Purification and Properties of an Allergenic Protein in Rice Grain

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A protein antigenic to the IgE antibody of allergic individuals was isolated from rice grain by ion-exchange and gel-filtration chromatographies. The molecular weight of the allergenic protein was estimated to be about 16,000 by sodiumdodecylsulfate-gel electrophoresis. The protein contained 7 mol of cystine residue per mole of protein and no cysteine residues, and its immunoreactivity was quite stable to heating at 100°C. This allergenic protein was mainly present in the endosperm portion of rice seeds.

Although many investigations have been reported on allergenic foods causing various hypersensitive reactions,[1～3] there have only been a few in which the pure allergenic components were isolated and characterized.[4～7] A variety of food hypersensitivity effects, especially the immediate type of reaction, are mediated by the IgE antibody.[8～10] Hence, *in vitro* methods using sera from allergic patients are effective for identifying and isolating specific allergenic components in some foods causing allergic diseases,[11] and the radio allergosorbent test (RAST) for analysing serum specific IgE is used widely for the diagnosis of food allergies.

Rowe *et al.*[12] suggested that cereal grains were a major cause of allergy. Hoffman[13] also reported that a substantial number of atopic children produced IgE antibodies reacting with cereal grains, although many of them produced IgE antibodies reacting with other common foods including egg and milk. Rice is a grain consumed in great quantity in Japan and the other Asian countries, and Shibasaki *et al.*[14] reported the allergenicity and lymphocyte-stimulating property of rice proteins. They prepared two protein fractions of rice grain, glutelin and globulin, and found that the globulin fraction was reactive with specific IgE and that the glutelin fraction showed an allergen potency lower than that of the globulin fraction on a weight basis.[14] Furthermore, rice proteins have been reported to be closely related antigenically with corn proteins as measured by the RAST inhibition test using sera from allergic individuals.[13]

Thus, some interesting results have been reported on rice allergenic proteins. However, the physical, chemical and immunological properties of rice allergenic proteins still remain unknown, because no allergenic proteins have been isolated. The aim of the present investigation is to isolate the allergenic protein in rice grain, using antigenicity to the serum IgE antibody as an index, and to characterize some physical and chemical properties of the isolated allergenic protein.

MATERIALS AND METHODS

Rice protein. Rice seeds of the Japonica variety were purchased locally and stored at 4°C until use. The bran and endosperm portions of the rice grain were used, the bran being prepared by polishing grains up to an 8% weight loss and the endosperm being prepared by polishing up to a further 10% weight loss. Both the rice grain and endosperm were ground into powder. The bran from powdered fractions of the rice grain and endosperm was defatted with cold acetone and thoroughly dried. The

proteins were extracted from the defatted powder (100 g) with 500 ml of 1.0 M NaCl solution for 16 hr at 20°C according to the method of Morita *et al.*[15] After centrifuging the extract at 9,000 × *g* for 20 min, solid ammonium sulfate was slowly added to the supernatant until the solution was saturated to 90%. After centrifuging at 9,000 × *g* for a further 20 min, the precipitate was dissolved in distilled water and dialyzed against distilled water or 20 mM phosphate buffer (pH 7.4) containing 0.15 M NaCl (PBS).

Allergenic activity measurement. The allergenic activity of rice proteins was measured by analyzing the reactivity with specific IgE antibody from allergic patients. The sera with high RAST scores against rice protein were selected from the sera of patients with known clinical histories of allergies. The reactivity of IgE antibody to rice proteins was determined by an enzyme-linked immunosorbent assay (ELISA).[16] Flat-bottomed microtiter plates were coated with an appropriate concentration (1 ~ 10 μg/ml) of the protein antigens. After incubating with human sera diluted appropriately (1 : 100 ~ 1 : 1000), the human IgE antibody which reacted with the plate-bound antigens was determined, using peroxidase-coupled anti-human IgE antibody (goat, ε-chain specific, Cappel Laboratories Inc., Cochranville, PA) with *o*-phenylenediamine as the enzyme substrate as described previously.[17]

