed with plasma at concentrations of 0–1.667 mg/L. Then, 100  $\mu$ L each of 3:10 diluted standards of plasma Gly m Bd 30K and plasma sample in PBS was applied to each well of antibody-coated plates and the plates were incubated for 1 h at room temperature. The wells were washed with PBST, and 100  $\mu$ L of a 1:100 diluted biotinylated rabbit polyclonal antibody against Gly m Bd 30K in PBS was added; the plates were then incubated for 1 h at room temperature. After the plates were washed with PBST, they were incubated with 100  $\mu$ L of a 1:10000 diluted horseradish peroxidase (HRP)-conjugated AMDEX streptavidin (Amersham Bioscience) in PBS. Each well was washed 7 times with PBST and incubated with 100  $\mu$ L of TMB Microwell peroxidase substrate (Kirkegaard and Perry Laboratories) for 2.5 min, and then 100  $\mu$ L of 1 mol/L phosphoric acid was added. The absorption at 450 nm was measured using the Multilabel Counter 1420 ARVOsx-1 (Perkin-Elmer Life Sciences).

**Immunoprecipitation and immunoblotting.** Fifty microliters of 1:2.5 diluted plasma in PBS was incubated with 5  $\mu$ L of Protein A-agarose (Sigma) at a low-speed rotation for 1 h at room temperature. The pellet of nonspecific binding materials was removed by centrifugation at  $14,000 \times g$  for 5 min at 4°C. The rabbit polyclonal antibody against Gly m Bd 30K was incubated with 100  $\mu$ L of Protein A-agarose for 90 min at 4°C and 5  $\mu$ L of incubated solution was added to the diluted plasma sample. The interaction between Gly m Bd 30K in plasma and the Protein A-agarose-conjugated polyclonal antibody was induced by low-speed rotation overnight at 4°C. The immunocomplexes were recovered by centrifugation at  $14,000 \times g$  for 5 min at 4°C and then washed 4 times with 500  $\mu$ L of RIPA buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS]. SDS sample buffer (30  $\mu$ L) was added and the reaction mixture was boiled for 5 min. SDS-PAGE was carried out on 12.5% polyacrylamide gel according to the method of Laemmli (23). For immunoblotting analyses, proteins separated by SDS-PAGE were electroblotted onto polyvinylidene difluoride membranes under semidry conditions (24), and the electroblotted membranes were blocked in PBST containing 5% skim milk. The membranes were incubated with the rabbit polyclonal antibody against Gly m Bd 30K diluted 1:100 in blocking solution overnight at 4°C. The bound IgG antibody was detected using the HRP-conjugated anti-rabbit IgG antibody and an enhanced chemiluminescence Western blotting reagent (Amersham Bioscience).

**Protein assay.** Protein concentration was measured by the Bradford method (25) with a protein assay kit (BioRad) using bovine  $\gamma$ -globulin (Sigma) as a standard.

**Measurement of triglycerides in plasma.** The plasma triglyceride concentration was measured enzymatically with a Triglyceride E-Test Wako kit, which was obtained from Wako Pure Chemicals.

**Statistical analysis.** Data are presented as means  $\pm$  SD. Differences in the plasma Gly m Bd 30K concentration among groups for each time point were analyzed by 1-way ANOVA and the Tukey-Kramer Multiple Comparison Test (Expts. 1 and 3). Differences in the plasma Gly m Bd 30K concentration between 24-d-old and 10-wk-old mice for each time point were tested by unpaired *t* test (Expt. 2). Differences from the previous time point in a group were analyzed by paired *t* test (Expts. 1, 2, and 3). The StatView program (SAS Institute) was used for the analysis. Differences were considered significant at  $P < 0.05$ .

