



Three-dimensional structure of Gly m 5 (β -conglycinin) plays an important role in its stability and overall allergenicity



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ABSTRACT

To date, there is insufficient knowledge regarding the relationship of the allergenicity and the three-dimensional structure of Gly m 5 (β -conglycinin), a major allergen in soybean. In the present study, allergen Gly m 5 was demonstrated to generate three major digestion-resistant fragments when it was subjected to *in vitro* digestion. The largest fragment corresponded to the main body of the monomer and two smaller fragments corresponded to the main bodies of two modules of the monomer. Two major protease cleavage sites were located in the regions near to the connection between two modules. Coincidentally, the major digestion-resistant fragments were demonstrated to contain intact IgE epitopes and be capable to induce basophil histamine release in Gly m 5 sensitised piglets, indicating that the three-dimensional structure of Gly m 5 afforded the molecule some protection from complete degradation into small peptides and amino acids, and contributed to its overall allergenicity.

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1. Introduction

Soybean proteins represent one of the most important sources of dietary proteins because of their relatively well-balanced composition of amino acids (Friedman & Brandon, 2001). However, soybean is also a dietary allergenic source for humans (Gagnon, Poysa, Cober, & Gleddie, 2010). Soybean allergy has been reported primarily among young children nourished with soybean protein-contained infant formula (Zeiger, Sampson, & Bock, 1999). The incidence of soybean allergies in adults was estimated at approximately 0.5% of the population in the United States (Sicherer & Sampson, 2006). Allergic symptoms to soybean may range from skin, gastrointestinal, or respiratory reactions to anaphylaxis (Ballmer-Weber, Holzhauser, & Scibilia, 2007; Mittag, Vieths, & Vogel, 2004). Allergen Gly m 5 (β -conglycinin), the main soybean storage protein, has been identified as one of the major allergens in soybean (Krishnan et al., 2009; Holzhauser et al., 2009).

Some characteristics of a dietary allergen increase the ability to induce a severe allergic reaction. These characteristics include its capacity to provoke high levels of IgE and to resist digestion in gastrointestinal tract to generate relatively large protein fragments containing multiple IgE epitopes. Small fragments containing single IgE epitopes by gastrointestinal degradation are unable to cross-link IgE-bound receptors on mast cells and, thus, to induce these cells degranulation. Therefore, the structural and biochemical properties may play an important role in the allergenicity of a protein allergen. Allergen Gly m 5 contains three kinds of subunits, namely, α , α' , and β (Wang, Qin, Sun, & Zhao, 2014). All these subunits of Gly m 5 which share extensive amino acid sequence homology (Maruyama et al., 1998) were demonstrated as potential dietary allergens for both humans and animals (Holzhauser, Wackermann, & Ballmer-Weber, 2009; Zheng, Qin, Tian, & Sun, 2014). The crystal structures of Gly m 5 monomer consists of two compact modules. Each module is subdivided into a core domain and a loop domain. The α helical bundles located on the ends of each monomer overlap to form the stable Gly m 5 trimer (Maruyama et al., 2001). Allergen Gly m 5 is more resistant to digestion than other proteins in soybean (Yang et al., 2016; Zhao,

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Qin, & Sun, 2008). Of the three subunits, the α -subunit exhibited remarkable digestive stability to pepsin digestibility and a computer-generated three-dimensional structure indicated that the IgG-binding epitopes were located on the molecular surface of the homology-modeled structure of the α -subunit (Fu, Jez, Kerley, Allee, & Krishnan, 2007). However, the digestive stability of native Gly m 5 needs to be further characterised when it is exposed to both simulated stomach and intestinal digestions. Besides, identifying IgG-binding protein rather than IgE binding protein is not enough to understand the allergenicity of an allergen. As a matter of fact, IgG-binding ability indicates that the protein is an antigen but unnecessarily an allergen.

To date, there is insufficient evidence linking Gly m 5 allergy and three-dimensional structure of the allergen as well as its digestion-resistant properties. The objective of this study was to investigate whether the three-dimensional structure of Gly m 5 played a role in soybean allergy by simulated gastrointestinal digestion of the allergen into peptide fragments, the analysis of the fragments structure and the protease cleavage sites, and determination of IgE-binding properties and histamine release activity of the digestion-resistant fragments using an experimental piglet model of Gly m 5 allergy.

