Rice (*Oryza sativa* L.) α-Amylase Inhibitors of 14−16 kDa Are Potential Allergens and Products of a Multigene Family

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Five proteins of 14−16 kDa (**I**−**V**), with cross-reactivity with antibodies specific for a previously isolated 16-kDa allergen, were purified from rice endosperm. The N-terminal amino acid sequences of these proteins suggested that four of them were products of previously isolated cDNA clones of a rice RA multigene family. All purified proteins showed inhibitory activity toward human salivary α-amylase, whereas they inhibited neither bacterial α-amylase nor bovine trypsin. Radioallergosorbent tests (RAST), using immunoglobulin class E (IgE) antibodies from patients allergic to rice, showed that the IgE from some patients reacted with all five proteins, whereas others specifically reacted with individual proteins. These results indicate that all five proteins characterized as α-amylase inhibitors are potentially allergenic.

Keywords: *Allergen; rice albumin; α-amylase inhibitor; storage protein; Oryza sativa*

INTRODUCTION

Rice grain proteins account for ∼8% of the dried endosperm. They consist of 5−10% prolamins, 4−10% albumins and globulins, and 80−90% glutelins (Juliano, 1972). Rice NaCl-soluble protein fraction (globulin and albumin), which includes a 26−28-kDa group and a 11−17-kDa group, has been reported to cause IgE-mediated food allergy (Hoffman, 1975; Shibasaki et al., 1979). Limas et al. (1990) reported that a 28-kDa protein and a 12.5-kDa protein were IgE-binding proteins. We have previously identified a 16-kDa salt-soluble protein as rice allergen (Matsuda et al., 1988). Other proteins showing microheterogeneity in their molecular masses (14−16 kDa) and isoelectric points (p*I* 6−8) revealed cross-reactivities with polyclonal and monoclonal antibodies raised against the rice 16-kDa allergen, suggesting structural similarity among them (Matsuda et al., 1991). These proteins have been suggested to be products of a multigene family, although the possibility that they are a result of post-translational modification still remains. Ten cDNA clones and two genomic clones encoding 14−16-kDa proteins have already been obtained from rice (Izumi et al., 1992; Adachi et al., 1993; Alvarez et al., 1995a), and our recent results suggested that some of their gene products (RA17, RA14, RA14B, and RA5B) are potential allergens (Alvarez et al., 1995b). Based on their deduced amino acid sequences, their gene products were considered to belong to the cereal α-amylase/trypsin inhibitor family (Izumi et al., 1992; Adachi et al., 1994). The amino acid sequences of some NaCl-soluble proteins in rice, as reported by Limas et al. (1990), are similar to the deduced amino acid sequences of the aforementioned cDNA and genomic clones (Adachi et al., 1993).

To date, proteins with inhibitory activity against α-amylase or protease have been detected in most cereals, such as wheat, barley, rye, sorghum, maize, oat, pearl millet, setaria, ragi, triticale, and others (Garcia-Olmedo et al., 1987; Barber et al., 1989; Feng et al., 1991a). Rice salt-soluble proteins of ∼14 kDa have been reported to inhibit insect and mammalian α-amylases (Feng et al., 1991b), but their primary structures and their relations to rice allergy have not been elucidated.

In this study, to clarify the relationship between α-amylase inhibitors and allergenic proteins in rice endosperm, the 14−16-kDa protein components of a salt-soluble protein fraction were isolated based on their reactivities to antibodies specific for a previously isolated 16-kDa allergen, and their reactivity to IgE antibodies from patients allergic to rice and their inhibitory activities against human salivary and bacterial α-amylases were determined. The N-terminal amino acid sequences of the isolated proteins were also compared with the deduced amino acid sequences from the previously isolated cDNA clones belonging to α-amylase/trypsin inhibitor gene family.

MATERIALS AND METHODS

Materials. For the isolation of rice albumins, commercially available dehulled grains of rice (*Oryza sativa* L. *Japonica* var. Nipponbare) were used. The rabbit antiserum and a mouse monoclonal antibody (mAb) 25B9 specific for the 16-kDa rice allergen were prepared as described previously (Matsuda et al., 1988). Human salivary α-amylase (Type IX-A) was purchased from Sigma Chemical Company. Bacterial α-amylase of *B. amyloliquefaciens* was a gift from Dr. H. Yamagata (Tokyo College of Pharmacy).

