

Disulfide-related proteomic studies on food allergens

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An increasing proportion of the population is affected by food allergy. Some food allergens ('incomplete' allergens) elicit clinical symptoms only owing to their homology with other allergens, while others ('complete') can both sensitize and elicit clinical symptoms. To challenge food allergy, it should be effective to invest more energy in tackling the complete ones. Post-translational modification proteomics allows a comprehensive investigation of complete allergens. It provides clues to elucidate mechanisms of the structures that contribute to allergenicity, which thus, in turn, to help alleviate food allergens. This review summarizes the recent proteomic challenges to identify and alleviate food allergens, especially through disulfide-related studies.

KEYWORDS: allergenicity • disulfide bond • protease resistance • proteomics • thioredoxin

Food allergy is defined as an adverse immunologic response to food [1]. A wide variety of systemic manifestation induced by food hypersensitivity responses shows the possibility of localized exposure to foods in the gastrointestinal (GI) tract to result in symptoms in distal target organs. Wheat allergy, for example, may cause atopic dermatitis, isolated urticaria, anaphylaxis, baker's asthma or celiac diseases [2]. On the other hand, major fruit allergen, Bet v 1, induces symptoms of the oral allergy syndrome. Disorders with acute onset of symptoms in individuals predisposed to developing allergies after ingestion are usually mediated by IgE. The immune system produces IgE antibodies against epitopes of allergic proteins. The IgE molecules arm tissue mast cells and blood basophils, resulting in a state termed sensitization. Upon re-exposure, the causal food proteins bind to the IgE molecules specific for them, and trigger the release of a number of chemical mediators, such as histamine, which cause allergy symptoms.

Conventional epidemiologic surveys estimate that 3–4% of any population has a food allergy [3,4], and an increasing number are being affected [5]. While a systematic survey of the prevalence of plant food allergies, taking into account double-blind, placebo-controlled, food-challenge tests, IgE sensitization and reported reactions, points to considerable heterogeneity in the prevalence estimates [6], the growing impact of food allergies demands further understanding on how some proteins become

allergens. This review introduces the challenges to investigate allergens in the viewpoint of disulfide-related studies.

Identification of allergens

Conventional 2DE/immunoblotting to identify allergens

Identification and characterization of allergens are essential to elucidate how they become allergenic. A combination of 2DE and immunoblotting is a powerful tool to identify allergens [7,8]. Recently, the term 'allergenomics' [9] or 'allergome' [10] has been used for a systematic and comprehensive analysis of putative allergens. In allergenomics, proteins in allergen sources, such as latex [9] and wheat [11], are separated with 2DE, and putative allergens are detected by IgE immunoblotting using a pooled (or each) serum of allergic patients. Subsequently, the IgE-interactive proteins are cut out from the gel and subjected to in-gel protease digestion. Then, the obtained peptides are analyzed with mass spectrometry. Based on the mass spectrometric data, the candidate allergens are identified through a database search. Allergenomics provides direct serologic evidence that patients have allergen-specific IgE, and allows identification of putative allergens. On the other hand, while specific IgE is necessary to cause allergenic disease, its presence in the sera does not necessarily indicate that the patient is clinically allergic to the protein [12]. In addition, as the reactivity of allergens varies among patients, and reactivity or potency may diminish

during the denaturing and blotting procedures, some allergens may not be detectable by this conventional method. Therefore, complementary methods based on different principles are needed to detect allergens that escaped the notice of conventional methods.

'Incomplete' & 'complete' allergens

Aalberse classified food allergens into two groups: 'incomplete' food allergens only elicit clinical symptoms in previously sensitized individuals, while 'complete' food allergens can both sensitize and elicit reactions in susceptible individuals [13]. Incomplete food allergens elicit clinical symptoms owing to their homology with other allergens. Conversely, the complete allergens survive the denaturing conditions of food processing or digestive action in the GI tract, and are, thereby, enabled to reach the immune system in the gut. Resistance to protease digestion is thought to be an important characteristic of food allergens [14] since the longer a significant portion of a protein remains intact, the more likely it is to encounter cells of the immune system [15]. Allergens or their protease-resistant fragments generally require a molecular mass of 3.5–5 kDa, as each should contain at least two IgE-binding epitopes in order to crosslink mast cells and elicit allergic reactions.

