

Structural Characterization of the 16-kDa Allergen, RA17, in Rice Seeds. Prediction of the Secondary Structure and Identification of Intramolecular Disulfide Bridges

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The 16-kDa rice allergen, RA17, belonging to the α -amylase/trypsin inhibitor family was isolated from rice seed and structurally characterized by identifying cystine-containing peptides and predicting the secondary structure and hydrophobic regions. Eight peptides, which constitute three sets of cystine-containing peptides, were purified by HPLC from a thermolytic digest of RA17 and identified by their amino acid sequence and composition, indicating five intramolecular disulfide bridges: Cys³⁴-Cys⁹⁴, Cys²⁶-(Cys⁵⁰ or Cys⁵¹)-Cys¹¹⁰ and Cys¹²-(Cys⁶² or Cys⁶⁴)-Cys¹²². Analyses of the CD spectrum and the Chou-Fasman prediction suggested that RA17 had some helical- and sheet-structure regions. Based on these experimental and predicted data, RA17 is proposed to be a globular molecule with a small hydrophobic core having folding restricted by five intramolecular disulfide bridges.

Key words: rice; allergen; food allergy; α -amylase/trypsin inhibitor; disulfide bridge

Cereal grains have been reported to sometimes be a causative of the food allergy.¹⁾ Several clinical studies in Japan have suggested that rice grains were responsible for severe atopic dermatitis in some adult patients, although the frequency of such rice allergy was lower than that of egg, milk and soybean allergies.^{2–4)} *In vitro* studies on the IgE-binding reaction to rice seed proteins have suggested that the NaCl-soluble proteins, globulin and albumin, were the major allergenic components recognized by IgE of sera from allergic patients.^{5,6)}

Several immunologically cross-reactive proteins with heterogeneity in their molecular mass (14–16-kDa) and isoelectric point (pI6–8) have been isolated and identified as allergenic components.⁷⁾ Furthermore, cDNAs encoding these allergenic proteins have been cloned and their nucleotide sequences determined.^{8–11)} The deduced amino acid sequences show that these 14–16-kDa allergens were structurally homologous and, in particular, that ten cysteine residues were conserved among these allergens. These sequences also show similarity to those of the α -amylase/trypsin inhibitors of other cereal species,

especially in the number and distribution of cysteine residues.¹²⁾ In fact, the isolated 14–16-kDa rice allergens showed inhibitory activity toward human salivary α -amylase.¹³⁾ The wheat and barley α -amylase/trypsin inhibitors have been identified as major allergens associated with baker's asthma disease.^{14,15)}

The allergenicity of these 14–16-kDa proteins in rice seeds has been ascribed to their stability against heat and proteolytic digestion.¹⁶⁾ Although such stability of the rice allergens is suggested to be due to their structural properties, the protein structure of these allergens has not yet been well-characterized, except for the primary structure.

In this paper, we describe the intramolecular disulfide bridges, the secondary structure and polypeptide folding of the 16-kDa allergen, RA17. All of the ten cysteine residues were found to form five intramolecular disulfide bridges, leading to stabilization of the protein conformation. Possible folding of the rice allergen is proposed on the basis of the disulfide bonding, and the secondary structure is predicted by statistical and CD analyses.

Materials and Methods

Isolation of a major rice allergenic protein. The 16-kDa allergen, RA17, was isolated from grains of rice (*Oryza sativa* L var. *japonica* cv. *Nipponbare*) by ammonium sulfate precipitation and anion-exchange chromatography as described previously.^{13,16)} The last peak of the DE52 chromatogram containing RA17 was collected, dialyzed against distilled water and lyophilized.

Enzymatic digestion and peptide separation. One milligram of RA17 was digested with 0.02 mg of thermolysin (Seikagaku Corporation, Japan) in 0.2 ml of a 50 mM Tris-HCl buffer at pH8.0 containing 5 mM CaCl₂ at 37°C for 15 h. The resulting thermolytic digest was applied to an ODS-HPLC column (Biofine RPC-PO, 4.6 × 150 mm; Jasco, Tokyo, Japan) that has been equilibrated with 0.1% TFA, and eluted with a linear

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Abbreviations: SBD-F, 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonic acid ammonium salt; HPLC, high-performance liquid chromatography; CD, circular dichroism; IgE, immunoglobulin class E; DTT, dithiothreitol

gradient of acetonitrile from 0 to 60% by using a two-pump gradient HPLC system (model 880-PU, Jasco, Tokyo, Japan). The UV absorbance of the eluate was monitored at 212 nm.

