Digestion and Gastrointestinal Absorption of the 14–16-kDa Rice Allergens

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The digestibility and gastrointestinal absorption of 14–16-kDa rice allergens (RAs) were investigated. RAs and bovine serum albumin (BSA) were first evaluated for their digestibility. BSA was digested completely by in vitro incubation with some proteases, but RAs remained almost intact. Administered orally (20 mg per mouse), intact RAs were clearly detected in the small intestine even 60 min after the administration, the amount of total RAs in the small intestine being estimated to be 0.59 mg. RAs were then biotinylated and infused into the duodenal lumen of anesthetized mice, and portal blood was collected. The RA concentrations in the portal plasma were respectively estimated to be 0.4–0.9 and 0.3–2.5 μg/ml for 0.4 and 4 mg doses. These results suggest that RAs are highly resistant to digestive enzymes and that about 1/100 of orally administered RAs remain intact in the small intestine, while at least 1/1,000–1/10,000 is absorbed and delivered into circulated blood.

Key words: 14–16-kDa rice allergen; digestion; absorption; biotinylated protein

IgE antibody-mediated food allergies are a common problem occurring in developed countries. The inappropriate immune responses that may result in an inflammatory reaction and tissue damage have been classified into four types by Coombs and Gell. Only type I hypersensitivity reactions involve the generation of an IgE antibody response toward a food allergen, the IgE then binding to the surface of histamine-containing mast cells. Following exposure to the same allergen on a subsequent occasion, the allergen becomes bound to several IgE molecules, cross-linking them. This event triggers the release of mediators such as histamine from the mast cells, which go on to trigger an acute inflammatory reaction. The normal function of IgE is to provide some protection against parasitic infestation, but the mechanism involved in triggering its production in allergic disease has not been defined.

Most food allergic patients are affected after the consumption of milk, eggs, wheat, peanuts, buckwheat, soybeans and rice. Rice is a cereal produced and consumed in large quantity in South and East Asian countries. On the one hand, several clinical studies have suggested that rice grains are responsible for severe atopic dermatitis in some adult patients.1–3) Some allergenic proteins, including the 14–16-kDa, 26-kDa and 33-kDa types, have been identified and characterized biochemically and immunochemically.4–9) In particular, 14–16-kDa proteins have been reacted most frequently with IgE in the serum of rice allergic patients. Furthermore, cDNAs encoding these allergenic proteins have been cloned and their nucleotide sequences determined.9–13) The deduced amino acid sequences show that RAs are structurally homologous the α-amylase/trypsin inhibitors of other cereal species, especially in the number and distribution of ten cysteine residues. RAs appear to be globular and compactly folded molecules with five intermolecular disulfide bridges,8) which would stabilize the folding of the ordered structures, resulting in heat stability and resistance to proteolytic degradation in the gastrointestinal tract.

The gastrointestinal tract serves to provide an encounter between the body and the external environment. The mouth, esophagus, stomach and small/large intestines are each part of the digestive system including the pancreas and liver.14) The primary function of the gastrointestinal tract is the digestion and absorption of dietary components from the intestinal lumen into the circulation. Another function is to act as a barrier, preventing infestation by harmful entities including microorganisms, luminal antigens and luminal inflam-
matory factors. This function is provided by immunologic mechanisms involving immunoglobulins and lymphocytes present in the intestinal mucosa and by non-immunologic mechanisms such as selective intestinal permeability.\(^\text{15}\) Dietary proteins are primarily digested within the gastrointestinal tract by the action of secreted proteinases or peptidases in the enterocyte microvillous membrane. However, some studies support the idea that a small, but significant amount of food allergens escape this enzymatic breakdown, and biologically and/or antigenically active peptides and proteins pass through the epithelial cells of the intestines\(^\text{16–20}\) and are sensitized via the gastrointestinal tract. It is clear that factors such as the level of exposure to allergens and genetic predisposition play important roles, although these mechanisms have not been well characterized in vivo.