Then, what are the characteristic structures to contribute in enabling food proteins to become allergenic? If a common structure of allergens is clarified, the knowledge may provide a clue on how to alleviate both complete and incomplete allergens. Although the 2DE/immunoblotting provides serologic evidence to show that the detected proteins are reactive with IgE, it does not make the difference between the complete and incomplete allergens. On the other hand, post-translational proteomics is useful for a comprehensive structural analysis of allergens.

Structure of epitopes

Investigation of epitope-containing regions of allergens is critical to study allergen–IgE interactions. Epitopes are generally categorized into two types. Conformational epitopes are recognized by IgE depending on the secondary or tertiary structure of allergens, whereas linear epitopes are recognized by the linear amino acid sequence of allergens.

Bannon discusses that, while conformational IgE-binding epitopes are prevalent and important to aeroallergen-mediated allergic reactions, linear epitopes are important to food allergens, mainly because the immune system encounters them only after they have been partially denatured and digested by the human GI tract [16]. Caseins are such an example. Patients with persistent milk allergy possessed higher detectable levels of IgE antibodies to linearized than to conformational (native) epitopes of caseins, while none of the patients who outgrew milk allergy had IgE binding to the linear epitope [17].

By contrast, several recent reports provide evidence for the importance of the tertiary structure of the epitopes even in the case of food allergens. 2S albumins are major allergens that are widely distributed in mono- and di-cotyledonous plants. After synthesis as a large precursor, the protein is modified post translationally to form four intramolecular disulfide bonds, followed by truncation

into a large and a small subunit. Finally, the protein has two intermolecular (between the two subunits) and two intramolecular (in the large subunit) disulfide bonds to form the 'disulfide skeleton' as a core scaffold structure conserved commonly in the 2S albumins. Trials for a linear epitope mapping of the 2S albumin family Jug r 1 [18] and Sin a I [19] revealed the presence of conformational epitopes along with the linear ones. Moreno *et al.* reported that *in vitro* digestion of 2S albumin Ber e 1 from Brazil nuts, using a physiologically relevant GI model system, yielded a 5-kDa fragment [20]. The fragment resistant to digestion conserved the characteristic disulfide skeleton [21], and contained potentially active B- and T-cell epitopes. Diaz-Perales *et al.* reported that simulated gastric fluid digestion of avocado allergen, Prs a 1, yielded peptides with hevein-like domain, in which intramolecular disulfide bonds held together the proteolytic fragments, and were reactive with IgE both *in vitro* and *in vivo* [22]. Using circular dichroism studies, Sen *et al.* reported that disulfide bonds play a critical role in the structural stability of Ara h 2, one of the most commonly recognized allergens in peanut [15]. Also reported is that protease treatment of Ara h 2 produced relatively large (10-kDa) fragments that contained intact IgE-binding epitope, and the reduction of the disulfide bonds rendered Ara h 2 susceptible to protease digestion [15]. Using a canine model [23], Buchanan and coworkers have shown that reduction of the intramolecular disulfide bonds rendered wheat [24] and milk [25] allergens less allergenic and susceptible to protease digestion. Kovacs-Nolan *et al.* reported that the protease-resistant fragments of egg white ovomucoid had IgE-binding activity and the cleavage of the disulfide bonds made ovomucoid susceptible to protease digestion [26]. Stability of major allergens, in relation to their structure, is listed in TABLE 1. These observations suggest that, even in the case of food allergens, conformational epitopes play critical roles in their allergenicity. In addition, the protease-resistant core structure, sustained by disulfide bonds, contributes significantly to allergenicity. Cheek *et al.* classified 3000 small, disulfide-rich protein domains into 41 fold groups on the basis of structural similarity [27]. Comprehensive experimental and database analyses of such scaffolding in relation to protease resistance or allergenicity should pave the way for a new classification of allergens. More recent approaches include *in silico* mapping of molecular surface features to identify allergen-specific motifs [28,29]. With accumulating data of detailed epitope structures, bioinformatics will help elucidate allergen–IgE relationships that are useful to develop strategies for alleviation of allergens as well as medical treatments.