The amount of allergenic protein was measured by a single radial immunodiffusion according to the method of Mancini *et al.*[18]

Rabbit antiserum to rice protein. A rabbit was subcutaneously immunized by injecting with 1 mg of rice protein in 1 ml of 0.15 M NaCl/0.01 M phosphate buffer, pH 7.2 (PBS), emulsified with Freund's complete adjuvant. The rabbit was then given two booster injections of the same antigen 14 and 28 days after the first immunization. Bleeding was performed 10 days after the last booster injection, the serum being separated by centrifugation and stored at −80°C.

Electrophoresis. Sodiumdodecylsulfate (SDS) polyacrylamide gel electrophoresis was performed according to the method of Laemmli,[19] the gel sheets being stained with Coomassie R-250 Brilliant Blue.

The protein transfer from the polyacrylamide gel sheets to nitrocellulose sheets was performed according to the procedure of Towbin *et al.*[20] Immediately following transfer, the nitrocellulose sheet was incubated with an antiserum solution and the reacted protein to the antiserum was visualized by ELISA as already described.

High-performance liquid chromatography (HPLC). The rice proteins extracted and dialyzed against PBS were fractionated by gel filtration chromatography using a column (7.5 mm × 60 cm) of TSK G3000SW gel (Toyo Soda, Tokyo). The proteins were eluted with PBS at a flow rate of 0.7 ml/min, and the elution profile was monitored by absorbance at 280 nm.

Amino acid analysis. The amino acid composition was determined with an amino acid analyser (JLC-6AH, Jeol, Tokyo) after hydrolysis in 6 N HCl at 100°C for 24 hr.

Protein determination. The protein concentration was determined by the method of Lowry *et al.*[21] using ovalbumin (SIGMA Chemical Co., Ltd.) as the standard.

RESULTS AND DISCUSSION

Purification of an allergenic protein

The water soluble fraction of the rice proteins (about 150 mg) was dialtzed against 20 mM Tris–HCl buffer (pH 8.6) containing 0.05% NaN_3. After centrifugating the solution at 9,000 × *g* for 20 min at 20°C, the protein in the supernatant was fractionated at 25°C by ion-exchange chromatography on a column (2 × 20 cm) of DEAE cellulose (DE 52, Whatman Biochemicals Ltd.) that had been equilibrated with the Tris buffer. After applying the sample solution, the column was washed thoroughly with the same buffer, and the proteins absorbed were eluted with a linear gradient of NaCl (0 ~ 0.1 M in 500 ml of the Tris buffer) (Fig. 1). The reactivity with IgE antibody of each fraction under the 7 major peaks was tested by ELISA, and the fractions under peaks 2 and 5 showed a positive reaction (Fig. 2), A relatively higher homogeneity of the proteins in fraction 5 was found by an SDS-gel electrophoretic analysis of each fraction, so that the fractions under peak 5 (the hached area in Fig. 1) were pooled, dialyzed against 20 mM Tris–HCl buffer (pH 8.6) containing 0.05% NaN_3, and then further purified by rechromatography on the same column. The fractions with reactivity to IgE antibody were pooled, dialyzed against distilled water and freeze-dried. The dried protein was dissolved in 0.1 M Tris–HCl buffer (pH 8.0) containing 0.05% NaN_3, and fractionated at 25°C by gel-filtration chromatography on a column (2 × 90 cm) that had been equilibrated with the same buffer (Fig. 3). The fractions (Nos. 65 ~ 77, the hached area in Fig. 3) with reactivity to IgE were pool-