Seventeen sera showing positive RAST values (Urisu et al., 1991) for rice were obtained from patients with atopic dermatitis with or without bronchial asthma. RAST values higher than the mean plus two standard deviations (SD) of those of control sera from healthy volunteer were judged as positive.

Extraction, Isolation, and Purification of Rice Salt-Soluble Proteins. Dehulled rice in 1 M NaCl was sonicated under cold conditions; the supernatant obtained after centrifugation at 10000*g* for 15 min was saturated to 70% ammonium sulfate; the precipitate was dialyzed against 20 mM Tris-HCl (pH 8.6); and the dialyzate (200 mL) containing ∼1 g of protein was subjected to DEAE ion-exchange chromatography (DE52, Whatman). The Tris-HCl buffer (20 mM, pH 8.6) was passed through the column until unabsorbed proteins

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were eluted. The column was subjected to a linear gradient of NaCl from 0 to 0.1 M in 1 L of the Tris-HCl buffer to fractionate the absorbed proteins. This procedure was followed with elution with 500 mL of the Tris-HCl buffer containing 0.5 M NaCl to recover the proteins remaining in the column. The elution profile was monitored by measuring UV absorbance at 280 nm, and the fractions comprising each peak were pooled and freeze-dried. The major components in each peak were further purified by reversed-phase HPLC (RP-HPLC) on a Biofine RPC-PO column (JASCO). The proteins were eluted with a linear gradient of acetonitrile from 0 to 60% in 0.1% trifluoroacetic acid (Uozumi et al., 1991). The N-terminal amino acid sequence of each purified protein was determined with a 476 Model ABI protein sequencer.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblotting. SDS-PAGE was carried out according to Laemmli (1970), and the proteins were electroblotted on PVDF membranes (Immobilon, Millipore; Towbin et al., 1979). After blotting, the membranes were incubated at 4 °C overnight in 3% bovine serum albumin (BSA), incubated with 25B9 for 2 h, and then reacted with peroxidase (POD)-linked anti-mouse IgG (Cappel) for 1.5 h. The color reaction was visualized by incubation of the membranes in phosphate buffered saline (PBS) containing 0.03% H_2O_2 and 0.05% 4-chloro-1-naphthol (Matsuda et al., 1991).

Assay of α-Amylase and Trypsin Inhibitory Activities. α-Amylase assays were performed with a Wako amylase test kit (Wako Pure Chemical Industries, Osaka, Japan). Five micrograms of rice proteins or control protein (BSA) were incubated with 0.2 mL of α-amylase solution (140 U) in 0.25 M phosphate buffer (pH 7.0) at 37 °C for 5 min. One milliliter of 0.25 M phosphate buffer (pH 7.0) containing 0.4 mg of soluble starch as substrate was added to the enzyme solution, and the solution was incubated at 37 °C for 7.5 min. The enzyme activity was determined by the change in absorbance (A_{660}) after adding 1 mL of iodine solution and 5 mL of distilled water. Inhibitory activity is reported as the percent of the enzyme activity inhibited by 5 μg of protein. Trypsin inhibitory activity was assayed by measuring the change in A_{420} of the enzyme reaction mixture containing α-*N*-benzoyl-L-arginine *p*-nitroanilide as substrate (Waheed and Salahuddin, 1975).

RAST and Enzyme-Linked Immunosorbent Assay (ELISA). RAST was done as described previously (Urisu et al., 1991). The purified proteins (1 μg), dissolved in 50 μL of 0.1 M sodium carbonate buffer (pH 9.8), were placed in each well of a microtiter plate (ELISA plate, E. Sumimoto Bakelite Company, Ltd.) for 12 h at 4 °C. The antigen-coated plates were blocked with 2% human serum albumin (HSA; Sigma Chemical Company)/0.1 M sodium carbonate buffer (pH 9.8) for 3 h, reacted with patients' sera diluted with 10% HSA/PBS (1:100) for 12 h, followed by reaction with ^{125}I-anti-human IgE antibody (Dynabot K.K.) for 12 h at 4 °C, in which 45 757 cpm of the radiolabeled antibody was added to each well. Radioactivity of each well was measured with a γ-counter (Packard Company Ltd.). ELISA was done using POD-linked anti-mouse IgG and *o*-phenylenediamine as described previously (Matsuda et al., 1988).