Disulfide proteome analysis of food allergen

We looked for selectively labeled disulfide bonds to see whether allergens were detected (FIGURE 1A) [30,31]. First, the proteins' free sulfhydryl (SH) groups were capped with nonfluorescent iodoacetamide. Then, proteins were treated with dithiothreitol to expose SHs taking part in the disulfide bonds. Finally, they were labeled by fluorescent probe, monobromobimane (mBBBr). The protocol was applied to the mixture of soybean trypsin inhibitor (containing disulfide bonds) and myoglobin (containing no disulfide bonds), which resulted in the fluorescent labeling of only the

former one (FIGURE 1B). Next, the procedure was applied to the salt-soluble fraction of rice [32] and buckwheat [33], which has been clinically shown to be allergenic [34,35]. The major fluorescent spots were subjected to the internal amino acid sequence analyses and confirmed that the fraction was composed of known allergens or their homologues (FIGURE 1C). In addition, using this technique, protease treatment of a saline-soluble fraction of buckwheat was shown to yield some resistant fragments, all of which contained disulfide bond(s) (FIGURE 2) [33]. These observations collectively suggest that although protease cleavable sites are distributed throughout the length of the protein, some disulfide-containing regions were inaccessible to protease. Thus, disulfide bonds often make a protein more stable to digestion in the GI tract, thereby enabling some fragments to reach the gut immune system intact.

Therefore, why do most cereal proteins contain such a recalcitrant structure? The answer lies in the stability disulfide bonds provide to the storage proteins. During maturation of grain, storage proteins are compacted into the protein body. Disulfide bonds act effectively to fold the proteins. In addition, disulfide bonds stabilize the molecular scaffold of some protease inhibitors to protect seeds against consumption by microbes or animals. On the other hand, during germination and early seedling development, these proteins require prompt degradation to supply amino acids and nitrogen for growth. Therefore, plants have an effective mechanism to make possible both compact packaging of the storage proteins and their ready mobilization. For example, in rice seed germination, glutelin, the major storage protein, undergoes degradation in an ordered fashion [36]. First, limited proteolysis truncates the individual glutelin molecules, thereby disrupting the original ordered macromolecular conformation, making the protein more soluble and the disulfide bonds of its fragments accessible to reduction. The intermediates are then reduced and become susceptible to complete proteolysis. Thus, in a way, plants untangle storage proteins prior to degradation, while animals, on the other, attempt to digest them without prior unfolding. It is similar to cutting a tangled yarn with scissors, and occasionally yields allergenic fragments. The natural untangling mechanisms of plants provide us with a clue for developing allergen-alleviating strategies, which will be discussed in the following section.

Trials to reduce food allergens

Reducing allergenicity of food proteins by cutting their disulfide bonds

The contribution of disulfide bonds to the allergenicity of some proteins suggests a new strategy to alleviate allergens by the cleavage of them. Buchanan and coworkers, who found and contributed significantly to clarifying the biochemical role of thioredoxin (Trx) in plants [37], have successfully applied the

Table 1. Major food allergens.

Allergen	Comment
<i>Plant allergens</i>	
Prolamins	α -helical proteins sustained by four-disulfide skeleton. Resistant to heat/protease
Vicilins	No disulfide, but forms stable trimer which is resistant to heat/protease
Legumins	Two subunits with inter-/intra-molecular disulfide bonds
Profilin, Bet v 1	Conformational epitopes that is not sustained by disulfide bonds; labile to heat/protease. Cause oral allergy elicited by raw foodstuffs
<i>Animal allergens</i>	
Lactoglobulin	β -barrel structure sustained by two disulfide bonds; heat causes destabilization as well as Maillard reaction with lactose
Casein	Heat treatment alone does not alter the allergenicity of the linear epitopes
Parvalbumin	Conformational epitopes sustained by calcium-binding domain; resistant to heat/protease

reductive capability of its disulfide bonds in order to alleviate allergenicity. Trxs are widely distributed 12-kDa proteins that are functional in the regulation of cellular processes throughout the animal, plant and microbial kingdoms. An h-type of Trx, reduced by NADPH via NADP-Trx reductase (NTR), reduces disulfide bonds of its target proteins.