As a first step to identify the mechanisms involved in the uptake of an allergen and the subsequently induced allergic reaction, we investigated the digestibility of RAs in vitro and in vivo. We assessed the degradation kinetics of RAs after an oral administration to mice, and measured the absorption of these allergens from the duodenal lumen to the portal vein of anesthetized mice by sandwich ELISA, using avidin and the mouse monoclonal antibody, 25B9,\(^\text{21}\) specific to RAs. We discuss the correlation between the dose and absorbed amount of allergens, and the mechanism for allergen transport.

### Material and Methods

**Antibodies.** To detect RAs by immunoblotting analyses, competitive ELISA and sandwich ELISA, the mouse monoclonal antibody, 25B9, specific to RAs and horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Jackson Immunoresearch, USA) were used. RAs are products of a multigene family and have sequence similarity in their amino acid level from 70% to 95%, especially near the C-terminus. 25B9 recognizes this site and, thus, this monoclonal antibody can detect all RAs. On the other hand, to detect BSA, the rabbit anti-BSA antibody (MP Biomedicals, USA) and HRP-conjugated anti-rabbit IgG (Bethyl Laboratories, USA) were used.

**Fraction containing RAs prepared from rice grains.** RAs were isolated from grains of rice (\textit{Oryza sativa} L. var. \textit{japonica} cv. Hoshinoyume) by precipitation with 70% saturated ammonium sulfate. The precipitate was collected and dissolved in PBS, with subsequent dialysis against distilled water. The insoluble proteins (mostly \(\alpha\)-globulin) were removed by centrifugation, and the supernatant (mostly albumin) was lyophilized and used for the subsequent experiments as the RA preparation.

**In vitro digestion of RAs and BSA.** One milligram of RAs and BSA (Wako Pure Chemicals, Osaka) were incubated with 25 \(\mu\)g of pepsin (Yoneyama Chemicals, Osaka) in 0.2 ml of 30 mM NaCl (pH 1.2 adjusted by HCl) and also incubated with trypsin or chymotrypsin (Wako Pure Chemicals, Osaka) in a 50 mM Tris–HCl buffer (pH 8.0) at 37°C for 0, 10, 30, 60, 120 min and 16 h. After this incubation to stop the enzyme reaction, the samples incubated with pepsin were added 75 \(\mu\)l of 160 mM Na\(_2\)CO\(_3\), and the other samples incubated with trypsin or chymotrypsin were boiled for 10 min. The digestibility of each sample was evaluated by an SDS–PAGE analysis.

**In vivo digestion of RAs and BSA.** The mice were fasted with free access to water for 16 h before the experiment. Twenty milligrams of RAs or BSA in 200 \(\mu\)l of PBS was administrated orally to the mice, the digestive organs being excised after 15, 30, 60 and 120 min from the mice in the RA group and after 15 min from the mice in the BSA group. The digestive organs were divided into the stomach, small intestine, appendix and large intestine, and the small and large intestines were further divided into six parts and two parts, respectively. The contents of the each part were collected by washing out with 1 ml of cold PBS, and the supernatant was recovered by centrifugation at 10,000 \(\times\) g for 10 min. One hundred microliters of the supernatant was mixed with 25 \(\mu\)l of a 5 \(\times\) SDS sample buffer and boiled for 5 min. The digestibility of these proteins in each sample was assessed by SDS–PAGE and immunoblotting analyses.

**Preparation of biotinylated RAs.** RAs purified by the ammonium sulfate precipitation method as already described were dissolved in PBS and incubated with Biotin Sulfo-OSu (13 mg/g of protein; Wako Pure Chemicals, Osaka) at 4°C overnight. Subsequently, the solution was applied to a Sephadex G-50 gel
filtration column (ϕ2 × 90 cm) and eluted with PBS. The fraction of the second UV peak was collected, dialyzed against distilled water and lyophilized.