2. Materials and methods

2.1. An experimental piglet model of Gly m 5 allergy

A piglet model of Gly m 5 allergy that mimicked the allergic symptoms of human beings had been previously reported (Zheng et al., 2014). Briefly, three pregnant Landrace sows were fed a soybean-free diet from day 108 of pregnancy until the end of their lactations to limit colostral transfer of maternally-derived anti-soybean antibodies to their piglets. A total of 12 crossbred piglets (Large White $\times$ Landrace), selected from the three litters, were divided into one of two treatment groups at 35 days of age. Each of the two treatment groups contained six piglets. The piglets in the control group were fed the soybean-free control diet at all times. The piglets in the exposed group were orally challenged with the diets containing 18% purified Gly m 5 at days 0 to 10, days 18 to 20 of the trial. Thereafter, piglets were challenged for 3 days at 7-day intervals. They were fed the control diet at all other times. After the final oral challenge (day 40), blood was taken via precaval vein. An aliquot of blood was used for the separation of serum. Sera from the piglets in the exposed group were individually assayed for IgE-binding response to allergen Gly m 5. Another aliquot of the whole blood was heparinized and used for basophil activation assay. As a result, transient diarrhea with lower average daily gain and feed conversion was observed, which was associated with positive skin prick test to purified Gly m 5 in the exposed group (Zheng et al., 2014). Sera from six piglets in the exposed group all exhibited IgE-binding to allergen Gly m 5. Equal aliquots of IgE-containing serum from each piglet in the exposed group and serum from the piglet in control group were pooled, respectively, and used to identify epitope-containing fragments produced by the digestion of allergen Gly m 5 with proteolytic enzymes.

2.2. Purification of native soybean allergen Gly m 5

Gly m 5 was purified from a soybean cultivar composed mainly of α - and α' -subunit essentially according to previously published methods (Liu, Wang, & Cui, 2007). Briefly, defatted soybean flour was extracted with 0.03 mol/l Tris-HCl buffer (pH 8.0). The supernatant after centrifugation was adjusted to pH 6.4 and kept at 4 °C overnight. After centrifugation, the supernatant was adjusted to pH 5.5. After being stirred for 30 min and centrifuged, the supernatant

was adjusted to pH 4.8 and then centrifuged. The precipitate mainly contained crude Gly m 5 fraction. The crude Gly m 5 fraction was then loaded onto a Sepharose CL-6B column, and the column was washed with phosphate buffer solution (pH 7.6). The fractions containing Gly m 5 were pooled and lyophilized. Purified Gly m 5 was stored in aliquots at -80 °C.

2.3. *In vitro* digestion of purified native Gly m 5

In vitro digestion was conducted essentially according to a previously published method (Astwood, Leach, & Fuchs, 1996). Briefly, purified Gly m 5 was dissolved in simulated gastric fluid (containing 100 mmol/l HCl, 30 mmol/l NaCl, pH 1.2) and incubated in the presence of 20 mg/ml pepsin (Sigma-Aldrich) at a ratio of 1:1 (w/w) of pepsin and substrate at 37 °C. Aliquots were taken at 1, 10, 30 and 60 min, respectively. The digestion reaction in each aliquot was stopped by adjusting the solution with 1 mol/l TRIS to pH 8.3. Thereafter, the samples were successively incubated in the presence of 10 mg/ml trypsin (Sigma-Aldrich) and 10 mg/ml chymotrypsin (Sigma-Aldrich) at a ratio of 1:10 (w/w) of enzyme and substrate at 37 °C, respectively. Then aliquots were taken at 5, 60 and 90 min. The hydrolysis reaction in each aliquot was then quenched by the addition of SDS-PAGE loading buffer and heating for 6 min. Meanwhile, bovine serum albumin (BSA), a nonallergenic protein obtained from Sigma-Aldrich, suffered the same *in vitro* digestions as a control.

2.4. Gel electrophoresis and immunoblotting analysis

The electrophoresis procedure was performed essentially according to Laemmli (1970). Briefly, ten microliter aliquot of the above samples with loading buffer was loaded onto a 12% polyacrylamide gel and resolved by SDS-PAGE. Next, gels were stained with Coomassie Blue R250. A quantitative assessment of Gly m 5 fragments of different molecular weights was carried out by scanning densitometry of SDS-PAGE gels and results were expressed as the percentages of optical density for each band among the total integral optical density for the lane, according to the method of Mujoo, Trinh, and Ng (2003).

For the detection of IgE-binding fragments of Gly m 5, SDS-PAGE-resolved proteins were blotted to PVDF membranes (Millipore Inc, USA.) electrophoretically (Zheng et al., 2014). The PVDF membranes were blocked with 3% BSA in 0.05% PBS-Tween (PBST) for peptide fragment detection, or 5% non-fat dry milk in 0.1% Tween-20 (PBST) for BSA detection, then incubated with an appropriate dilution of pooled piglet sera for 18 h at 4 °C. After four washes with PBST, the membranes were incubated with biotinylated anti-pig IgE (RapidBio) and then horseradish peroxidase (HRP)-conjugated Streptavidin (Beijing Biosynthesis Biotechnology). After washes, the membranes were visualised by chemiluminescence (Super Enhanced Chemiluminescence Detection Kit, Appligen Technologies).

2.5. Separation of digestion-resistant fragments of Gly m 5

Preparative SDS-PAGE was performed as described previously (Saboori, Rose, & Yuhasz, 1999; Wang, Zhang, & Wang, 2005). Briefly, after gel electrophoresis, the digestion-resistant fragments in the gel were compared with the molecular weight standards. The gel section containing each peptide fragment was cut from the gel and the peptide fragment in the gel strip was eluted. After being dialysed and concentrated, the eluted peptide fragments were checked by SDS-PAGE to determine the purity. The purified peptide fragments were lyophilized and used for basophil activation assays.