## RESULTS

**Expt. 1.** A regression line for the sandwich ELISA was obtained for the concentrations of Gly m Bd 30K in plasma between 0.016 and 1.667 mg/L (data not shown). Gly m Bd 30K in plasma of 24-d-old mice was detected at its maximum concentration of  $0.391 \pm 0.055$  mg/L 30 min ( $P < 0.05$ ) after the administration at 2000 mg/kg BW, and its plasma concentration gradually decreased to  $0.127 \pm 0.052$  mg/L by the end of the observation period (Fig. 1A). The plasma Gly m Bd 30K concentration vs. time curve after 500 mg/kg BW administra-

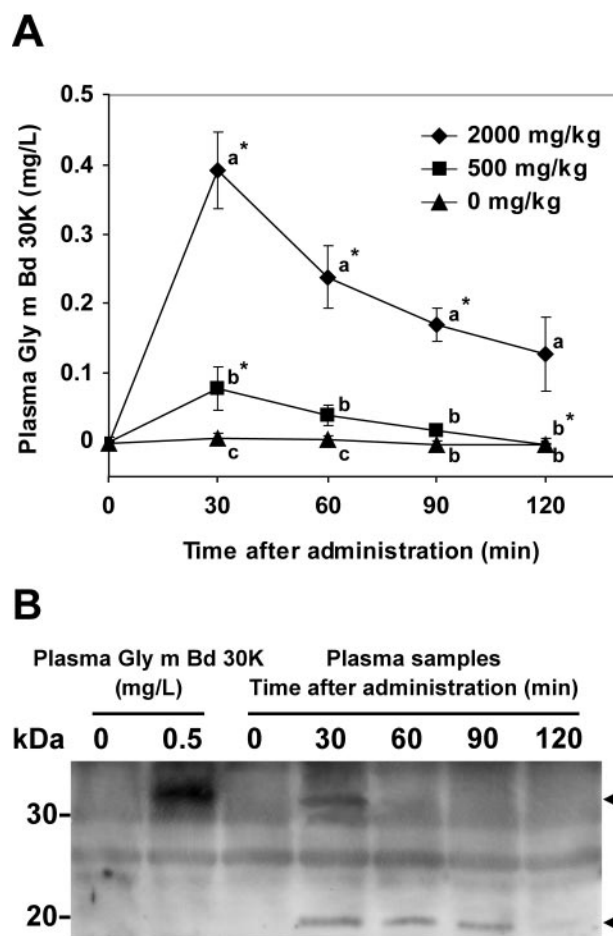


**FIGURE 1** Dose-dependence of gastrointestinal absorption of Gly m Bd 30K in 24-d-old mice (Expt. 1). Food-deprived 24-d-old mice were orally administered 1 mL of PBS containing Gly m Bd 30K (0, 500, or 2000 mg/kg BW). *Panel A* is a curve of plasma Gly m Bd 30K concentration vs. time, which was determined by sandwich ELISA using the rabbit polyclonal antibody. *Panel B* contains immunoblots of plasma samples after the oral administration of PBS containing Gly m Bd 30K (2000 mg/kg BW). Data are means  $\pm$  SD,  $n = 5$ . Means at a time without a common letter differ,  $P < 0.05$ . \*Different from the previous time point in that group,  $P < 0.05$ .

tion had a pattern similar to that after administration of 2000 mg/kg BW. Plasma Gly m Bd 30K reached its peak concentration of  $0.077 \pm 0.031$  mg/L at 30 min ( $P < 0.05$ ) and decreased to  $0.003 \pm 0.001$  mg/L at 120 min after the administration at 500 mg/kg BW. Plasma Gly m Bd 30K concentrations differed between mice administered 500 and 2000 mg/kg BW until 120 min ( $P < 0.05$ ). Gly m Bd 30K was not detected in plasma during the experimental period when PBS alone was administered orally as the control ( $P < 0.05$ ). These results indicate that the gastrointestinal absorption of Gly m Bd 30K is dose dependent.

To determine whether the primary structure plays any role in the absorption of Gly m Bd 30K molecules, it was essential to determine whether Gly m Bd 30K remained intact in plasma when orally administered to mice. Intact Gly m Bd 30K was detected by immunoblotting plasma obtained 30 min after its administration at 2000 mg/kg BW (Fig. 1B). We also detected truncated fragments of a molecular mass of  $\sim 20$  kDa in plasma obtained from 30 to 120 min postadministration.