- (1) $\text{NADPH} + \text{H}^+ + \text{Trx ox (S-S)} \rightarrow \text{NADP}^+ + \text{Trx red (-HS, SH-)}$
- (2) $\text{Target ox (S-S)} + \text{Trx red (-SH, HS-)} \rightarrow \text{Target red (-SH, HS-)} + \text{Trx ox (S-S)}$

Available evidence supports that Trx plays an important role in seed germination. Trx exists in the aleurone cells of germinating wheat [38]. During germination of the seed, Trx is converted from the oxidized (inactive) to the reduced (active) form [39]. In germinating wheat, Trx activates thiocalsin, a serine protease specific for indigenous seed storage proteins [40]. Overexpression of Trx in barley endosperm enhances the activity of pullulanase [41] and α -amylases [42], and also accelerates germination [41]. All these observations suggest that Trx acts as a 'wake-up call' in seed germination: it should be responsible for the detangling of the storage proteins, as well as activation of enzymes to digest them. Buchanan, Frick and colleagues have successfully applied Trx in reducing allergenicity of wheat [24] and milk [25] allergens, respectively.

FIGURE 3 shows an application of the disulfide proteome technique to investigate the relationship between Trx-dependent reduction of disulfide bonds and protease susceptibility of allergens [43]. FIGURE 3A shows the saline-soluble allergenic fraction of rice [44], which contains allergenic 14- to 16-kDa trypsin inhibitors. The left panel shows a control (untreated) sample, and the right panel shows a Trx-treated sample. There is little difference between the Coomassie brilliant blue-stained gel profiles. By contrast, when the free SH groups of the protein were labeled with mBB, a specific fluorescent probe, and the gels were observed