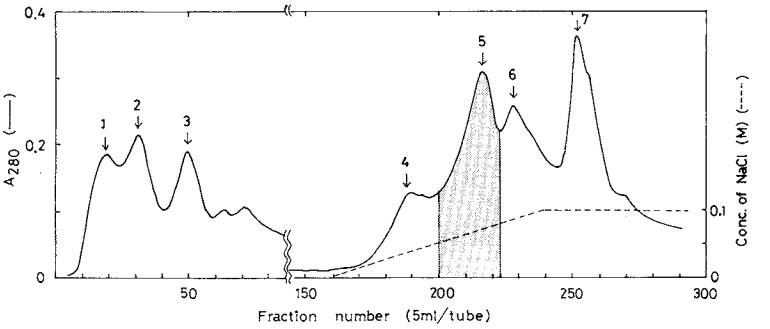

FIG. 1. Ion-exchange Chromatography of Rice Soluble Proteins on a Column of DEAE Cellulose (DE 52). The arrows (1 ~ 7) indicate the peaks whose reactivity to IgE was examined.

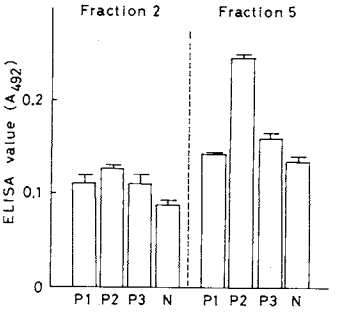

FIG. 2. ELISA Value of the Two Fractions (Fractions 2 and 5 of Fig. 1) from the Column of DEAE Cellulose.

P1, P2 and P3 show the sera obtained from three allergic individuals (T.K., S.M. and S.I.), and N shows that from a non-allergic individual (H.S.). The incubation time for the ELISA enzyme reaction was 60 min, and each point represents the mean of two determinations.

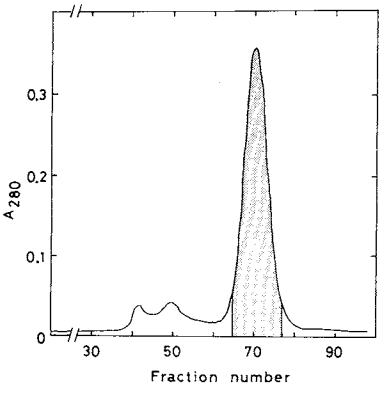

FIG. 3. Purification of Rice Allergenic Protein by Gel Filtration on a Column of Sephadex G-50.

ed, dialyzed against distilled water and freeze-dried.

Figure 4 shows the electrophoretic patterns

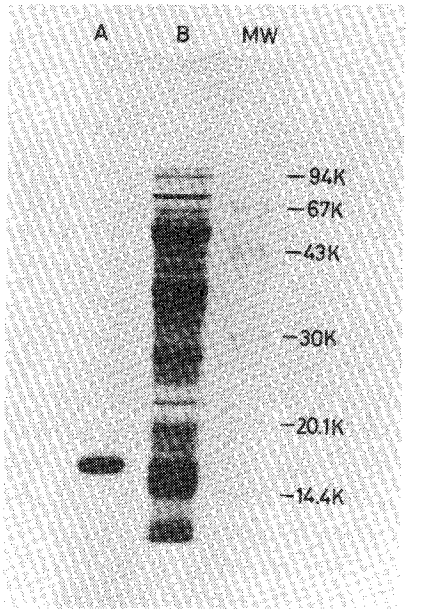

FIG. 4. SDS-gel Electrophoresis of the Purified-rice Allergenic Protein.

Lanes A and B are the purified protein and rice-PBS soluble proteins, respectively. The standard proteins (MW) also were applied for molecular weight estimation.

of the allergenic protein preparation, one protein band being observed in the lane for protein preparation, indicating that the protein prepared was homogeneous enough to be used for the following experiments. The molecular weight of the protein was estimated to be about 16,000 based on the electrophoretic mobilities of standard proteins (Pharmacia Fine Chemicals) on the same gel, suggesting that this protein corresponded to a

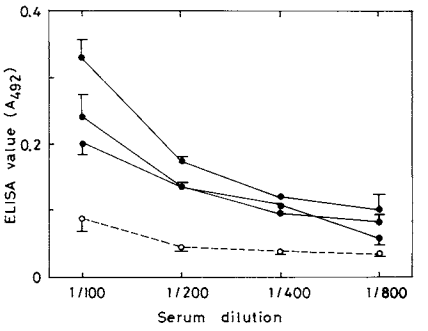

FIG. 5. Reactivity of the Purified Allergenic Protein to IgE Antibodies from Three Allergic Individuals (●—● S.M., T.K. and S.I.).