**Competitive ELISA.** Each sample solution and a standard RA solution were appropriately diluted with PBS, mixed with a fixed amount of the anti-RA antibody, 25B9, in 1% BSA/PBS, and pre-incubated at 4°C overnight. ELISA plates (96 wells, Nunc, Denmark) were incubated with RAs (10 μg/ml of PBS) at 4°C for 16 h and blocked with 1% BSA/PBS at 37°C for 2 h. The wells were washed 4 times with PBS containing 0.05% (v/v) Tween 20 (PBST). The sample and standard solutions that had been pre-incubated with the anti-RA antibody, 25B9, were applied to each well of the antigen-coated plates and incubated at 37°C for 2 h. After washing with PBST, they were incubated with HRP-conjugated anti-mouse IgG in 1% BSA/PBS at 37°C for 2 h. The HRP activity was measured by adding 0.04% o-phenylenediamine and 0.003% H2O2 in a substrate buffer (0.05 M citrate/0.1 M disodium hydrogenphosphate at pH 5.0). The absorption at 492 nm was measured with an MTP-650FA microplate reader (Corona Electric, Ibaragi, Japan).

**Infusion of biotinylated RAs into the intestinal lumen and blood sampling.** The biotinylated RAs (0.4 or 4 mg per mouse) were infused directly by needle into the duodenal lumen of mice with an opened abdominal cavity under Nembutal anesthesia. The blood of the mice was collected by cannulation from the portal vein 5 min after the infusion, and plasma was recovered by centrifugation at 3,000 × g for 10 min. Each sample was stored at −20°C until needed.

**Sandwich ELISA for biotinylated RAs.** ELISA plates were coated with 2 μg/ml of streptavidin at 4°C overnight and blocked with 1% BSA/PBS at 37°C for 1 h. Purified RAs used as a standard were dissolved in mouse normal plasma at concentrations of 0–20 μg/ml. After the wells had been washed with PBST, 50 μl each of 1:10-diluted plasma samples as well as the standard RAs in 1% BSA/PBS were added to each well of the streptavidin-coated plates and incubated at 37°C for 1.5 h. The plate-bound RAs were detected by using the anti-RA antibody, 25B9, and HRP-conjugated antimouse IgG as already described.

**Results**

**Resistance of RAs to proteolytic hydrolysis both in vitro and in vivo**

To examine the stability of the allergens, RAs and BSA as a control were evaluated for their digestibility to pepsin, trypsin and chymotrypsin in vitro by SDS-PAGE. They were incubated with each enzyme (an enzyme/substrate weight ratio of 1/40) at 37°C for 0, 10, 30, 60, 120 min and 16 h. The gel sheet was stained with CBB and a molecular mass standard (M) was also applied to the same gels.
allergenic protein administrated orally to a mouse was almost completely digested but 40 mg of the protein was not. The two proteins in the intestinal contents were then assessed by SDS–PAGE/immunoblotting analyses (Fig. 2). BSA was not detected in the gastrointestinal tract, apart from the stomach, by the immunoblotting analysis, suggesting that BSA had been broken down by digestive enzymes in the small intestine. On the other hand, intact RAs and their fragments were clearly detected in the intestinal contents. These results indicate that RAs were much more resistant than BSA to proteolytic hydrolysis in vitro and in vivo.

Degradation kinetics of orally administrated RAs in the mouse gastrointestinal lumen

To analyze the degradation kinetics of RAs in the gastrointestinal lumen, RAs remaining in the gastrointestinal tract were assessed in samples with various in vivo incubation times. Using the same methods as those just described, 20 mg of RAs were administrated orally to the mice, and the gastrointestinal contents were recovered at various times (15–120 min) after the oral administration. RAs and their fragments remaining in the luminal contents were then assessed by SDS–PAGE and immunoblotting analyses (Fig. 3). Strong protein bands corresponding to intact RAs and their proteolytic fragments were clearly detected in the contents after 15, 30 and 60 min of incubation that were recovered from the proximal, middle and distal parts of the small intestine, respectively.