**Expt. 2.** After the administration of 1 mL of soy milk, plasma Gly m Bd 30K concentration in 24-d-old mice in-

creased continuously, reaching  $0.996 \pm 0.109$  mg/L by 120 min, the end of the observation period ( $P < 0.05$ ) (Fig. 2). In 10-wk-old mice, the level of Gly m Bd 30K absorbed reached its peak of  $0.150 \pm 0.036$  mg/L at 30 min ( $P < 0.05$ ) and then gradually decreased to  $0.013 \pm 0.013$  mg/L at 120 min. Plasma Gly m Bd 30K concentrations differed between the age groups from 60 to 120 min postadministration ( $P < 0.05$ ).

**Expt. 3.** The plasma Gly m Bd 30K concentration in 24-d-old mice gradually increased to  $0.588 \pm 0.055$  mg/L at 90 min ( $P < 0.05$ ) and was  $0.568 \pm 0.112$  mg/L at 120 min after coadministration with 5% corn oil (Fig. 3). Absorption was enhanced at 90 and 120 min compared with the control group, which was administered Gly m Bd 30K alone ( $P < 0.05$ ). When 30% corn oil was coadministered with Gly m Bd 30K, the plasma Gly m Bd 30K concentration reached a plateau of  $0.825 \pm 0.092$  mg/L within 30 min ( $P < 0.05$ ) and remained constant until the end of the experimental period. The plasma triglyceride concentration peaked at  $\sim 1.6$ – $2.0$  mmol/L after 30 to 60 min and gradually decreased until the end of the observation period after the coadministration of Gly m Bd 30K and corn oil (5 and 30%). The plasma triglyceride concentration did not change during the experimental period when the allergen alone was administered orally (data not shown).

In mice administered Gly m Bd 30K and 3% sucrose fatty acid ester, the plasma Gly m Bd 30K concentration increased significantly to  $0.631 \pm 0.105$  mg/L at 30 min ( $P < 0.05$ ) and increased slightly ( $P = 2.76$ ) until 120 min (Fig. 4A). The concentration was greater than in the controls from 30 through 120 min ( $P < 0.05$ ). When 30% corn oil and 3% sucrose fatty acid ester were coadministered with Gly m Bd 30K, the plasma Gly m Bd 30K concentration reached a plateau at  $1.168 \pm 0.126$  mg/L at 30 min ( $P < 0.05$ ). The concentration in this group was greater than in all other groups until 120 min ( $P < 0.05$ ).

Intact Gly m Bd 30K and truncated fragments ranging in size from 15 to 20 kDa were visible on immunoblots of plasma with the rabbit polyclonal antibody against Gly m Bd 30K from 30 to 120 min after the coadministration of Gly m Bd 30K with 30% corn oil and 3% sucrose fatty acid ester (Fig.

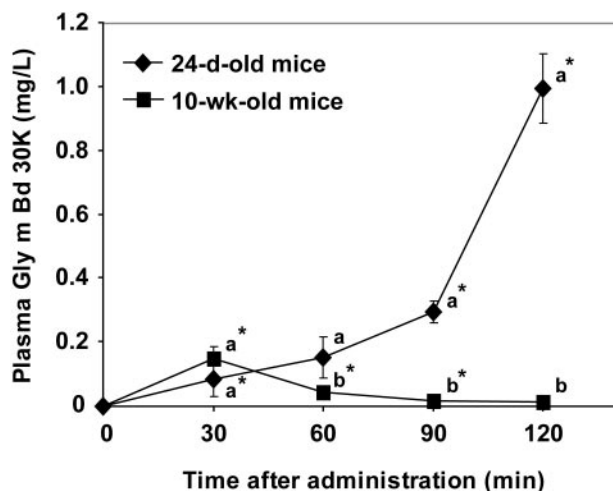


**FIGURE 2** Gastrointestinal absorption of soy milk Gly m Bd 30K in young and older mice (Expt. 2). Food-deprived 24-d-old and 10-wk-old mice were orally administered 1 mL of soy milk. The plasma Gly m Bd 30K concentration was determined by sandwich ELISA using the rabbit polyclonal antibody. Data are means  $\pm$  SD,  $n = 5$ . Means at a time without a common letter differ,  $P < 0.05$ . \*Different from the previous time point in that group,  $P < 0.05$ .