Detection of cystine-containing peptides. Cystine-containing peptides in each fraction of the HPLC eluent were detected according to the method of Sueyoshi *et al.*¹⁷⁾ with 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonic acid ammonium salt (SBD-F, Dojindo Lab., Kumamoto, Japan). The fluorescence intensity of the reaction mixture was measured at 25°C with excitation at 385 nm and emission at 515 nm by an FP-550A fluorescence spectrophotometer (Jasco, Tokyo, Japan).

Reduction of the inter-peptide disulfide bridges. The cystine-containing peptides isolated by reversed-phase HPLC were dried and reduced by adding 50 μ l of 2 mM DTT and then incubating at room temperature for 5 min. The reduced peptides were applied again to the ODS column which was eluted with the acetonitrile gradient as already described.

Amino acid analyses. The isolated peptides were hydrolyzed with 6N HCl at 110°C for 24 h and analyzed by an automated amino acid analyzer (JLC300, Joel, Tokyo, Japan). The N-terminal amino acid sequence of each peptide was determined by a protein sequencer (type 610A, Applied Biosystems).

CD analysis. The isolated RA17 was dissolved in a 20 mM phosphate buffer at pH 7.4 containing 0.15 M NaCl, and the far-ultraviolet CD spectrum of the RA17 solution (0.51 mg/ml) was measured in a 1-mm-path length cuvette at 25°C with a J-500C spectropolarimeter (Jasco, Tokyo, Japan). The results are given in terms of $[\theta]$, the mean residue ellipticity, in units of $\text{deg cm}^2/\text{decimol}$. The mean residue weight of RA17 was taken as 109 which was calculated from the deduced amino acid sequence.⁸⁾ The secondary structure composition was estimated according to the equation of Chang *et al.*¹⁸⁾ as described previously.¹⁹⁾

Hydropathy analysis and secondary structure prediction. Consecutive hydropathy averages were calculated for a five-residue window according to the method of Kyte and Doolittle.²⁰⁾ The regions with an ordered secondary structure, α -helix, β -sheet and β -turn, were predicted according to the method of Chou and Fasman.²¹⁾

Results

To determine whether RA17 contained free cysteine residues, sulfhydryl groups were analyzed by the DTNB method.²²⁾ No sulfhydryl group was detected, even at a high protein concentration and after heating in the presence of EDTA (data not shown). These results prompted us to assume that the cysteine residues formed disulfide bridges, and to identify the cystine-containing peptides of RA17.

Isolation of the cystine-containing peptides

Purified RA17 was hydrolyzed with trypsin, chymotrypsin and thermolysin, and the resultant digests were preliminarily analyzed by SDS-PAGE. RA17 was found to be resistant to trypsin and chymotrypsin, whereas it was well-hydrolyzed with thermolysin (data not shown). Therefore, RA17 was digested with thermolysin and applied to HPLC to isolate the cystine-containing peptides. A typical elution profile of the thermolysin digest from the ODS-HPLC column is shown in Fig. 1. Each peak was collected, and its cystine content was estimated by the SBD-F fluorescence method. As shown in Fig. 1, four peaks were markedly positive to the fluorescence assay for cystine residues. These fractions, termed A, B, C and D, which were assumed to have cystine-containing peptides, were dried, reduced with DTT and applied again to the same column. Each fraction was separated into three or four peaks after the DTT treatment. The typical HPLC elution profiles of the three fractions are shown in Fig. 2. These peaks were separately collected and used for the subsequent amino acid analyses. The HPLC profiles of the RA17 peptides were reproducible, indicating that no sulfhydryl/disulfide-exchange reaction occurred during the sample preparation.

Peptide identification by amino acid analyses

The N-terminal amino acid sequence and the amino acid composition of each isolated peptide were determined. The amino acid compositions obtained are summarized in Table 1. These peptides all contained one or two cysteine residues. Based on these sequence and com-