2.6. Basophil activation assay

The histamine-releasing capability of the digestion-resistant fragments was measured essentially according to the method of Batanero, Crespo, and Monsalve (1999). Briefly, heparinized whole blood was diluted 1:7 in PIPES buffer (pH 7.35) and 100 μ l of diluted blood was incubated with 50 μ l of the purified peptide fragments (3 μ g/ml) at 37 °C for 45 min in 96-well microplates. The reaction was quenched by placing the plates on ice. After centrifugation, histamine in the cell-free supernatants was measured using a pig histamine ELISA kit (RapidBio Lab, Calabasas, California, USA). Spontaneous release was determined by incubation with a buffer control under the same condition. Total histamine release into the supernatant was determined after cell lysis in distilled water and freeze thawing of the blood cells. The basophil histamine release ratio was calculated by the following equation: histamine release ratio (%) = [(allergen-stimulated histamine release – spontaneous release) / (total histamine release – spontaneous release)] $\times$ 100%.

2.7. Amino-terminal sequencing of digestion-resistant peptides

Aliquots of the products of Gly m 5 digestion were electrophoresed on 12% SDS-PAGE gels and blotted onto a PVDF membrane (Immobilon PVDF, Millipore Inc., USA) in CAPS transfer buffer (pH 11.0). The protein bands were excised and sequenced by Automated Edman sequencing using an Applied Biosystems Protein Sequencer Procise 492 cLC (Zheng et al., 2012).

2.8. Molecular model of Gly m 5 homotrimer and the location of the protease cleavage sites

A molecular model of Gly m 5 α' -homotrimer published previously, representing aa 125–517, was utilized to locate protease cleavage sites on three-dimensional structure of Gly m 5 (Maruyama, Maruyama, Mikami, & Utsumi, 2004). The protease cleavage sites were labeled using the free Cn3D macromolecular structure viewing program from the NCBI databases (<https://www.ncbi.nlm.nih.gov/Structure/pdb/1UIK>).

2.9. Statistical analysis

Differences in histamine release rates between two groups and amongst digestion-resistant fragments were analysed by using Student's *t* test and the analysis of variance (ANOVA) procedure of SAS system, respectively. *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. The digestion-resistant fragments of native Gly m 5

The native Gly m 5 was exposed to three major proteolytic enzymes (20 mg/ml pepsin, 10 mg/ml trypsin and 10 mg/ml chymotrypsin) successively for different lengths of time. The concentrations of proteolytic enzymes were such that the tested nonallergenic proteins (BSA) were degraded to small fragments (<10 kDa) in a short period of time (<5 min, data not shown). The products of Gly m 5 digestion were then electrophoresed on SDS-PAGE gels. Peptide fragments ranging in size from 10 kDa to 52 kDa were observed on Coomassie-stained gels as shown in Fig. 1. The contents of Gly m 5 fragments were determined by densitometry and the results were summarized in Table 1. Among these peptide fragments, three digestion-resistant fragments, of molecular weight $\sim$ 25 kDa, $\sim$ 30 kDa, and $\sim$ 52 kDa, were dominant

except for the two dispersed bands of $\sim$ 10 kDa and $\sim$ 15 kDa fragments, suggesting that both of them were peptide mixtures with similar molecular weight.

3.2. The IgE-binding properties of the major digestion-resistant fragments

Immunoblotting analysis was performed to evaluate the IgE-binding properties of the long-lived digestion-resistant fragments. A pool of serum from six piglets in exposed group was utilized. For control purposes, a pool of serum from six piglets in control group was also included. The result was shown in Fig. 2. Three major digestion-resistant fragments, of molecular weight $\sim$ 25 kDa, $\sim$ 30 kDa and $\sim$ 52 kDa, revealed strong IgE-binding capacities to the pooled serum from six piglets in exposed group who were allergic to Gly m 5. However, no allergen-specific IgE binding was detected using a pool of serum from six piglets in control group (data not shown). This observation indicated three major digestion-resistant fragments of Gly m 5 contained intact IgE-binding epitopes and perhaps survived the degradation in the gastrointestinal tract.

3.3. Basophil histamine release response of the major digestion-resistant fragments

Three digestion-resistant fragments of Gly m 5 were purified to apparent homogeneity by preparative SDS-PAGE. Histamine release rates from whole blood induced by the three digestion-resistant fragments were significantly higher in the piglets in the exposed group relative to the control group (*P* = 0.008, 0.009 and 0.005 for 52 kDa peptide fragment, 30 kDa peptide fragment and 25 kDa peptide fragment, respectively) (Table 2). The results indicated that the digestion-resistant fragments contained multiple IgE-binding epitopes and could activate a blood effector cell response.