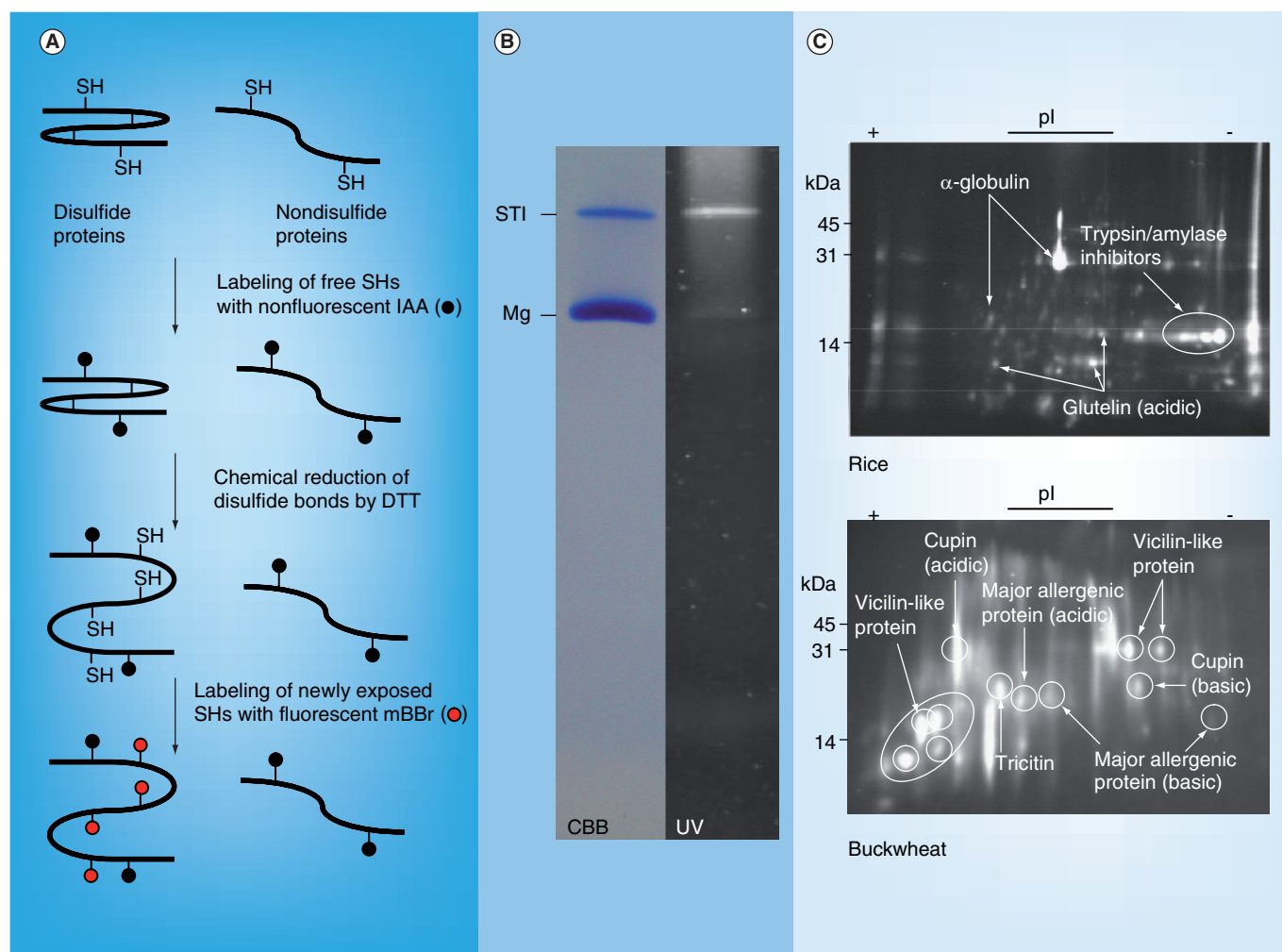


Figure 1. Selective fluorescent labeling of disulfide proteins. (A) Explanatory panel of the protocol. (B) Application of the protocol to the mixture of STI and Mg. (C) Fluorescent labeling of disulfide proteins in the allergenic fraction of rice and buckwheat.

CBB: Coomassie brilliant blue; DTT: Dithiothreitol; mBBR: Monobromobimane; Mg: myoglobin; SH: Sulfhydryl; STI: Soybean trypsin inhibitor; UV: Ultraviolet.

Modified with permission from [30,32,33].

under the UV lamp (365 nm), the fluorescence increased in the case of Trx-treated sample. This shows that Trx cleaved the disulfide bonds of the proteins. When the control sample was subjected to trypsin digestion, many protease-resistant fragments appeared (FIGURE 3B). On the other hand, when the Trx-treated extract was subjected to trypsin, few fragments remained. This shows that cleavage of the disulfide bonds of allergens by Trx increased their susceptibility to protease.

Practical application of Trx to food processing is in progress. Waga *et al.* treated wheat gliadin with active (reduced) Trx, and the immunoreactivity of gliadins, as estimated by ELISA against allergic patients' sera, was shown to be decreased by half [45]. Nevertheless, the dough rheological properties were not affected significantly. The results confirm and extend the usefulness of Trx in wheat flour modifier [24], decreasing allergenicity without adversely affecting the technological properties. By overexpression of Trx in barley [41], Wong *et al.* found that disulfide bonds of hordeins in the dry seed were reduced significantly [42], and recovery

of the extractable and soluble proteins increased. Recently, Li *et al.* reported increased solubility of disulfide proteins and lower allergenicity of the gliadin fraction in transgenic wheat seed, in which Trx was overexpressed in the endosperm [46]. Such trials will open the door to develop less-allergenic cereals, which will be utilized as major ingredients for less-allergenic foods. Meanwhile, some recent studies based on IgE binding of sera from patients with bakers' asthma suggest the possibility that Trx itself is an allergen [47,48]. Although the role of Trx in allergies is expected to be resolved in the future studies [49], industrial application of Trx would have large potential to alleviate allergens.

Effective immunotherapy by incorrect disulfide bonding

Allergen-specific immunotherapy (SIT) is the practice of gradually administrating increased doses of an allergen extract to an allergic patient to relieve symptoms. While allergic reactions can be alleviated by pharmacotherapy, SIT is the only causative approach and has proven successful, for example, in insect venom