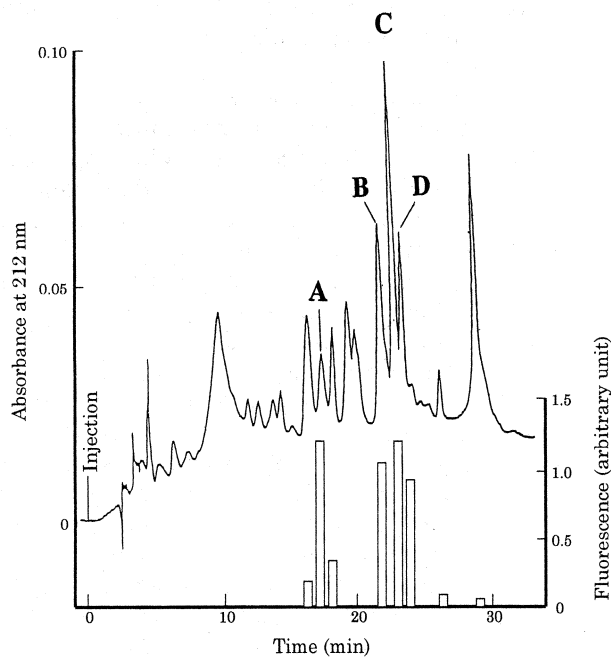


Fig. 1. Separation of the Thermolytic Peptides of RA17 by Reversed-phase HPLC.

The conditions for thermolytic digestion and HPLC are described in the Materials and Methods section. The fluorescence intensity of each fraction detected by the SBD-F method is indicated by solid bars below the chromatogram.

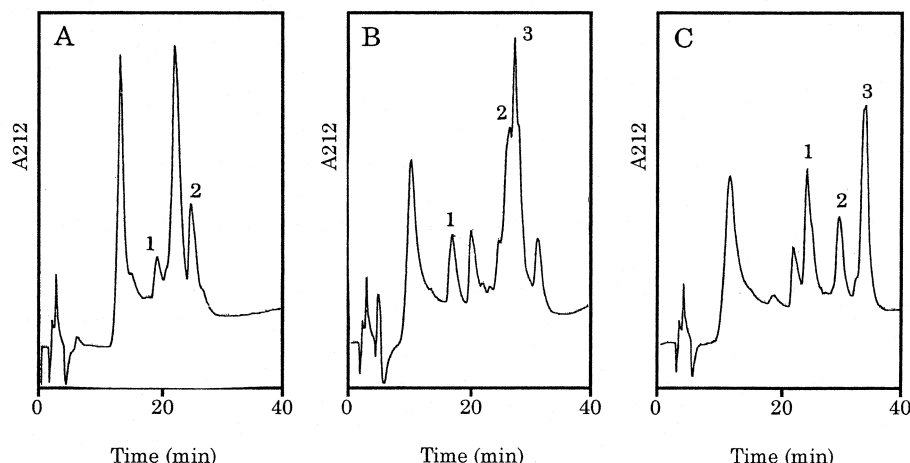


Fig. 2. HPLC Separation of Peptides Constituting the Cystine-containing Peptides after Reduction with DTT.

The cystine-containing peptides of peak fractions A, B and C (Fig. 1) were reduced with DTT, and applied to the same HPLC column. Each peak fraction was collected, dried and applied to amino acid- and peptide-sequence analyses.

Table 1. Amino Acid Composition of the Cysteine-containing Peptides Isolated from the Thermolytic Digest of RA17

Amino acid	A-1	A-2	B-1	B-2	B-3	C-1	C-2	C-3
Asx		0.85	1.10		1.06			1.95
Thr				0.90				
Ser				0.86		0.87		
Glx	1.02			1.18	2.13	2.12		
Gly		1.63				2.04		2.24
Ala								1.03
Cys	0.83	0.89	0.88	0.75	1.80	0.87	0.90	1.70
Val	1*	1*			1*	1*	1*	1*
Met								
Ile								
Leu				1*				
Tyr						1.01	0.96	
Phe		1.02	1*					
His								
Lys								
Arg	1.90	2.10		1.11	1.12	1.08		1.24
Pro		1.20		1.11		2.27		
Total	4.75	8.69	2.98	6.91	7.11	11.26	2.86	9.16

* The Amino acid composition of each peptide was estimated in a molar ratio relative to Val, Leu or Phe equal to 1.0.

position data, the eight isolated peptides were identified in the deduced amino acid sequence of RA17⁸⁾ as follows: peptides A-1 and A-2 were Val³⁰-Cys³⁴ and Val⁹⁰-Asp⁹⁸, respectively; peptides B-1, B-2 and B-3 were Phe¹⁰⁹-Asn¹¹¹, Ser²²-Thr²⁸ and Val⁴⁶-Gln⁵³, respectively; and peptides C-1, C-2 and C-3 were Val⁵-Gly¹⁵, Val¹²¹-Trp¹²⁴ and Val⁵⁷-Ala⁶⁶, respectively. The peptides of peak D were identical to those of peak B, except that some amino acid residues were added to the N- or C-terminus of the peak B peptides (data not shown). Each peptide contained one or two cysteine residues, and all the ten cysteine residues of RA17 were contained in either of the identified peptides. Thereby, three sets of cystine-containing peptides of the RA17 thermolytic digest were determined as shown in Fig. 3. Since two cys-