The control serum from a non-allergic individual (○---○ H.S.) also was analyzed for comparison. The incubation time for the ELISA enzyme was 60 min.

protein in α-glubulin or to the Gl-3 fraction reported by Morita *et al.*[15] and by Shibasaki *et al.*,[14] respectively. The relatively low molecular weight of the allergenic protein seems to be a common factor among some allergenic proteins reported earlier.[5]

The reactivity to IgE of the isolated protein was quantitatively analyzed using sera from three individuals allergic to rice (Fig. 5). The protein reacted well with IgE antibodies in all of the three sera, indicating that the protein was a common allergen to the three individuals. The IgE antibody titer to the protein of the patients' sera was calculated to be about 8 times or more the titer of serum from a non-allergic individual.

Distribution of the allergenic protein in rice grain

Figure 6 shows the electrophorogram of the total extractable proteins from bran and endosperm. The constituent proteins from each part of the rice grain were greatly different, with much larger amounts of low molecular-weight protein being present in the endosperm. Western blotting of the extractable proteins clearly shows that a larger amount of allergenic protein was present in the endosperm (Fig. 7). To confirm this, the allergenic protein content of both fractions of the rice grain was measured by a single radial immuno-diffusion

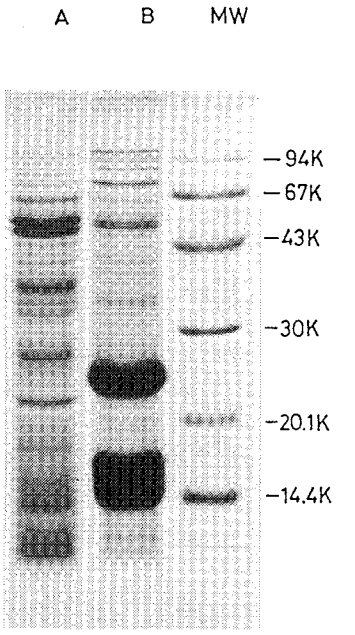

FIG. 6. SDS-gel Electrophoresis of the Total Extractable Protein from Endosperm and Bran.

A, bran; B, endosperm.

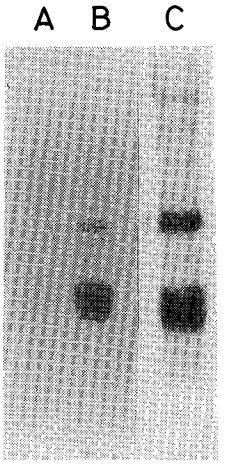

FIG. 7. Western Blotting of the Total Extractable Protein from Endosperm and Bran.

A, bran; B, endosperm; C, SDS-gel electrophoretic pattern of the total extractable protein from endosperm.

(Table I). Although the amount of total extractable proteins from the bran was much larger than that from the endosperm, that of the allergenic protein from the endosperm was much larger than that from the bran.

TABLE I. DISTRIBUTION OF THE ALLERGENIC PROTEIN BETWEEN ENDOSPERM AND BRAN OF RICE GRAIN

	Total extractable protein (mg/g of dry matter)	Allergenic protein (mg/g of dry matter)
Endosperm	7.83	0.39
Bran	31.5	0.07

A large part of the protein in endosperm has been shown to be present as protein bodies.[22,23] Since allergenic protein can be easily extracted with a low concentration of NaCl such as 0.1 M or less (data not shown), this protein might not be included in the protein bodies. Although further studies are needed on how this allergenic protein is distributed in endosperm, it is certain that removal of the allergenic protein by polishing rice grains will be rather difficult.