Furthermore, residual amounts of RAs in the contents recovered at various times after the oral administration were estimated by competitive ELISA (Fig. 4). In the

Fig. 2. Resistance of RAs to Mouse Digestive Enzymes in Vivo.

RAs or BSA (20 mg per mouse) were administrated orally into the mouse stomach, and the gastrointestinal tract was excised after 15 min. The obtained tract was then divided into the stomach (1), small intestine (2–7; divided into 6 parts numbered from proximal to distal), appendix (8) and large intestine (9 and 10; divided into 2 parts numbered from proximal), and the contents in each part were recovered by washing out with 1 ml of PBS. The proteins in the contents were subjected to SDS–PAGE/immunoblotting analyses. The gel sheets were stained with CBB (upper two panels), and the blotted membrane was immunostained with the anti-RA antibody, 25B9, (right-hand panel) or anti-BSA antibody (left-hand panel).

Fig. 3. Kinetics for the Proteolytic Degradation of Orally Administered RAs in the Mouse Gastrointestinal Lumen.

Twenty milligrams of RAs were administrated orally to the mice, and the gastrointestinal contents were recovered after 15, 30, 60 and 120 min as described in the legend to Fig. 2. RAs and their fragments were then detected by SDS–PAGE/immunoblotting analyses. Numbered lanes show the stomach (1), small intestine (2–7; divided into 6 parts numbered from proximal to distal), appendix (8) and large intestine (9 and 10; divided into 2 parts numbered from proximal).
case of the 20-mg administration, the RA amounts remaining in the stomach were estimated to be from 0.2 to 5.5 mg, and those in each small intestinal section were from 0.03 to 0.9 mg per section 15 min after the administration. The location of RAs changed with time from the stomach to the distal part of the small intestine, the amount of total RAs in the small intestine being estimated to be from 0.07 to 0.59 mg 60 min after the oral administration. These data obtained by two distinct immunochemical methods reveal that considerable amounts of RAs remained intact in the intestinal lumen for at least 60 min after the oral administration.

Absorption of biotinylated RAs from the intestinal lumen and transportation into the portal vein of mice

To examine the amount of RAs absorbed from the intestinal lumen, purified and biotinylated RAs were infused into the duodenal lumen of anesthetized mice (0.4 or 4 mg per mouse). The dose (0.4 mg of protein per mouse) was adopted from the amounts (0.1–0.9 mg) of residual RAs in a prior experiment (Fig. 4), and a tenfold dose (4 mg) was also adopted for comparison. The portal blood was collected 5 min later, and plasma RAs were measured by sandwich ELISA. Figure 5A shows that RAs purified by gel filtration had no change in molecular weight by biotinylation and could be detected by the anti-RA antibody, 25B9. The standard curve for RAs shown in Fig. 5B demonstrates that biotinylated RAs in plasma could be successfully determined at a level of 50–1,000 ng/ml by sandwich ELISA. The amounts of RAs estimated by ELISA were plotted against the dose of RAs administered to the mice (Fig. 5C) and against the plasma volume obtained from the portal vein of the mice (Fig. 5D). The concentrations of RAs in the portal plasma tended to be increased dose-dependently, being 0.4–0.9 μg/ml with a 0.4-mg administration and 0.3–2.5 μg/ml with a 4-mg administration, although a large deviation was observed within a group. Thus, the RA concentrations in the portal plasma were increased in a dose-dependent manner. On the other hand, there was no significant correlation between the volume of a blood sample collected and the allergen concentration in the sample.

Discussion

We investigated in this study the digestibility and gastrointestinal absorption of RAs in an attempt to elucidate the mechanism for the uptake of food allergens and the subsequently induced allergic reaction.