RESULTS

Protein Purification and Characterization. Total salt-soluble proteins in rice endosperm were fractionated by DEAE ion-exchange chromatography. Five proteins (**I**−**V**) were isolated (Figure 1). Three protein fractions (**I**−**III**) were separated by isocratic elution, whereas two others (**IV** and **V**) were eluted with a salt gradient of 0−0.1 M NaCl. Proteins remaining in the column were eluted with 0.5 M NaCl. All of the five fractions were positive to mAb 25B9, as measured by ELISA (data not shown).

An aliquot (20 μL) containing ∼1 μg of protein from each protein fraction (**I**−**V**) was subjected to SDS-PAGE and immunoblotting. As shown in Figure 2A, the major

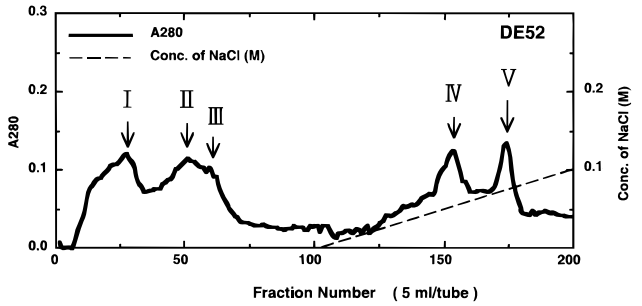

Figure 1. Separation of rice salt-soluble proteins by DEAE ion-exchange chromatography. The five peaks used for further studies are indicated by the arrows.

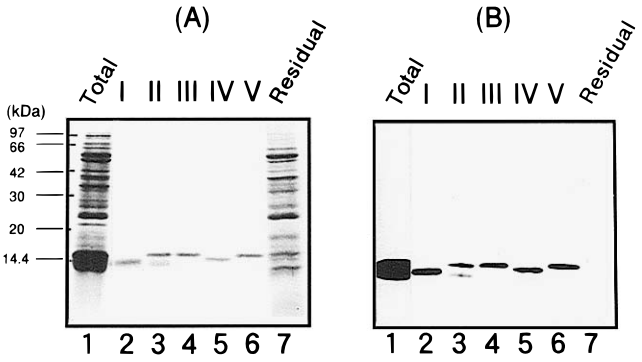

Figure 2. SDS-PAGE and immunoblotting of isolated rice salt-soluble proteins. After SDS-PAGE, gels were stained with Coomassie brilliant blue (A) or immunoblotted with mAb 25B9 (B). Key: (Lane 1) total salt-soluble proteins; (lane 2) protein **I**; (lane 3) protein **II**; (lane 4) protein **III**; (lane 5) protein **IV**; (lane 6) protein **V**; (lane 7) proteins eluted with 0.5 M NaCl.

components in two fractions (**I** and **IV**) had similar apparent molecular masses of ∼14−15.5 kDa, and the other three (**II**, **III**, and **V**) were ∼16 kDa. All five proteins reacted strongly with the mAb 25B9 (Figure 2B). Although a 16-kDa protein is the major protein in fraction **II**, a small amount of ∼14-kDa protein was also present. Both of the protein fractions reacted with mAb 25B9. The major proteins in the five fractions were also positive to a rabbit polyclonal antiserum specific for the 16-kDa allergen (data not shown). The fractions that eluted with 0.5 M NaCl (residual proteins) also contained some proteins of 14−16 kDa, but they did not react with mAb 25B9, indicating that there is no protein recognized by mAb 25B9 in the acidic proteins eluted with 0.5 M NaCl.

The N-terminal sequences (Figure 3) of the major components in each fraction isolated by RP-HPLC revealed that three proteins (**II**−**IV**) were identical in this region of the N-terminal sequences, and that their sequences were identical to the deduced N-terminal sequence of previously isolated cDNA clones belonging to the RA14 subfamily (Adachi et al., 1993; Alvarez et al., 1995a). Also, protein **V** had the same N-terminal sequence with that of RA17 (Izumi et al., 1991). Although protein **I** is similar to RA16 (Alvarez et al., 1995a), no cDNA clone corresponding to this protein has yet been obtained.