Properties of the allergenic protein

The amino acid composition of the allergenic protein is shown in Table II, the protein containing a relatively high proportion of proline and cystine residues as compared with rice glutelin[24] or rice γ-globulin.[25] The cystine residues would probably form intramolecular disulfide bonds because no sulfhydril groups of the protein were detected even in the presence of 0.5% SDS, and because no dimeric or oligomeric forms of the protein were observed by gel filtration (Fig. 3) and by SDS-gel electrophoresis under the condition without a reducing reagent (data not shown).

The heat stability of the protein allergenic activity was examined (Fig. 8), and compared with the earlier results that boiling rice for 1 hr did not remove but did decrease its reactivity with IgE antibodies,[13] and that the RAST activity of the globulin fractions was reduced to 60~70% of the native ones by heating at 100°C for 10 min.[14] In the present study, about 60% of the reactivity of the isolated protein remained even after heating at 100°C for 60 min, which seems to be in good agreement with the earlier results obtained using crude allergenic proteins.[14]

TABLE II. AMINO ACID COMPOSITION OF THE RICE ALLERGENIC PROTEIN

Amino acid	mol/mol
Asp	11
Thr	5
Ser	6
Glu	13
Pro	15
Gly	17
Ala	17
1/2 Cys-Cys	14
Val	9
Met	3
Ileu	4
Leu	9
Tyr	6
Phe	3
His	5
Lys	2
Arg	14

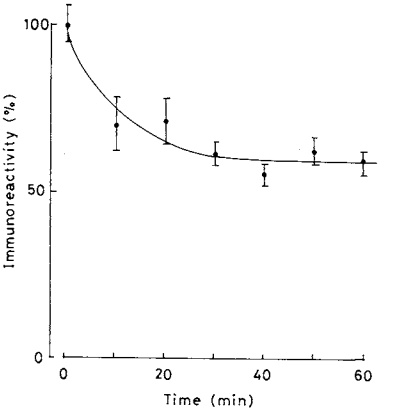

FIG. 8. Effect of Heating (100°C) on the Reactivity to IgE Antibody from an Allergic Patient (S.M.) of the Purified Allergenic Protein as Measured by ELISA.

To determine whether the isolated protein was the major allergenic protein in rice soluble proteins, the PBS-soluble fraction of the rice protein was subjected to gel-filtration HPLC, and the reactivity with IgE antibody was examined for each separated protein. Figure 9 shows the HPLC elution profiles of the PBS-soluble proteins and of the isolated allergenic protein. A relatively high reactivity to IgE antibody was detected for the smaller-molecular size fractions (peaks D~G), whose

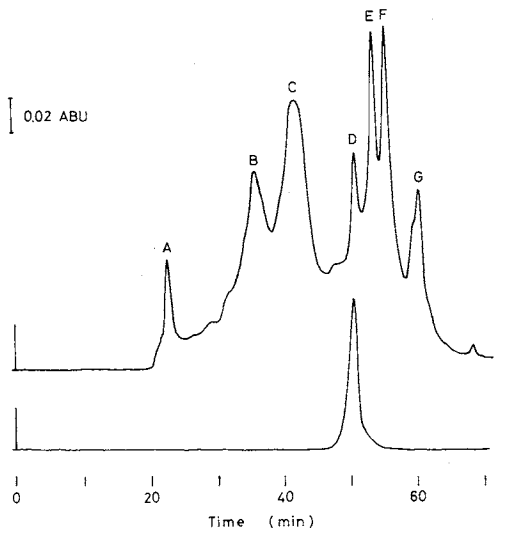

FIG. 9. Gel Filtration Chromatography of Rice-PBS Soluble Proteins (A) and the Purified Allergenic Protein (B) on a Column TSK-gel G3000 SW.

molecular weights estimated by SDS-electrophoresis were almost the same (about 15,000 ~ 16,000). The allergenic protein isolated in the present study was expected to correspond to the protein in the fraction from HPLC elution under the same conditions, indicating that the isolated protein was one of the major allergenic proteins in rice grain. Reactivity with IgE antibody was also detected in another fraction from the ion-exchange chromatography (peak 2 in Fig. 1), and this fraction was found to contain proteins with a molecular weight of about 16,000 by SDS-electrophoretic analysis (data not shown). The isolation and characterization of another low molecular weight allergen is now in progress.