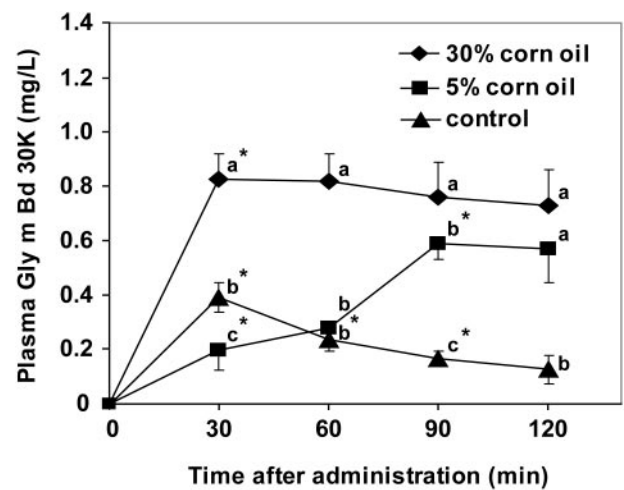


**FIGURE 3** Effect of a dietary fat on gastrointestinal absorption of Gly m Bd 30K in 24-d-old mice (Expt. 3). Food-deprived 24-d-old mice were orally coadministered 1 mL of sonicated PBS containing Gly m Bd 30K (2000 mg/kg BW) and corn oil [5 or 30% (v:v)]. The control was PBS containing Gly m Bd 30K (2000 mg/kg BW) alone. The plasma Gly m Bd 30K concentration was determined by sandwich ELISA using the rabbit polyclonal antibody. Data are means  $\pm$  SD,  $n = 5$ . Means at a time without a common letter differ,  $P < 0.05$ . \*Different from the previous time point in that group,  $P < 0.05$ .

4B). These results suggest that intact Gly m Bd 30K and large fragments survived digestion by gastrointestinal digestive enzymes and were absorbed into the circulation.

## DISCUSSION

In this study, we demonstrated for the first time that both purified and natural forms of Gly m Bd 30K in soy milk are absorbed from the gastrointestinal tract into the blood of young and older mice. In addition, the effects of corn oil and/or sucrose fatty acid ester on the absorption of Gly m Bd 30K were investigated in vivo. In Expt. 1, plasma Gly m Bd 30K concentration peaked 30 min after the administration of the purified allergen (Fig. 1A) and absorption occurred in the upper small intestine in a dose-dependent manner. Plasma Gly m Bd 30K concentration decreased continuously until the end of the observation period due to catabolism and degradation in the body. Generally, allergenic proteins seem to be more resistant to digestion than nonallergenic proteins (26–29). Astwood et al. (30) demonstrated that intact Gly m Bd 30K is not as stable as nonallergenic proteins in a gastric model (simulated gastric fluid), whereas the digested fragments of Gly m Bd 30K are stable for at least 8 min. This result suggests that Gly m Bd 30K has a unique property in the sensitizing system of an allergic reaction. In the present study, Gly m Bd 30K was identical not only to intact proteins, but also to digested fragments in plasma with a molecular mass of  $\sim 20$  kDa as determined by immunoblotting with the rabbit polyclonal antibody (Fig. 1B). Unfortunately, the nonspecific binding proteins from plasma were between 20 and 30 kDa. We did not detect fragments of molecular masses smaller than 10 kDa because we ran our SDS-PAGE experiments on 12.5% polyacrylamide gels.