3.4. The protease cleavage sites of allergen Gly m 5 that tended to be easily cut off

The amino-terminal sequencings of the digestion-resistant fragment indicated that the 25 kDa peptide fragment began at HKNKN, and the 30 kDa peptide fragment began at TISSE (or KTISS), and the 52 kDa peptide fragment began at HKNKN. Because the soybean cultivar used in this experiment was composed predominately of α - and α' -subunit (data not shown), the protease cleavage sites were located on the α - and α' -subunit of Gly m 5. The results were shown in Fig. 3. There were two major protease cleavage sites on allergen Gly m 5 which tended to be easily cut off. One of protease cleavage sites was located at R125–H126 for α' -subunit or at R189–H190 for α -subunit, and the other was located at K333–T334 (R332–K333) for α' -subunit or at K397–T398 (R396–K397) for α -subunit. The cleavage site of R–H was located in the region between N-terminus and N-terminal strand Z. The cleavage sites of K–T (R–K), however, were located on a relatively loose helix between the tight helix 6 and C-terminal strand Z. These two cleavage sites may represent the fragile sites on allergen Gly m 5 and tend to be cut off during *in vivo* digestion. The 25 kDa digestion-resistant fragment, therefore, appeared to correspond to the region between the two cleavage sites, and the 30 kDa digestion-resistant fragment that contained the carbohydrate moieties corresponded to the region between the cleavage site of K–T (R–K) and the C-terminus. The 52 kDa fragment, however, corresponded to the region between the cleavage site of R–H and the C-terminus.

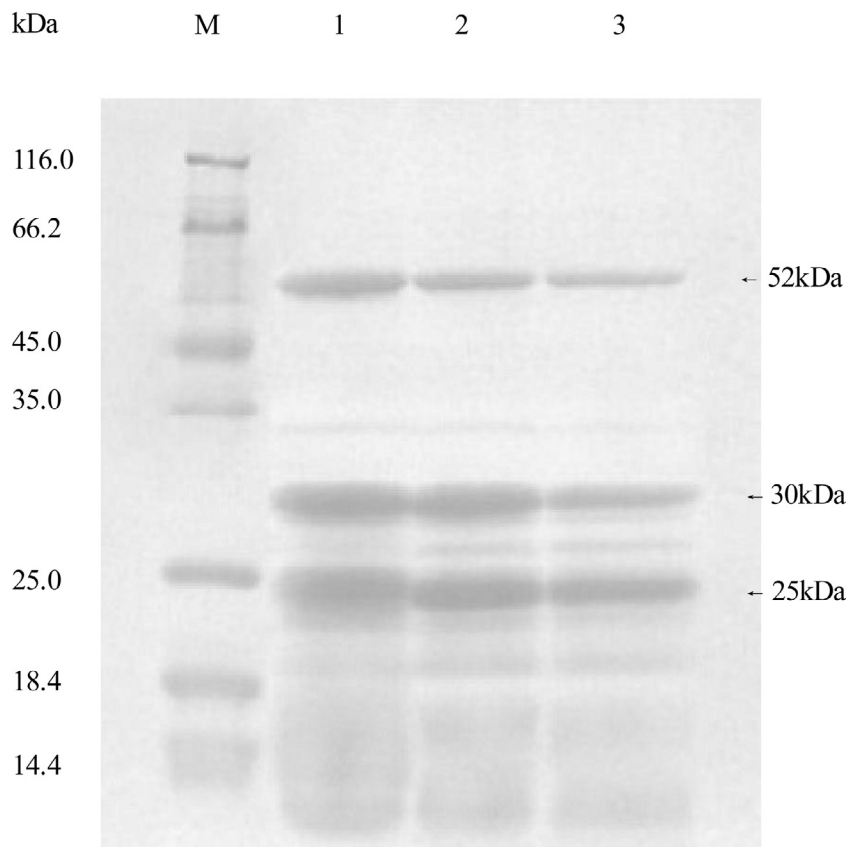


Fig. 1. SDS-PAGE of the peptide fragments of native Gly m 5 digested with major proteolytic enzymes. (M) Molecular weight standards. (1) Native Gly m 5 was digested with pepsin for 60 min, then trypsin and chymotrypsin for 5 min, respectively. (2) Native Gly m 5 was digested with pepsin for 60 min, then trypsin and chymotrypsin for 60 min, respectively. (3) Native Gly m 5 was digested with pepsin for 60 min, then trypsin for 90 min and chymotrypsin for 90 min, respectively. The arrowheads showed the presence of 52 kDa, 30 kDa, and 25 kDa digestion-resistant fragments, which were dominant, when native Gly m 5 was subjected to *in vitro* digestion with major proteolytic enzymes.

Table 1

The contents of peptide fragments derived from *in vitro* digestion of native Gly m 5 (%).