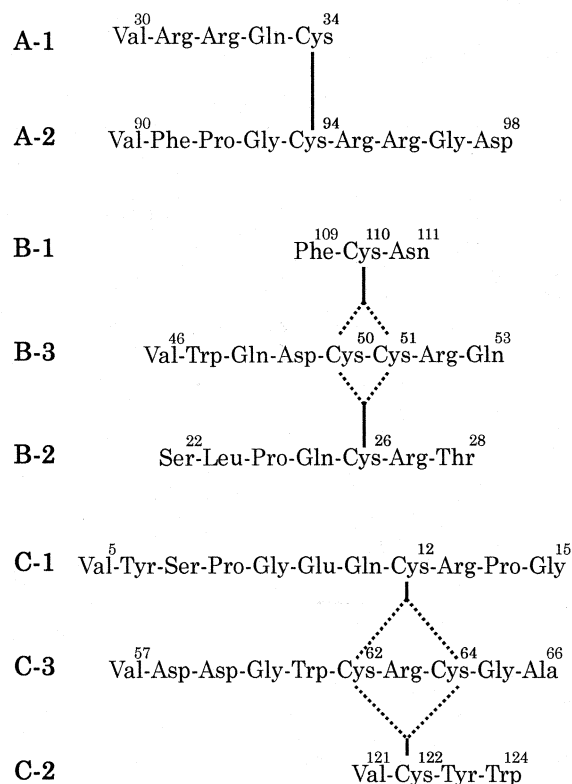


Fig. 3. The Cystine-containing Peptides Identified in the Thermolytic Peptides of RA17.

The single-chain peptides purified by HPLC (Fig. 2) were identified by comparing the sequence and composition data with the deduced amino acid sequence of RA17.⁸⁾ The solid and broken lines indicate intrapeptide-disulfide bridges, and broken ones are those which could not be determined in the present study.

teine residues were contained in each of the B-3 and C-3 peptides, interpeptide disulfide bridges could not completely be assigned for the B and C peptides. Nevertheless, the intramolecular disulfide cross-linking of RA17 predicted from these cystine-containing peptides is simi-

lar, and probably identical, to that reported for the wheat α -amylase inhibitor,²³⁾ suggesting that the disulfide cross-links in addition to the ten cysteine residues are well-conserved within the cereal α -amylase/trypsin inhibitor family.

Secondary structure prediction

The secondary structure of RA17 was predicted from the deduced amino acid sequence by the Chou-Fasman method (Fig. 4). About 30% and 20% of the total amino acid residues of mature RA17 could be expected to be in the α -helix and β -sheet regions, respectively. Seven β -turn structures were predicted, most of them being located at positions adjacent to the α -helix or β -sheet regions.

To estimate the secondary structure content of RA17, a CD analysis was done on the isolated RA17. As shown

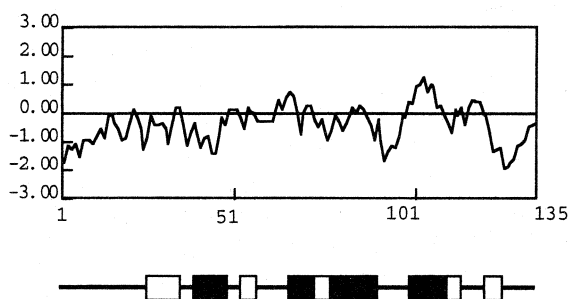


Fig. 4. Hydropathy Profile and Secondary Structure Prediction for RA17.

Hydropathy (top) was calculated along the deduced amino acid sequence of RA17. The location of the α -helix (■), β -sheet (□), and β -turn (—) regions (bottom) was predicted by the method of Chou and Fasman.