α-Amylase Inhibitory Activities. Inhibitory activities of purified proteins against two α-amylases (human saliva and *B. amyloliquefaciens*) are summarized in Figure 4. All isolated proteins (**I**−**V**) showed inhibitory activity toward human salivary α-amylase, whereas they did not inhibit the bacterial α-amylase, suggesting that the 14−16-kDa proteins exhibited selectivity for the mammalian enzyme. The total albumin fraction

Peak	N-Terminal Amino Acid Sequence	Corresponding cDNA clone
I	D - H R G Q V V Y T P G Q L X A A G R G Y P M P	
II	D H H K D Q V V Y S L G	RA14 subfamily
III	D H H K D Q V V Y S L G E R X Q P G M G	RA14 subfamily
IV	D H H K D Q V V Y S L G E	RA14 subfamily
V	D H H - - Q - V Y S P G E Q X R P G I S Y P T	RA17
RA14	D H H K D Q V V Y S L G E R C Q P G M G	
RA17	D H H - - Q - V Y S P G E Q C R P G I S Y P T	

Figure 3. N-terminal amino acid sequences of isolated salt-soluble proteins. Corresponding cDNA clones are shown on the right side of the sequences. Unidentified amino acid residues are shown by X. Identical amino acid residues (at least three of five proteins) are boxed. Deduced amino acid sequences from cDNA clones, RA14 and RA17 (Adachi et al., 1993), are aligned at the lower position. Gaps (−) are introduced for maximum alignment.

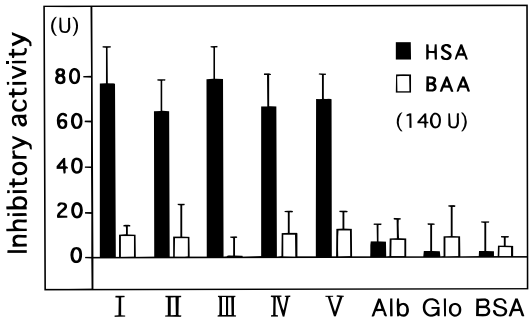

Figure 4. α-Amylase inhibitory activities of isolated salt-soluble proteins. Five micrograms of each purified protein **I**−**V**, total albumin (Alb), total globulin (Glo), and bovine serum albumin (BSA) were used for the inhibition assay against α-amylase (140 U) from human saliva (HSA) or *B. amylolique-faciens* (BAA). Inhibitory activity is shown as the α-amylase activity (U) inhibited by each protein added. Values are means ± SD of four experiments.

(Alb in Figure 4) did not show significant inhibitory activity toward these two α-amylases. These proteins showed no inhibitory activity against bovine trypsin (data not shown).

Reactivity of Purified Proteins to Patients' IgE. Allergenic reactions to rice are due in part to the production of IgE antibodies against certain antigenic components in rice grain (Limas et al., 1991; Shibasaki et al., 1979; Urisu et al., 1991). To determine the antigenic reactivity of the isolated proteins to human IgE, RAST was done with sera of patients allergic to rice. Sera samples from 17 patients were tested for reactivity against the five purified proteins (**I**−**V**) and the total salt-soluble proteins. Eleven (64%) of 17 patients tested were RAST-positive against protein **I**, and seven (41%) and five (29%) were positive to proteins **IV** and others (**II**, **III**, and **V**), respectively. Several representative patients' IgE reactions to the proteins are shown in Figure 5. The background radioactivities (cpm) obtained for each protein using two cord blood sera were in a range 24−79 cpm.

Reaction patterns were roughly classified into three groups: that is, the serum IgE antibodies reacted to (i) all of the isolated proteins (four patients including patient 17), (ii) one or some protein (s) of them (patients 1, 13, 14, 21), or (iii) none of the proteins (three patients including patient 16). The serum IgE level specific for