	Digestion-resistant fragments							
	52 kDa peptide	34 kDa peptide	30 kDa peptide	27 kDa peptide	25 kDa peptide	19 kDa peptide	15 kDa peptide (dispersed)	10 kDa peptide (dispersed)
The fragments of native Gly m 5 digested with pepsin (60 min), trypsin (5 min) and chymotrypsin (5 min)	8.6 ± 1.0	2.2 ± 0.5	16.8 ± 0.5	2.5 ± 0.5	26.1 ± 0.8	9.2 ± 0.4	13.9 ± 0.2	20.7 ± 1.9
The fragments of native Gly m 5 digested with pepsin (60 min), trypsin (60 min) and chymotrypsin (60 min)	8.2 ± 1.2	2.5 ± 0.7	13.6 ± 0.5	2.7 ± 0.4	27.2 ± 1.4	9.1 ± 0.4	17.9 ± 0.3	18.8 ± 2.0
The fragments of native Gly m 5 digested with pepsin (60 min), trypsin (90 min) and chymotrypsin (90 min)	8.1 ± 1.2	3.1 ± 0.6	12.2 ± 2.4	3.0 ± 1.1	22.0 ± 2.7	8.0 ± 0.6	21.4 ± 4.2	22.2 ± 2.2

Note: Data in the table were mean ± SD of 3 samples.

The contents of digestion-resistant fragments were estimated as the percentages of optical density for each band among the total integral optical density for the lane in gels by optical densitometry, as described by Mujoo et al. (2003).

3.5. Location of the cleavage sites and digestion-resistant fragments on the three-dimensional structure of allergen Gly m 5

To locate the protease cleavage sites on three-dimensional structure of Gly m 5, a molecular model of Gly m 5 α' -homotrimer (Maruyama et al., 2004) was used. Because the α - and α' -subunit shares 90.4% amino acid sequence identity, the three-dimensional structure of α -subunit was very similar to that of α' -subunit and was not shown. The molecular models of the Gly m 5 α' monotrimer were presented in Fig. 4. The three-dimensional structure of the molecule consisted of three monomers. Each monomer contained two compact modules. Each mod-

ule is subdivided into a core domain and a loop domain. The α helical bundles located on the ends of each monomer overlap to form the stable Gly m 5 trimer. The two cleavage sites were mapped on the opposite surfaces of the protein and predicted to be exposed to solvent and easily to be hydrolysed by proteolytic enzymes. The two major cleavage sites were in the regions near to the connection between two modules. The 25 kDa peptide fragment corresponded to the main body of N-terminal module of the monomer, and the 30 kDa peptide fragment containing the carbohydrate moieties corresponded to the main body of C-terminal module. The 52 kDa peptide fragment, however, corresponded to the main body of the monomer of Gly m 5. The results suggested

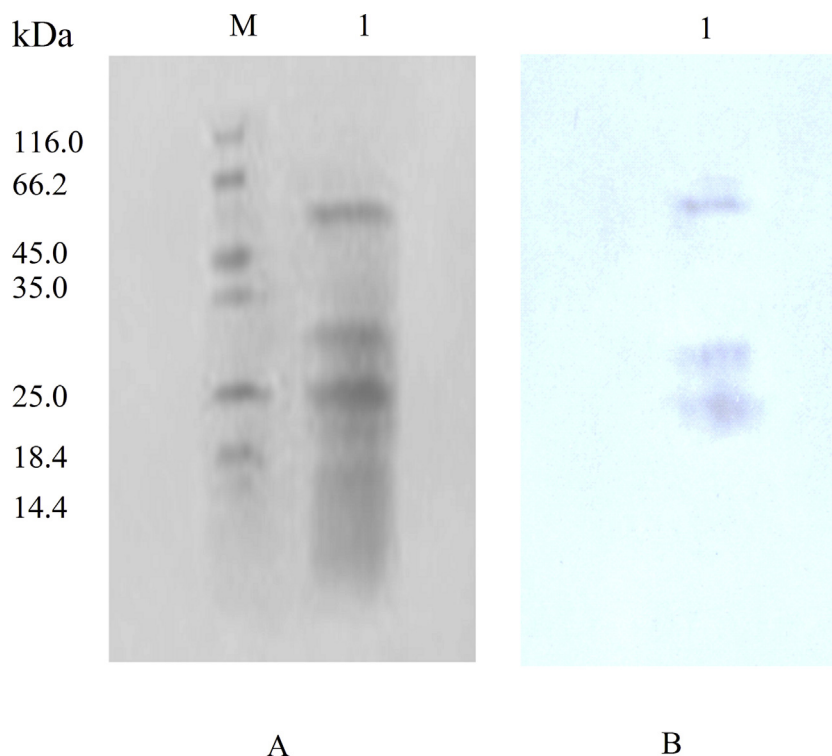


Fig. 2. IgE-immunoblot analysis of digestion-resistant fragments of allergen Gly m 5. Native Gly m 5 was subjected to *in vitro* digestion with major proteolytic enzymes and fractionated by SDS-PAGE on a 12% gel and stained with Coomassie Blue R250 (A). Proteins shown in panel A were transferred to a PVDF membrane and probed with a pooled serum from Gly m 5 sensitised piglets, and IgE-binding proteins were detected by biotinylated anti-pig IgE and then HRP-conjugated Streptavidin. The membrane was visualised by chemiluminescence (B).