Hoffman reported that the demonstration of specific IgE antibodies reacting with an allergen did not necessarily indicate a clinical allergy to that allergen.[13] On the other hand, Bjoksten *et al.*[8] suggested that the specific IgE antibodies were not truly "false" positive reactions but a sequence from a previous allergy. Further clinical investigations using isolated proteins reacting with IgE antibodies would be necessary to identify the allergenic components actually causing various allergic symptoms at a clinical level.

REFERENCES

1) F. Speer, "Food Allergy," PSG Publ. Comp., Massachusetts, U.S.A., 1978, p. 109.
2) D. D. Metcalfe, *J. Allergy Clin. Immunol.*, **73**, 749 (1984).
3) S. L. Taylor, *Food Technol.*, **39**, 98 (1985).
4) D. R. Hoffman, *J. Allergy Clin. Immunol.*, **71**, 481 (1983).
5) T. T. King, *Adv. Immunol.*, **23**, 77 (1976).
6) M. Shibasaki, S. Suzuki, S. Tajima, H. Nemoto and K. Takayoshi, *Int. Archs. Allergy Appl. Immun.*, **61**, 441 (1980).
7) E. Elsayed and J. Apold, *Allergy*, **38**, 449 (1983).
8) B. Bjorksten, S. Ahlstedt, F. Bjorksten, B. Carlsson, P. S. Fallstorm, K. Juntuneu, M. Kajossari and A. Kober, *Allergy*, **38**, 119 (1983).
9) Y. Y. Chua, K. Bremner, N. Lakdamalla, J. L. Lobet, H. L. Kokubu, R. Orange and C. Collins-Williams, *J. Allergy Clin. Immunol.*, **58**, 299 (1976).
10) D. R. Hoffman and Z. H. Haddad, *J. Allergy Clin. Immunol.*, **54**, 165 (1974).
11) J. Anet, J. F. Back, R. S. Baker, D. Barnett, R. W. Burley and M. E. H. Howden, *Int. Archs. Allergy Appl. Immun.*, **77**, 364 (1985).
12) A. H. Rowe, A. Rowe, Jr. and E. J. Yound, *J. Am. Med. Assoc.*, **169**, 1158 (1959).
13) D. R. Hoffman, *Immunochemistry*, **12**, 535 (1975).
14) M. Shibasaki, S. Suzuki, H. Nemoto and T. Kuroume, *J. Allergy Clin. Immunol.*, **64**, 259 (1979).
15) Y. Morita and C. Yoshida, *Agric. Biol. Chem.*, **32**, 664 (1968).
16) E. Engvall and P. Perlmann, *Immunochemistry*, **8**, 871 (1971).
17) T. Matsuda, K. Watanabe and R. Nakamura, *Biochem. Biophys. Res. Commun.*, **110**, 75 (1983).
18) G. Mancini, J. Vaerman, A. O. Carbonara and J. F. Heremans, *Immunochemistry*, **2**, 235 (1965).
19) U. K. Laemmli, *Nature*, **227**, 680 (1970).
20) H. Towbin, T. Staehelin and J. Gordon, *Proc. Nat. Acad. Sci. U.S.A.*, **76**, 4350 (1979).
21) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
22) K. Tanaka, T. Sugimoto, M. Ogawa and Z. Kasai, *Agric. Biol. Chem.*, **44**, 1633 (1980).
23) M. Horikoshi and Y. Morita, *Agric. Biol. Chem.*, **46**, 269 (1982).
24) H. Sawai, H. Nikaido and Y. Morita, *Agric. Biol. Chem.*, **34**, 1039 (1970).
25) H. Sawai and Y. Morita, *Agric. Biol. Chem.*, **34**, 61 (1970).