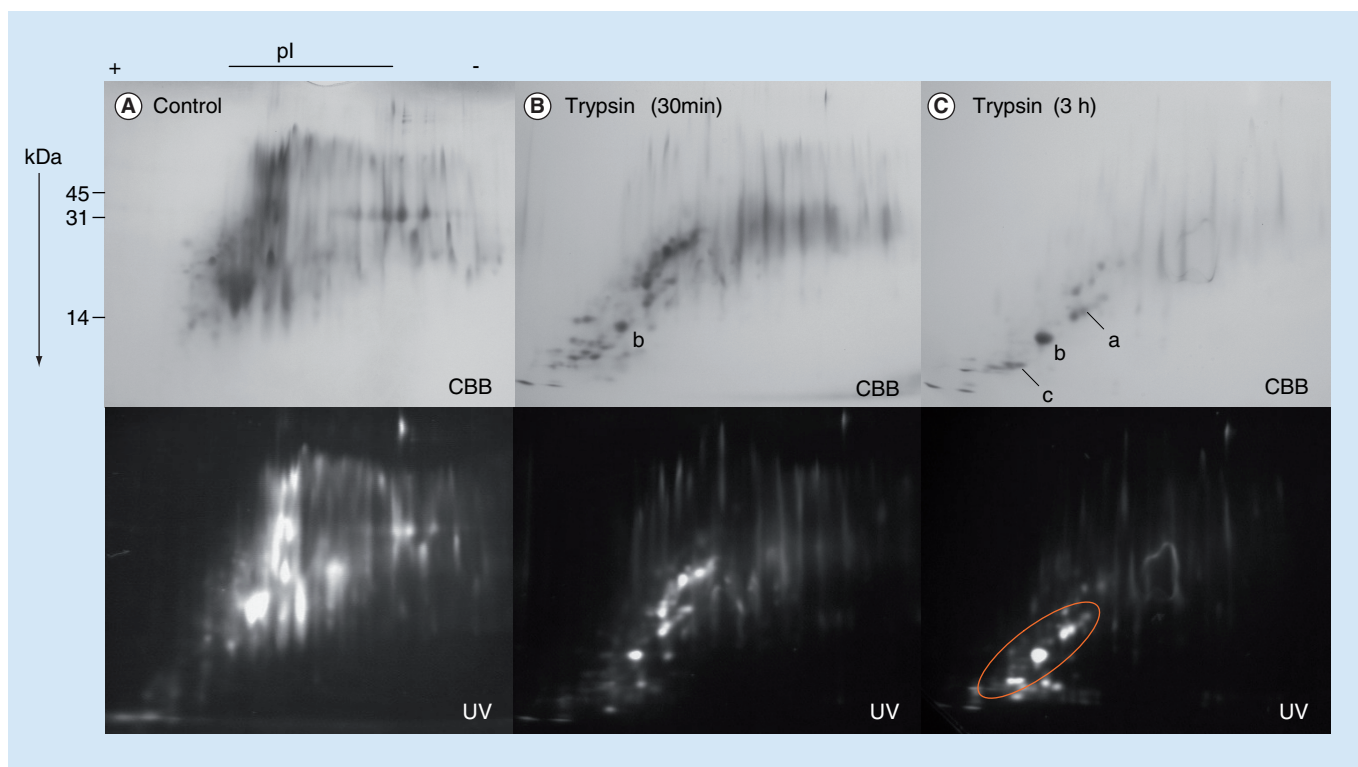


Figure 2. Digestion of dilute acetic acid-soluble fraction of buckwheat seed with trypsin reveals protease-resistant fragments. (A) Before digestion. Digestion with trypsin for **(B)** 30 min and **(C)** 3 h. Modified with permission from [33].

allergy [50] and allergic rhinitis or rhinoconjunctivitis [51]. On the other hand, owing to a rare but serious side effect of anaphylaxis on therapeutic administration, its use has been limited. A basic requirement for an ‘allergen vaccine’ [52] in achieving successful SIT without risk of anaphylaxis is to use a protein that still possesses T-cell epitopes, which induce T-cell tolerance and lack antibody-binding sites that mediate IgE crosslinking (for schematic figure, see [53]) [52]. B cells recognize their cognate antigens in their native forms, whereas T cells recognize processed fragments of antigens presented by antigen-presenting cells in association with MHC molecules. Accordingly, targeting T cells and bypassing IgE by modified allergens may enable the administration of higher doses of allergens, without the risk of anaphylaxis [54]. Immunotherapy using peptides (PIT) fits this strategy but it has some shortcomings, such as the instability of peptides and the requirement to prepare several recombinant allergen fragments. To overcome these problems, genetically engineered recombinant hybrid molecules that span the whole T-cell repertoire but do not bind IgE have been developed [52]. Recombination of disulfide bonds results in a conformational change of the allergen, which may lead to misrecognition by the conformation-dependent B-cell epitopes. Kusbebi *et al.* produced a fusion protein composed of the two major bee venom allergens Api m 1 and Api m 2 [52]. They predicted that the fusion protein, which has 14 cysteine residues, should form incorrect disulfide bonds and result in incorrect folding and conformation. They reported decreased allergenicity with preserved T-cell tolerance

of the fusion protein, and concluded that it represented a novel vaccine prototype for allergen-SIT. Thus, the authors took advantage of the disulfide bonding of allergens in developing allergen-alleviating strategy.