Table 2

Basophil histamine release responses of Gly m 5 sensitised and control piglets to digestion-resistant fragments¹ (%).

Groups	Digestion-resistant fragments			SEM ²	P value
	52 kDa fragment	30 kDa fragment	25 kDa fragment		
Gly m 5 sensitised group	38.6	28.2	25.8	2.86	0.63
Controls	11.9	10.2	10.9	0.65	0.19
P value	0.008	0.009	0.005	–	–

¹Data are presented as the means of histamine release ratio of six piglets per group. Histamine release ratio (%) = [(allergen-stimulated histamine release – spontaneous release)/(total histamine release – spontaneous release)] × 100%.

²Standard error of the means.

that the compact β barrel structures of core domains and the formation of a trimeric complex between loop domains of monomers may afford the module some protection from degradation.

4. Discussion

Dietary allergens have some biochemical characteristics that seem to be shared by most food allergens. One of the prominent characteristics is that they are rather stable proteins resistant to proteolytic enzymes and denaturation. The longer peptide fragments of an allergen protein survive in the digestive tract, the more possible it is to trigger an immune response (Stanley & Bannon, 1999). So resistance to digestion is considered to be the most critical property that makes a protein to be a food allergen. It has been demonstrated that many food allergens remain intact for longer periods of time in simulated gastric fluid when compared with non-allergic food proteins (Astwood et al., 1996). A digestion model using pepsin was adapted to assess the allergenic potential of food proteins (Astwood et al., 1996). Resistance to pepsin digestion became an accepted method for evaluating the allergenicity of a novel protein and was used as criterion for distinguishing dietary

allergens from non-allergenic food proteins (Aalberse, 2000; Konig, Cockburn, & Crevel, 2004). Although many allergens could not remain intact after digestion with the proteolytic enzymes, several high molecular weight polypeptides persisted. These relatively large fragments containing IgE-binding epitopes retained an allergenic potential and could induce allergenic response, which was another key feature of food allergens (Eiwegger, Rigby, & Mondoulet, 2006). In this study, after being digested by the major proteolytic enzymes, native Gly m 5 was hydrolysed into a number of peptide fragments that were resistant to further enzymatic digestion. Among them, three major fragments were observed whose molecular weights (MW) were ~25 kDa, ~30 kDa, and ~52 kDa, respectively. Our data suggested that allergen Gly m 5 was more resistant to digestion than other protein, and the fragments of the allergen were most likely to survive the longest in gastrointestinal tract. These impart an increased probability that relatively large fragments reach the intestinal mucosa, where absorption can occur, and induce allergenic response.

Whether a peptide fragment can bind IgE antibody and induce an allergic reaction is related to the molecule mass of the peptide fragment. One of the findings indicated that the minimal molecular