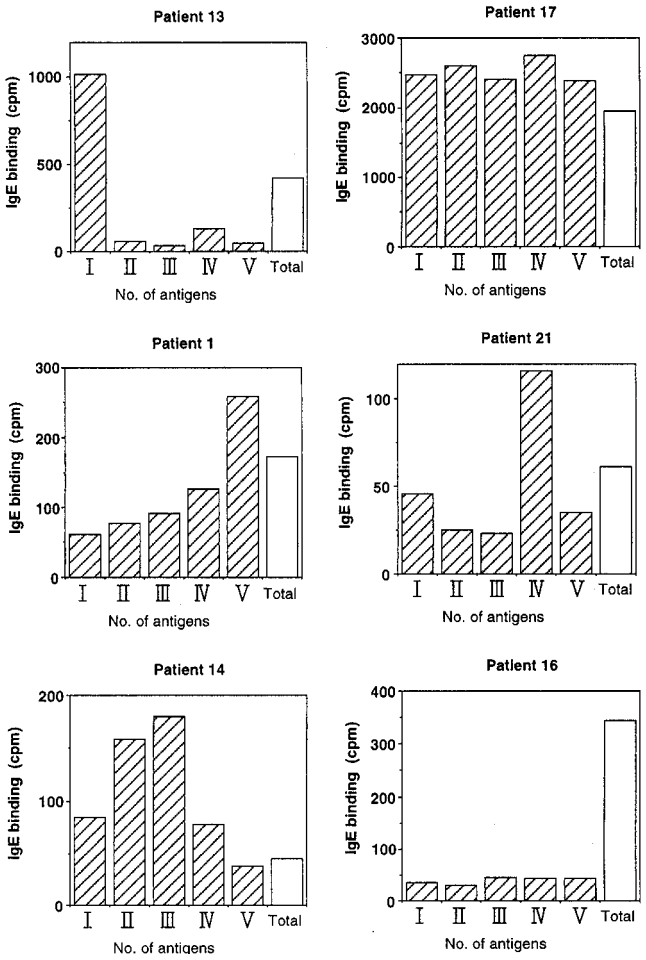

Figure 5. RAST of isolated salt-soluble proteins with the sera from patients allergic to rice. Reaction patterns of several representatives are shown. Isolated proteins (**I**−**V**) and total salt-soluble proteins were used as the antigen for RAST.

these antigens was significantly higher in group (i) patients than in other groups. In particular, there were seven patients whose serum IgE reacted specifically only with protein **I**. These allergenic proteins may have various epitopes recognized by human IgE, in which some of the epitopes are shared by the five proteins, whereas the others are recognized individually by a limited number of the patients.

DISCUSSION

Low-molecular-weight proteins from cereals such as wheat and barley have been known to be α-amylase and/or trypsin inhibitors (Garcia-Olmedo et al., 1987). These inhibitor proteins have also been known as major allergenic components associated with Baker's asthma (Barber et al., 1989; Gomez et al., 1990; Mena et al., 1992). In the present study, the rice low-molecular-weight proteins of 14−16 kDa were found to be α-amylase inhibitors as well as antigens recognized by IgE antibodies from patients allergic to rice. A relationship between the antigens eliciting specific IgE production in these allergic diseases and the proteins belonging to the α-amylase/trypsin inhibitor family has not been established, although the inhibitory activity would not be expected to directly relate to allergenic activity.

In spite of the structural and immunological similarities among the rice proteins isolated in this study, variable reactivities among the sera of several patients allergic to rice were observed (Figure 5). All of the isolated proteins were recognized by the sera used, suggesting that these proteins are potential allergens. Certain patients (group ii) reacted with only one of the purified proteins, indicating that the patients' IgE may recognize slight structural differences among the proteins. Our previous sequence data for these rice proteins showed that a sequence at the C-terminal proximal region is highly conserved among the cDNA clones isolated (Alvarez et al., 1995a). The conserved region near the C-terminus might not be antigenic for these patients. In contrast, four patients (group i) recognized all of the five proteins, suggesting that these patients have IgE against epitopes specific for each protein or against a common epitope shared by the five proteins. It is noteworthy that IgE from several sera specifically recognized only protein **I**. It will be of interest to determine the structure of epitopes of protein **I** because this protein might have the characteristic structure or sequence of an allergen.

We have previously reported that homologous cDNA clones are classified into at least four subfamilies (Alvarez et al., 1995a). The N-terminal sequences of the isolated proteins suggest that proteins **II**, **III**, and **IV** corresponded to the RA14 subfamily (Figure 3). Although three cDNA clones belonging to the RA14 subfamily have been obtained (Alvarez et al., 1995a), correspondence of each cDNA to protein **II**, **III**, or **IV** is still uncertain, because the N-terminal amino acid sequences determined were identical among the three proteins (Figure 3). Nevertheless, the similarity of their N-terminal amino acid sequences and correspondence to previously isolated cDNA clones suggest that rice α-amylase inhibitors of 14−16 kDa are products of a multigene family.

ABBREVIATIONS USED

RAST, radioallergosorbent test; IgE, immunoglobulin class E; mAb, monoclonal antibody; RP-HPLC, reversed-phase HPLC; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; BSA, bovine serum albumin; POD, peroxidase; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay.

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