Identification of Disulfide Proteins in the Salt Soluble Fraction of Rice (*Oryza sativa*) Seed

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ABSTRACT

Evidence has been accumulating to suggest that disulfide bonding is one of the key causes of allergenicity. Recently we developed the "disulfide proteome", a technique for the comprehensive analysis of disulfide bonding of proteins. We applied this new technique to the rice seed's salt-soluble fraction, which has long been known to be allergenic. Most proteins in the fraction, including α-amylase/trypsin inhibitor, α-globulin, and glutelin fragments, have formed intramolecular disulfide bonds. Also, unknown proteins, including one sharing similarities with known allergens, had disulfide bonds, from which we can infer possible allergenicity. This is a preliminary study to screen allergens from the basis of disulfide bonding.

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Some cereal proteins cause allergic reactions such as baker's asthma and severe atopic dermatitis in certain populations. As avoidance is thus far the only practical way to escape the effects of these allergens, there is an urgent need to study ways to mitigate the allergenicity.

Identifying the common characteristics of allergens is a necessary step toward lessening their allergenicity. General characteristics of allergens such as heat stability (Duranti et al 2000) and resistance to enzymatic digestion (del Val et al 1999) are attributed to intramolecular disulfide bonds. The allergenicity of wheat gliadins and glutenins was mitigated by the enzymatic reduction of the disulfide bonds (Buchanan et al 1997). The allergenic potentials of ovomucoid were decreased by exchanging disulfide bonding from intramolecular to intermolecular (Kato et al 2001). All these observations suggest that intramolecular disulfide bonding contributes positively to allergenicity. However, there has been little comprehensive analysis of cereal proteins from the standpoint of disulfide bonding (Izumi et al 1999).

We recently developed the "disulfide proteome" technique (Yano et al 2001a, 2002) to visualize the redox status of proteins comprehensively. We applied this technique in vitro (Yano et al 2001a) to find new targets for thioredoxin (Schürmann et al 2000), a ubiquitous enzyme that governs redox regulations in both plants and animals, and in vivo (Yano et al 2001b) to characterize the mobilization of rice glutelin. In this study, we applied this technique to the salt-soluble fraction of rice seed to observe the relationship between disulfide bonding and allergenicity. Rice is one of the most relevant cereals to use in studying allergenicity as it is a staple diet for 60% of the world's population. The salt-soluble fraction of rice is known to be especially allergenic (Shibasaki et al 1979).

First, we investigated the extent of disulfide bonding in the allergenic fraction by nonreducing/reducing 2D-PAGE (Allore and Barber 1984; Yano et al 2001a). Next, disulfide proteins were identified. Free sulfhydryl groups of proteins in the extract were modified in advance with nonfluorescent iodoacetamide (IAA), followed by the complete reduction of disulfide bonds with dithiothreitol (DTT), fluorescent labeling of newly exposed sulfhydryl groups by monobromobimane (mBBr), and separation of proteins by isoelectric focusing (IEF)/SDS 2D-PAGE. Finally, disulfide-forming proteins, labeled fluorescently on the 2D-gel, were identified by internal amino acid sequence analysis. This strategy, as a preliminary study to screen allergens, has broad application not only to cereals but also to pollen, house dust mites, and other allergens.

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MATERIALS AND METHODS

To isolate the salt-soluble fraction from rice seed, dehulled rice seeds (0.5 g) were ground with a mortar and pestle in 10 mL of 1*M* NaCl that contained 1 m*M* PMSF and 5 m*M* EDTA. The homogenate was stirred constantly for 1 hr and then was centrifuged at 14,000 × *g* for 5 min. The supernatant was dialyzed against water overnight on ice. The contents in the dialysis tube, including precipitations, were freeze-dried until use (salt-soluble fraction).

A portion of the salt-soluble fraction of rice was dissolved in the SDS sample buffer free of reducing agents, followed by the first 10–20% acrylamide gradient SDS-PAGE (Laemmli 1970). The gel strip was cut out, immersed in Laemmli sample buffer containing 100 m*M* DTT for 20 min, and subjected to the second 10–20% acrylamide gradient SDS-PAGE.

For specific fluorescent labeling of disulfide proteins, a portion of the salt-soluble fraction was dissolved in DTT-free lysis buffer containing 5 m*M* IAA and was incubated for 1 hr at room temperature. Dithiothreitol was added to a final concentration of 5 m*M* and the mixture was stored at room temperature for 1 hr. Then mBBr was added to a final concentration of 10 m*M* and the solution was stored for 15 min.