Other studies

Several important topics complementary to disulfide-related studies are introduced in the following sections.

Could glycosylation of food proteins contribute to allergenicity?

Some reports suggest possible allergenicity of glycosylated proteins. By comparative studies using native and deglycosylated celery allergen, Bublin *et al.* showed that Api g 5 reacted with human IgE and, thus, activated allergic patients’ basophils, exclusively via its crossreactive, plant-specific N-glycans [55]. Foetisch *et al.* reported that approximately a third of the crossreactive carbohydrate determinant (CCD)-positive sera from patients with tomato allergy contained carbohydrate-specific IgE, which induces *in vitro* basophil histamine release [56]. They conclude that the general claim that CCD-specific IgE is clinically irrelevant has to be reconsidered critically, and the use of natural allergens should be preferred over the use of recombinant allergens expressed in prokaryotic organisms. On the other hand, Breiteneder claims that, in these two cases, the allergen concentration needed to achieve the mediator release were relatively high [57]. In addition, van der Veen reported that crossreactive IgE, directed to the

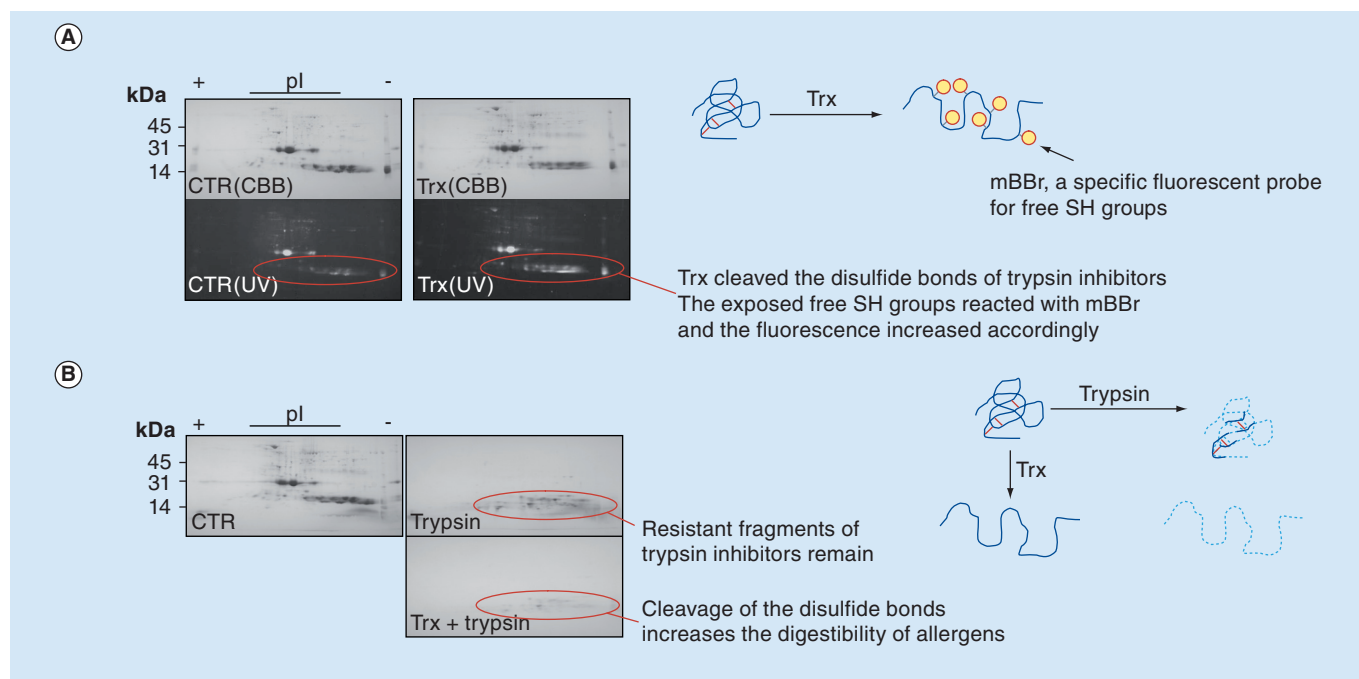


Figure 3. Monitoring of thioredoxin-dependent reduction of allergens and their susceptibility to protease. (A) Cleavage of the disulfide bonds of allergens by Trx. **(B)** Reduction of protease resistance by unfolding of allergens. CBB: Coomassie brilliant blue; CTR: Control; mBBr: Monobromobimane; SH: Sulfhydryl; Trx: Thioredoxin.