Many studies have been reported on the isolation, identification and structural analysis of food allergens, or on the antigenicity and binding affinity to IgE in vitro. In addition, several studies have attempted to find the mechanism for the absorption of intact protein and the induction of food allergy by using model proteins which are commercially available. It would therefore be important to analyze already identified allergens such as RAs and to compare their digestibility, absorbability and strength of the subsequently induced immune responses with those of non-allergenic model proteins.

It has been assumed that proteins that are stable to gastrointestinal digestion are inherently more hazardous than those which are susceptible, especially in relation to allergy. To validate this concept for allergens, we evaluated the digestive stability of RAs and BSA as a control in vitro and in vivo. SDS–PAGE/immunoblotting analyses revealed that BSA was digested completely in vitro and in vivo by digestive enzymes, whereas...
RAs were stable and remained almost intact under the same conditions. RAs appeared to be globular and compactly folded molecules with five intramolecular disulfide bridges. Moreover, RAs are members of the plant α-amylase/trypsin inhibitor family, and no less than ten cysteine residues in a small molecule consisting of about 150 amino acid residues are well-conserved within this family. The similarity of the intramolecular disulfide bridges and the sequence homology suggest that the proteins belonging to this inhibitor family have a common folding profile. Such a stabilized structure of this family might also contribute to the resistance to proteolytic degradation in the gastrointestinal tract, resulting in these proteins being allergenic. In fact, the α-amylase inhibitors of wheat and barley seeds have also been identified as major allergens in asthma, a respiratory allergy to cereal flour.34,35

When administrated orally into the mouse stomach (20 mg per mouse), intact RAs could be clearly detected in the contents recovered from the small intestine even 60 min after the administration, the amounts of total RAs in the small intestine being estimated to be from 0.07 to 0.59 mg by competitive ELISA. In this study, we used RAs purified from rice grains, but rice and its processed foods are usually cooked before eating. It is conceivable that the proteins in rice grains interact with such other nutrients as starch by heating, resulting in the digestibility and residual amount of RAs in the gastrointestinal tract being changed. Further studies using the natural form RAs are required to reveal their actual digestion and absorption in cooked rice grains of various foods.

To examine the amount of RAs absorbed from the intestinal lumen, purified and biotinylated RAs were infused into the duodenal lumen of anesthetized mice (0.4 or 4 mg per mouse). The portal blood was collected 5 min later, and the concentration of plasma RAs was measured by sandwich ELISA, using avidin and the anti-RA antibody, 25B9. Panel A shows RAs at each step of the purification and biotinylation processes. RAs prepared from rice grains by extraction and ammonium-sulfate precipitation (A-1) and purified by Sephadex G-50 gel filtration after biotinylation (A-2) were analyzed by SDS–PAGE. The biotinylated RAs were detected by the anti-RA antibody, 25B9 (A-3). Panel B shows a standard sandwich-ELISA curve for RAs obtained by using known concentrations of RAs added to the portal blood of normal mice. The RA concentrations estimated by ELISA were plotted according to the dose of RAs administrated to the mice (Panel C) and the plasma volume obtained from the portal vein of mice (Panel D). The unshaded and shaded circles represent the mice of the low-dose (0.4 mg) and high-dose (4 mg) groups, respectively (n = 10 in each group).
on average in about 0.4 ml of plasma (Figs. 5C and D), the absolute amount of absorbed allergens is calculated to be at least 200 ng, which respectively corresponds to 1/2,000 and 1/20,000 of 0.4 and 4 mg of allergens infused into the intestinal lumen. It is interesting to note that the highest content of total allergens in the intestinal lumen was estimated to be 0.59 mg among those mice examined in this study. These results suggest that RAs are highly resistant to digestive enzymes and, on some occasions, about 1/100 of orally administrated RAs remained in the small intestine, while about 1/1,000–1/10,000 was absorbed and delivered into the blood circulation.

References


25) Baur, X., and Posch, A., Characterized allergens causing