An immobilized pH gradient gel (IPG) strip (pH 3–10) was swollen by a protein sample, and the IEF was performed using the Protean IEF cell (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Then, the IPG strip was immersed in the Laemmli sample buffer (DTT+) followed by 10–20% acrylamide gradient SDS-PAGE. The resultant gel was stored in 30% methanol and 5% acetic acid solution, and was examined under a 365 nm UV light (FAS-2525, Toyobo, Tokyo, Japan) to detect mBBr-labeled proteins. Next, the gel was incubated overnight at room temperature in 30% methanol and 5% acetic acid containing 0.05% Coomassie Blue R-250 (CBB) and was destained by 30% methanol and 5% acetic acid until the protein spots were visible.

To identify the amino acid sequence of disulfide proteins, each spot of fluorescent protein was cut out from the 2D gel. In-gel digestion by trypsin was performed as described by Rosenfeld et al (1992). The amino acid sequence was determined by a protein sequencing system (Procise 494 HT, Applied Biosystems, Foster City, CA).

RESULTS

Intramolecular or Intermolecular Disulfide Bonds

Figure 1 shows the nonreducing/reducing 2D PAGE (Allore and Barber 1984) profile of the salt-soluble fraction of rice endosperm. On the nonreducing/reducing 2D gel, proteins that contain neither intra- nor intermolecular disulfide bonds appear on the diagonal line, whereas proteins with intra- or intermolecular disulfide

TABLE I
Internal Amino Acid Sequence Analysis of Disulfide Proteins of Rice Seed

No.	Internal Sequence	Homologous Protein	Matches
1	C^{mBBr}DALSVLVR	Trypsin/amylase inhibitor	7/9
	QLLEP$C^{mBBr}C^{mBBr}$R	nd	
	C^{mBBr}NLQHTGFFGC^{mBBr}PMFGGGM	nd	
2	LSEALGVSSQVA	Glutelin acidic chain	12/12
3	LQAFEPIR	Glutelin acidic chain	8/8
	DFLLAGNK	Glutelin acidic chain	8/8
4	SQAGTTEFFDVS	Glutelin acidic chain	12/12
5	AGYGGYGDVGEY	Unidentified QR310003 protein	12/12
6	VEPQQC^{mBBr}SIFAAG	α-Globulin	12/12
7	VIQPQGLLVPR	Glutelin acidic chain	11/11
8	GSMGLTFPGCPA	Glutelin acidic chain	12/12
	SEAGVTEYFDEK	Glutelin acidic chain	12/12
9	VEHGLSLLQPYA	Glutelin acidic chain	12/12

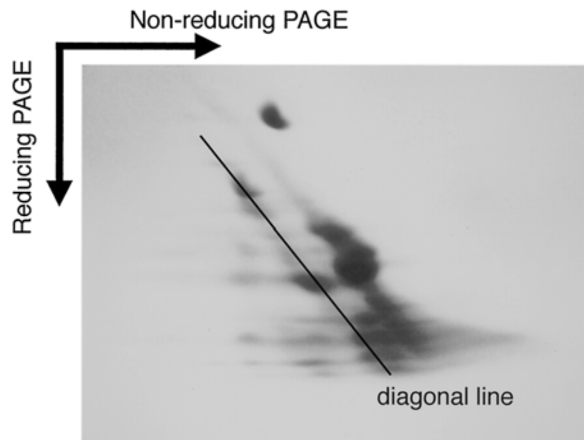

Fig. 1. Nonreducing/reducing 2D-PAGE profile of the salt-soluble fraction of rice seed protein (50 μg).

bonds migrate to above or below the diagonal line, respectively (Allore and Barber 1984). As shown in Fig. 1, most proteins migrate to above the diagonal line of the gel, while few migrated to the diagonal line or below it. This shows that the allergenic fraction is dominated by intramolecular disulfide proteins.

Specific Labeling of Disulfide Proteins

To identify disulfide proteins at higher resolution, the proteins were specifically labeled with fluorescence using IEF/SDS 2D-PAGE. First, free sulfhydryl groups of the proteins in the salt-soluble fraction were modified by nonfluorescent iodoacetamide. Next, disulfide bonds of proteins were completely reduced by DTT, and newly exposed sulfhydryl groups were labeled by fluorescent mBBr. Thus, disulfide-forming sulfhydryl groups were labeled selectively by fluorescent mBBr.