glycosyl portion of glycoproteins, found in grass pollen-sensitized patients, had poor biological activity: although the IgE cross-reacted with peanut proteins at a significant level, the patients were not allergic to peanuts [58]. Thus, the significance of glycosylation in allergenicity should be confirmed by further studies. Other than direct interaction with IgEs, some reports suggest that glycosylation increases protein stability [59,60], which may contribute to allergenicity.

Development of less-allergenic food/plants/cereals

Processing can influence the allergenicity of food proteins. Respective allergens react to a processing differently. For example, mere storage of apple increases its allergenicity [61] but not for most of other dry fruits. Proteases, such as trypsin, actinase and bromelain decreased the allergenicity of hazelnuts [62], rice [63] and wheat [64], respectively. Hypoallergenic baby formula is made by treating milk with protease followed by a filtration of the residual products [65]. Washout of soluble rice allergen from the polished rice grain under high pressure reduced allergenicity [66]. Interestingly, thermal processing of allergen reduces or increases its allergenicity. The conformational epitope of Bet v 1 allergen family in fruits and vegetables is readily destroyed by heat, which results in decreased allergenicity. On the other hand, in roasted peanuts, Maillard reaction between free amino group and sugar can crosslink Ara h 1 and Ara h 2, yielding high-molecular-weight aggregates that bind IgE more effectively than unmodified allergen [67]. In hot milk, reaction between β -lactoglobulin and lactose increases allergenicity [68]. By contrast, when peanuts are boiled, some allergens dissolve into the water [69]. Thus, processing has a variety of impacts on food allergen.

Genetic engineering of hypoallergenic plants is in progress. By antisense approaches, the expression of 15- to 16-kDa allergens from rice [70] and Gly m Bd 30 kDa from soybean [71] was successfully downregulated. RNA interference is also applied to suppress the expression of Mal d 1 in apples [72], Ara h 2 in peanuts [73] and Lyc e 3 in tomato [74]. On the other hand, to develop less-allergenic cereals by crossbreeding, breeders must select a limited number of allergens to be removed, since it is not technically easy to remove a large number of allergens. Alternatively, lessening allergenicity by restricting allergens' common features may work more effectively to render them less allergenic.

Expert commentary

Processing and genetic engineering are effective for a limited portion of food/plant allergens. If common structures of allergens are clarified, it helps the development of methods to lessen numerous allergens, including unknown ones. Disulfide bonds occasionally contribute to a cereal protein's protease resistance and allergenicity. Researches in the viewpoint of disulfide structure are critical for the mitigation of allergens, as well as for allergy treatment. Alleviation of the allergenicity of allergens by cleavage of the disulfide bonds and its industrial application are in progress, and the disulfide proteome technique is a useful tool to monitor the processes.

Five-year view

Tertiary structure analysis of the disulfide-rich domain of allergens will be accelerated. Databases that contain information on the disulfide domain structure, linear and conformational epitope mapping will be enriched and contribute eventually to the development of low-cost, safe strategy to alleviate allergens.

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Key issues

- A growing body of evidence suggests that the disulfide bonds are often related to a protein's allergenicity.
- Although protease-cleavable sites are distributed throughout the length of the protein, some disulfide-containing regions are inaccessible to proteases.
- Even in the case of food allergens, conformational epitopes play critical roles in their allergenicity. The protease-resistant core structure, sustained by disulfide bonds, also contributes significantly to the allergenicity.
- While the possibility of thioredoxin's allergenicity should be addressed in the future studies, industrial application of thioredoxin in reducing the disulfide bonds of allergens would have large potential to alleviate allergens.
- The disulfide proteome will be a useful tool in the investigation of allergens.

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