Food-specific IgE antibodies bind to high-affinity FcεR1 receptors on mast cells, basophils, macrophages, monocytes, eosinophils, and platelets. When food allergens containing  $>2$  IgE-binding epitopes pass through the mucosal barrier and

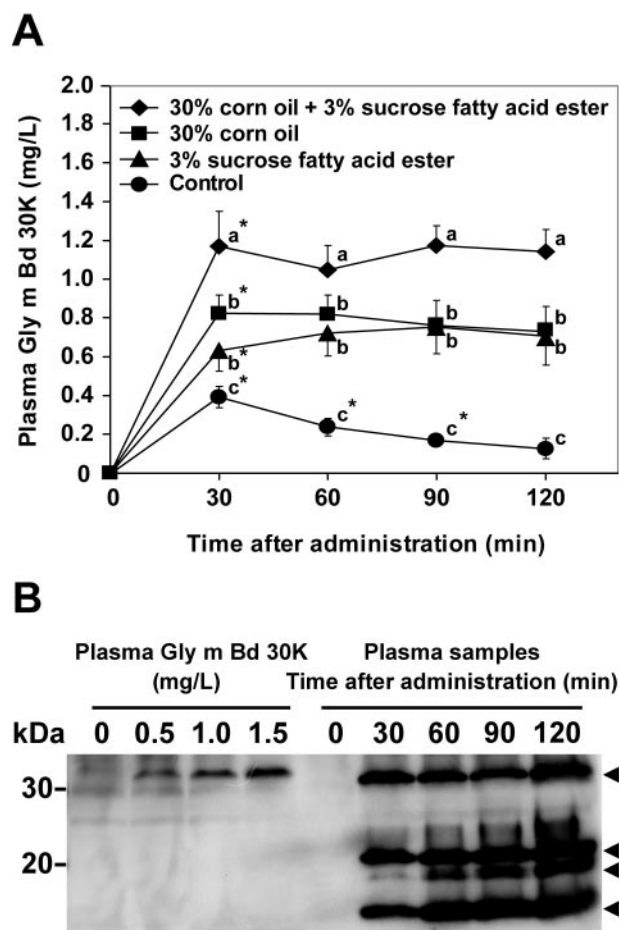


**FIGURE 4** Effects of dietary fat and/or exogenous emulsifier on the gastrointestinal absorption of Gly m Bd 30K in 24-d-old mice (Expt. 3). Food-deprived 24-d-old mice were orally coadministered 1 mL of sonicated PBS containing Gly m Bd 30K (2000 mg/kg BW) with 30% (v:v) corn oil, 3% (wt:v) sucrose fatty acid ester, or 30% (v:v) corn oil and 3% (wt:v) sucrose fatty acid ester. The control was PBS containing Gly m Bd 30K (2000 mg/kg BW) alone. *Panel A* is a curve of plasma Gly m Bd 30K concentration vs. time, which was determined by sandwich ELISA using the rabbit polyclonal antibody. *Panel B* contains immunoblots of plasma samples after coadministration of 1 mL of sonicated PBS containing Gly m Bd 30K (2000 mg/kg BW), 30% (v:v) corn oil, and 3% (wt:v) sucrose fatty acid ester. Data are means  $\pm$  SD,  $n = 5$ . Means at a time without a common letter differ,  $P < 0.05$ . \*Different from the previous time point in that group,  $P < 0.05$ .

contact IgE antibodies on mast cells or basophils, chemical mediators such as histamines and leukotrienes are released from cells and elicit immediate hypersensitivity (31). Van Beresteijn et al. (32) demonstrated the immunological properties of hydrolysates of whey protein concentrate and found that the minimum molecular mass necessary to elicit immunogenicity and an allergic response is between 3 and 5 kDa. Protease-resistant fragments generated by the *in vitro* digestion of the major peanut allergen, Ara h 1, with trypsin, chymotrypsin, or pepsin, ranged from 16 to 61 kDa and were identified by immunoblotting using sera IgE from pooled peanut-sensitive patients (33). We assume that intact Gly m Bd 30K and truncated fragments in plasma retain IgE-binding epitopes and can sensitize an allergic reaction.