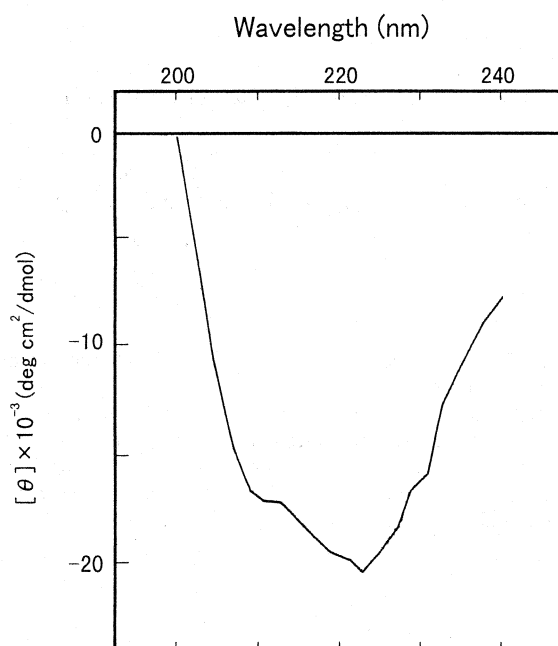


Fig. 5. CD Spectrum of RA17.

RA17 was dissolved in PBS, and the far-ultraviolet CD spectrum was measured.

in Fig. 5, the CD spectrum of RA17 was characterized by the negative extremum at 222 nm, with ellipticity of -19000 and one shoulder at about 210 nm. The α -helix content of RA17 estimated on the basis of the values at 208 and 222 nm¹⁸⁾ was in the range of 37–47%. This result and the prediction by the Chou-Fasman method already described suggested that RA17 contained more helical regions than sheet regions.

Hydropathy analysis

As shown in Fig. 4, the hydropathy plot for RA17 suggests that the greater part of the RA17 polypeptide was hydrophilic, except for one hydrophobic region, Leu⁹⁹-Phe¹⁰⁹, with a hydropathy score above 1.0. This hydrophilic nature would account for the high solubility of RA17 in an aqueous solution.^{7,16)}

Discussion

According to the results obtained in the present study and to the previously reported disulfide cross-linking of the wheat α -amylase inhibitor,²²⁾ the polypeptide folding of RA17 was predicted and is schematically drawn in Fig. 6. RA17 appeared to be a globular and compactly folded molecule with five intramolecular disulfide bridges and several ordered structure regions. The hydrophobic region, Leu⁹⁹-Phe¹⁰⁹, which was predicted to be helical, could be expected to form the hydrophobic core of the RA17 molecule. The N- and C-terminal hydrophilic regions might cover this hydrophobic core region, and contribute to the antigenic and/or allergenic epitopes of RA17. Such a prediction would agree with the result that rabbit and mouse antibodies against RA17 recognized the N-terminal region of recombinant RA17 molecules.²⁴⁾

RA17, as well as the other 14–16-kDa allergens of rice seeds, are the members of the plant α -amylase/trypsin inhibitor family.^{7–9)} Ten cysteine residues are well-con-

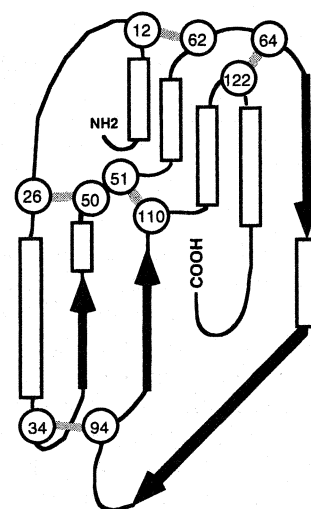


Fig. 6. Schematic Representation of the Peptide Folding and the Disulfide Bridges of RA17.

The open circles represent cysteine residues with residue numbers, and gray bars indicate disulfide bridges. The open squares and black arrows indicate the predicted sheet and helical structure regions, respectively.

served within this family.⁷⁾ The similarity of the intramolecular disulfide bridges and the sequence homology suggests that the proteins in this inhibitor family have a common folding profile, and conformational and physicochemical properties. RA17 has been identified as an allergen in severe atopic dermatitis caused by rice injection,^{2,6)} and α -amylase inhibitors of wheat and barley seeds have also been identified as major allergens associated with baker's asthma caused by the inhalation of cereal flour.^{14,15)} Allergenic proteins in food have been suggested to be stable against heat and proteases.¹⁶⁾ The five intramolecular disulfide bridges of RA17 would stabilize the folding of the ordered structures, resulting in heat stability and reversibility in heat-induced denaturation. Such a stabilized structure of RA17 might also contribute to resistance to proteolytic degradation in the alimentary tract. This stability against heat denaturation and proteolytic degradation would increase the allergenic potential of RA17.

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