Identification of Disulfide Proteins

Figure 2 shows the IEF/SDS 2D-PAGE profile of the salt-soluble fraction. Most proteins were labeled with mBBr, supporting the result of Fig. 1 (that the allergenic fraction is rich in disulfide proteins). Some of the proteins (1–9) were subjected to internal amino acid sequence analyses, and the amino acid sequences were subjected to the SWISS-PROT and TrEMBL databases using the FASTA algorithm (Table I). Some of the proteins were known allergens: 2–4 and 7–9/acidic chain of glutelin (Shibasaki et al 1979), 6/globulin (Usui et al 2001). As a complete molecule of glutelin is salt-insoluble, 2–4 and 7–9 are considered to be fragments of the acidic chain of glutelin. Previously (Yano et al 2001b), we reported that the acidic chain of glutelin is susceptible to truncation, while the basic chain is relatively resistant. It is speculated that the truncation of glutelin occurred during storage of rice grains and yielded fragments of the acidic chain of glutelin. The

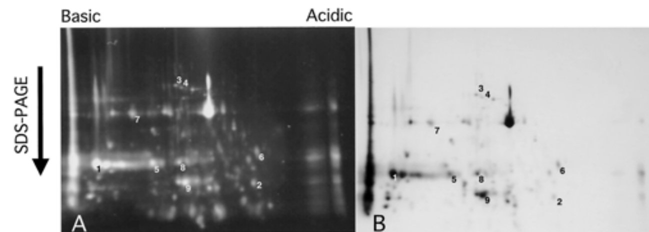

Fig. 2. Isoelectric focusing/SDS 2D-PAGE profile of the salt-soluble fraction of rice seed protein (100 μg). **A,** Detection of mBBr-labeled proteins. **B,** Coomassie Blue staining of the whole proteins.

amino acid sequence of one of the three fragments (1) was homologous to trypsin/amylase inhibitor, while the other two did not share similarities with any known proteins. These facts suggest that fragment 1 is a new candidate for an allergen homologous to the trypsin/amylase inhibitor. The 5 protein had no similarity with known proteins except for an unidentified QR 310003 protein from rice (PIR accession no. PC4269).

DISCUSSION

Screening of Allergens

Sen et al (2002) reported that disulfide bonding rendered Ara h2, a major peanut allergen, resistant against gastrointestinal (GI) proteases. They reported that treatment of GI proteases of Ara h2 yielded a protease-resistant fragment that contained IgE-binding epitopes. Also, the reduction of disulfide bonds made Ara h2 susceptible to proteases. By using canine models, Buchanan and colleagues have shown that the reduction of disulfide bonds of wheat (Buchanan et al 1997) and milk (del Val et al 1999) allergens rendered them less allergenic. Although we cannot conclude that all disulfide proteins are allergens, these findings suggest a significant correlation between disulfide bonding and allergenicity.

Here, we tried a comprehensive analysis on disulfide bond formation in the rice seed salt-soluble fraction, which has long been known as an allergen to some populations (Shibasaki et al 1979). We have shown that the allergenic fraction is composed mostly of intramolecular disulfide proteins (Fig. 1), supporting a correlation between allergenicity and disulfide bonding.

As even small amounts of some proteins cause allergic reactions, and considering that reactivity to allergens varies among patients, development of a comprehensive screening method for allergens is indispensable to the effort to identify new ones. Although allergens share a wide range of biochemical characteristics, none of the characteristics include all allergens as a class, making it difficult to screen allergens based on biochemical traits (Huby et al 2000). Our goal was to identify candidate allergens based on disulfide bond formation. As the protein 1, newly found here, shared similarities with known allergens, the strategy presented in this study is useful for screening allergen candidates. Confirmation

of the allergenicity of new candidates found here (1 and 5) is in progress in our laboratory. A combination of 2D and immunoblotting (Weiss et al 1997) has been used to screen major allergens. However, allergens expressed in small amounts may be lost in the blotting procedure. As mBBr-labeled proteins can be detected in gel, our strategy will complement 2D/immunoblotting by allowing the detection of small amounts of allergens.

Lessening Allergenicity by Genetic Engineering

The allergenicity of rice has been mitigated for some allergic patients by the introduction of anti-sense DNA for respective allergens (Nakamura et al 1996; Tada et al 1996). However, as the number of allergens is very large, and includes unknown ones, decreasing the number of allergens by the anti-sense method may not be effective. Alternatively, lessening allergenicity by hindering common features of allergen should work as a fundamental strategy. In this study, disulfide bonding was confirmed as one of the common features of cereal allergens. So manipulation of the thiol status of cereal (Hofgen et al 2001), such as overexpression of thioredoxin in seed (Cho et al 1999), may work to reductively cut some allergen disulfide bonds, rendering them less allergenic.

CONCLUSIONS

The disulfide proteome technique was used to identify disulfide proteins in rice endosperm. This work has confirmed and strengthened the hypothesis that disulfide bonding is one of the common features of allergens. Also, this work has made it feasible to screen possible allergens on the basis of disulfide bonding.

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