In Expt. 2, plasma Gly m Bd 30K concentration in 24-d-old mice was significantly higher than that in 10-wk-old mice after the oral administration of 1 mL of soy milk (Fig. 2). Ekstrom

et al. (34) reported that the intestinal uptake of macromolecules such as bovine serum albumin, bovine IgG, and fluorescein isothiocyanate-labeled dextran 70,000 is enhanced in young guinea pigs due to intestinal closure that occurs after the replacement of fetal absorptive cells with adult cells lacking this ability. In addition, Chen et al. (35) found that the digestive and absorptive functions of the rat jejunum at different ages are highly dependent on the state of villous morphology such as the villous plexus, the capillary networks, the number of endothelial holes, and the thickness of the basal membrane. The enzymatic activities of chymotrypsin, trypsin, amylase, and gastric proteases increased with age in piglets (36). A decrease in pancreatic enzymatic activity was found during wk 1 postweaning (37). On the basis of these observations, we suggest that the absorption of Gly m Bd 30K is age dependent and is enhanced in postweanling mice due to the immaturity of the intestinal morphology and enzymes.

In developed countries, the incidence of "lifestyle-related" diseases such as diabetes, hyperlipidemia, hypertension, and cancer has increased over the last few decades. The consumption of a high-fat diet was proposed to be a risk factor for these diseases. Recently, several studies showed an enhancing effect of dietary fats on the absorption of low-molecular-weight drugs and food constituents in *in vivo* and *in vitro* models (38–41). Azuma et al. (42) observed that the coadministration of dietary fats (soybean oil or lecithin) and emulsifiers (such as the bile constituent taurocholate or sucrose fatty acid ester) enhanced the intestinal absorption of quercetin, a plant flavonoid, in rats. In Expt. 2, plasma Gly m Bd 30K concentration gradually increased when soy milk was administered orally as a natural source of Gly m Bd 30K in 24-d-old mice (Fig. 2). One possible explanation for this finding could have been the ~3.5% dietary fat content of soy milk. Therefore, we conducted the current study to determine the influence of dietary fats, using corn oil, and to examine the absorption of Gly m Bd 30K.

In Expt. 3, the plasma Gly m Bd 30K concentration was significantly greater when coadministered with 5 or 30% (v:v) corn oil, compared with administration of allergen only (Fig. 3). When Gly m Bd 30K was administered with 5% corn oil, it was partly incorporated into lipid micelles after sonication. The lipid micelles could have promoted its solubility, transported through the unstirred water layer, and played an adjuvant role through the brush boarder membrane of intestinal epithelial cells. Our results suggest that lipophilicity is an important determinant for the incorporation of Gly m Bd 30K into intestinal epithelial cells. It is likely that a portion of Gly m Bd 30K was absorbed through the blood circulation due to the incorporation of a dietary fat. When the corn oil concentration was increased to 30%, the protein could be completely incorporated into lipid micelles by the lipophilic part from a dietary fat. These conditions could have effectively enhanced the resistance to gastrointestinal proteases and rapidly enhanced the participation of lipid micelles and epithelial cells in the small intestine. We presume that the enhancement of absorption of the oil-body-associated protein Gly m Bd 30K and digested fragments occurred via fat carrier-mediated transport and was enhanced with an increase in the percentage of corn oil (Fig. 3). This could be explained by the observation that plasma Gly m Bd 30K concentrations reached a plateau early ( $0.825 \pm 0.092$  mg/L), at 30 min in mice administered Gly m Bd 30K with 30% corn oil, whereas in the mice coadministered 5% corn oil, plasma Gly m Bd 30K concentration reached a plateau ( $0.588 \pm 0.055$  mg/L) after 90 min.