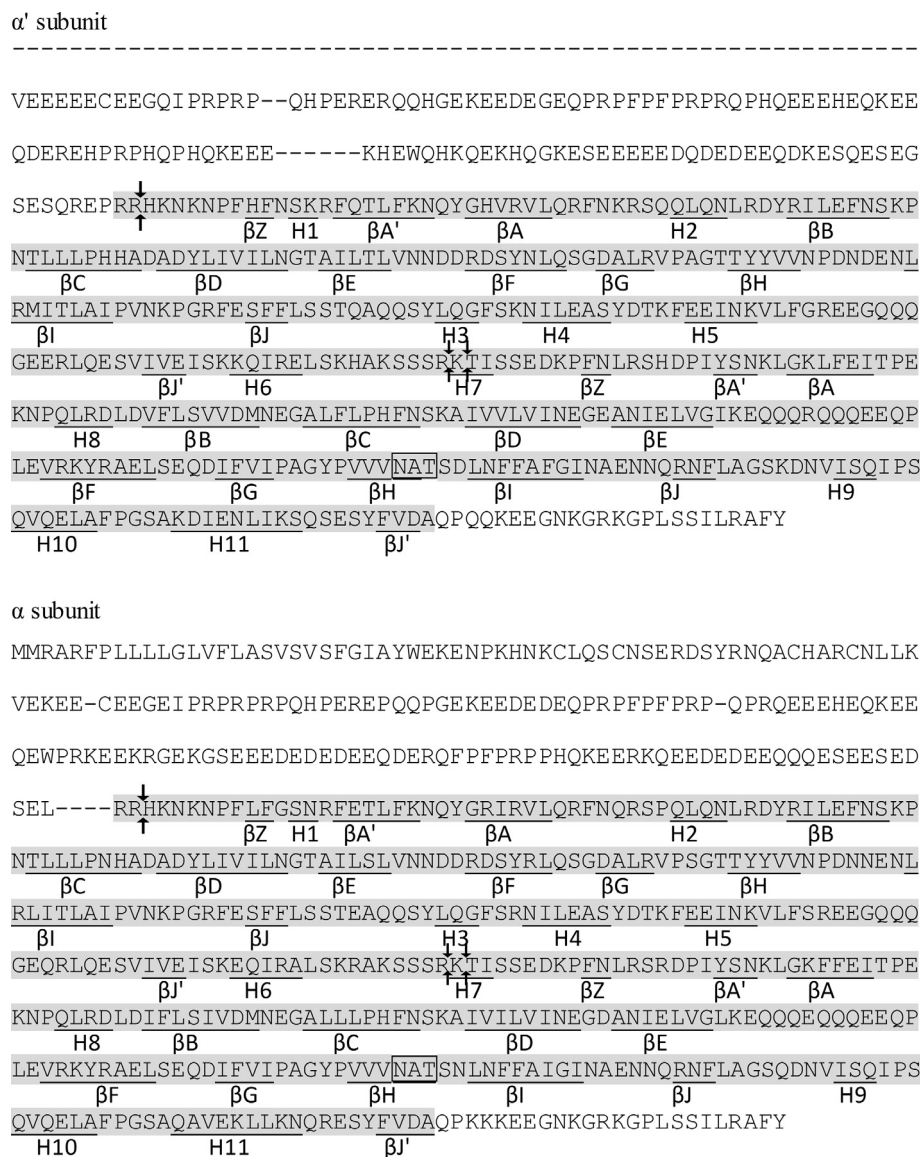


Fig. 3. Multiple protease cleavage sites and digestion-resistant fragments located on the α' - and α -subunit of Gly m 5. Amino acid sequences of α' - and α -subunit of Gly m 5 were obtained from Swiss-Prot protein knowledgebase (accession number P13916, P11827) and presented as the one-letter amino acid code. Dashes (-) were introduced for maximal alignment of the sequences. Shadows indicated the core regions of Gly m 5. The box showed the glycosylation site. Arrows indicated protease cleavage sites. H, helix; β , β strand.

weight for IgE binding seemed to be situated between about 970 and 1400 Da, and peptide fragments of >1400 Da were demanded for skin reaction (Van Hoeyveld, Escalona-Monge, de Swert, & Stevens, 1998). In food allergy, degranulation of mast cells, or basophilic cells, requires the binding of at least two epitopes to two adjacent membrane bound IgE on the surface of effector cells. This indicates that the peptide fragment, including at least two epitopes, can elicit an allergic reaction (Van Bostel, Koppelman, van den Broek, & Gruppen, 2008). Because each of the epitopes is a minimum of about 15 amino acid residues long, this implies that a lower size limit for peptide fragment which can induce an allergic response is about 30 amino acid residues (molecular mass of about 3000 Da) (Huby, Dearman, & Kimber, 2000). It was demonstrated that most allergens or their digestion-resistant fragments had more than two IgE epitopes and thereby these allergens had the ability to induce an allergic response in susceptible individual (Rabjohn, Helm, & Stanley, 1999; Shin, Compadre, & Maleki, 1998).

The physiological and immunological similarity between pigs and human beings makes the pig an important animal model for biomedical study (Huang, Xu, Yu, Gao, & Liu, 2010; Rupa, Hamilton, Cirinna, & Wilkie, 2008). Young pig has similar hypersensitivity reactions that are more closely aligned with human response (Helm et al., 2002), and sensitization/tolerance of piglets to soybean proteins that are similar to what is seen in young children (Wilson, Stokes, & Bourne, 1989). Some problems regarding allergen structure, food-induced allergic sensitization and serum IgE level can be readily investigated in the pig model (Helm et al., 2002). The pig food allergy model is also useful for studying the induction and mechanism of IgE and IgG response in young children and adults (Sun, Li, Li, Dong, & Wang, 2008). In this study, a piglet model of Gly m 5 allergy that was developed by systemic oral sensitization with Gly m5 was used. For detection of IgE-binding fragments of native Gly m 5, immunoblotting analysis was conducted using a pooled serum from Gly m5 sensitised piglets. The results suggested the major digestion-resistant fragments,