Based on our result in Expts. 2 and 3, the plasma Gly m Bd 30K concentration ( $0.996 \pm 0.109$  mg/L) 120 min after the

administration of soy milk (~1 mg of Gly m Bd 30K and 3.5% dietary fat) in 24-d-old mice did not reach a plateau and was 75% higher than that in mice coadministered (~20 mg of Gly m Bd 30K) 5% corn oil ( $0.569 \pm 0.122$  mg/L). We suggest that the absorption kinetics of Gly m Bd 30K in these cases are different. In soy milk, Gly m Bd 30K is most likely bound to the surface of oil bodies, which are lipoparticles (triacylglycerol matrix surrounded by a phospholipids/oleosin monolayer). Kalinski et al. (22) reported that a 34-kDa oil-body-associated protein (Gly m Bd 30K) has 6 mol of cysteine/mol from the DNA sequence. Samoto et al. (43) demonstrated that a portion of Gly m Bd 30K in soy milk specifically forms a complex through a disulfide bond with the  $\alpha'$ - and  $\alpha$ -subunits of conglycinin, which are other allergenic proteins. The specific binding of Gly m Bd 30K to food allergens and the other components in soy milk could be efficiently promoting the absorption of Gly m Bd 30K. In addition, the other nutrient-nutrient interactions in soy milk would influence intestinal protein absorption. However, because no data are available concerning the nutrient-nutrient interaction effects on the absorption of Gly m Bd 30K, these remain speculative at present. In the present study, soy milk and the 5% corn oil solution were not isoenergetic due to differences in total protein, carbohydrate, dietary fiber, and other nutrient contents. It was demonstrated that a high viscosity, a high fiber content, and a high energy content delay gastric emptying (44,45). On the basis of these reports, we conclude that the rate of gastric emptying after the administration of soy milk is determined to be slower than that after administration of a 5% corn oil solution. The absorption of Gly m Bd 30K was delayed in mice administered soy milk so that the plasma Gly m Bd 30K concentration increased rapidly 120 min after the administration of soy milk.

Emulsifiers are commonly added to food products such as confectionaries, bread, ice cream, and margarine to improve food texture and to stabilize emulsions. In Expt. 3, we used sucrose fatty acid ester, which is a nonionic surfactant for the stabilization of protein-fat emulsions, for coadministrations in mice. We found that a combination of sucrose fatty acid ester and corn oil enhanced Gly m Bd 30K absorption, likely because the emulsifier facilitated the formation of the emulsion and the stabilization of micelles (Fig. 4A). However, sucrose fatty acid ester was reported to increase the paracellular uptake of ovomucoid by the induced shortening of microvilli, actin disbandment, and structural separation of tight junctions in human intestinal epithelial Caco-2 cells (46). These effects could explain why the absorption of Gly m Bd 30K was significantly enhanced when Gly m Bd 30K was coadministered with sucrose fatty acid ester compared with the control solution (Fig. 4A).

The plasma triglyceride concentration increased after coadministration of Gly m Bd 30K with corn oil, suggesting that dietary fats were also absorbed into the blood circulation. It was demonstrated that dietary fats from the intestines are incorporated into chylomicrons with cholesterol and apolipoproteins and move from the intestinal mucosa into the lymphatic system (47,48). On the basis of this information, we suggest that a portion of Gly m Bd 30K and truncated fragments might by-pass the liver by becoming incorporated with dietary fats and subsequently absorbed into the peripheral blood via the lymphatic system. When ingested in a food containing soybean protein, Gly m Bd 30K would behave as a hydrophobic group and associate with dietary fats from soybeans and other hydrophobic food components; this behavior is characteristic of an oil-body-associated protein. These phenomena would enhance not only protease resistance in the

stomach but also the transit time of the protein in the small intestine. Accordingly, the absorption of Gly m Bd 30K from an epithelial cell membrane of the small intestine to the blood circulation would be enhanced so that a sensitization might occur. The association of Gly m Bd 30K with the oil body is the critical factor that renders it an effective allergenic protein.

In conclusion, we showed that intact Gly m Bd 30K and digested fragments were dose dependently absorbed *in vivo*. When Gly m Bd 30K was coadministered with dietary fats, absorption was enhanced via fat carrier-mediated transport. Finally, we demonstrated that plasma Gly m Bd 30K concentration in 24-d-old mice was significantly higher than that in 10-wk-old mice after the oral administration of soy milk. These observations suggest that the absorption of Gly m Bd 30K decreases with age. Further studies are required to determine the molecular mechanism(s) underlying the intestinal absorption of Gly m Bd 30K and to identify an absorption inhibitor that could potentially prevent food allergies.

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