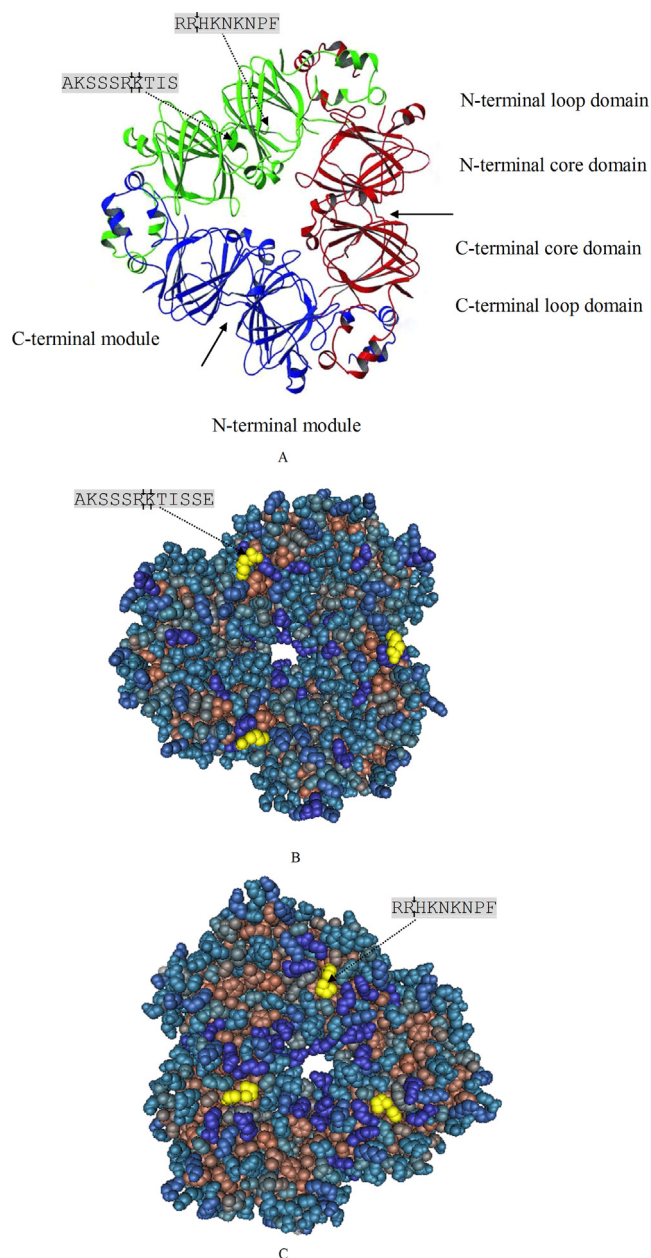


Fig. 4. Mapping of protease cleavage sites on the three-dimensional structure of Gly m 5 α' -homotrimer, representing aa 125–517, by using the free Cn3D macromolecular structure viewing program from the NCBI databases. (A) The ribbon diagram indicated the secondary structure of Gly m 5 α' monotrimer. α -Helices were shown as coils, and β -Strands were shown as arrows. (B) and (C) Space-filled model of Gly m 5 α' monotrimer with the protease cleavage sites colored in yellow. The atoms of the hydrophobic amino acids were shown in brown, and the remaining atoms of the hydrophilic amino acids were shown in blue, indigo, and yellow. The views in (A) and (B) were depicted as threefold symmetry axis ran perpendicular to the paper and the depiction in (C) was the opposite face of the view depicted in (A) and (B).

the 25, 30, and 52 kDa fragments, were recognized by IgE antibodies. To further investigate the biological potency of the digestion-resistant fragments, basophil activation assay was also performed. Our results showed that three digestion-resistant fragments could induce *in vitro* basophil histamine release, which suggested the digestion-resistant fragments contained multiple IgE-binding epitopes and had the ability to activate blood effector cell response.

Allergen Gly m 5 is a trimeric protein analogous to that of other the vicilin proteins (Shewry, Napier, & Tatham, 1995). The three-

dimensional structure of Gly m 5 monomer contains two modules, the amino-terminal and carboxyl-terminal module. Each module contains two domains, a core domain consisting mainly of β -barrels and a loop domain containing α -helical bundle (Maruyama et al., 2001, 2004). In the native form, allergen Gly m 5 forms a stable trimer consisting of three monomers, held together by inter-twining α -helices at the end of each monomer. The hydrophobic interactions are primarily responsible for Gly m 5 trimer formation in addition to hydrogen bonds (Maruyama et al., 2001, 2004). This compact structure may contribute to the stability of trimer and protect the area of monomer–monomer contact from degradation just like another vicilin allergen Ara h1 (Maleki, Kopper, & Shin, 2000). The β -barrel in the amino-terminal or carboxyl-terminal module is a large β -sheet that coils and twists to form a compact structure. The strands in the β -barrels, being arranged in an anti-parallel manner, consist of both hydrophobic and polar amino acid residues, and the hydrophobic residues are oriented toward the inside of the barrel to form a hydrophobic core, the polar residues, however, are oriented toward the exterior of the barrel on the water-exposed surface. These compact structures may prevent these areas from being degraded by proteolytic enzymes. In this study, the soybean cultivar which contained allergen Gly m 5 composed mainly of α - and α' -subunit was utilized. Although α - and α' -monomer had lots of pepsin, trypsin and chymotrypsin cleavage sites throughout the whole molecule, including the monomer–monomer contact area and the β -barrels of amino-terminal or carboxyl-terminal module, two major cleavage sites were observed only in the regions near to the connection between two modules of the α - and α' -monomer when it was subjected to *in vitro* digestion. Some studies indicated that many food allergens were proteins possessing compact structures that were significant to their allergenicity and protected IgE-binding epitopes from degradation, which had led to the hypothesis that protein structure played an important role in an allergen stability to resist degradation (Maleki et al., 2000). In our study, we had shown that native Gly m 5 had compact structure in monomer and could form a stable trimer complex. The structural characteristics of Gly m 5 afforded the molecule some protection from complete degradation into small peptides and amino acids and permitted passage of peptide fragments containing intact IgE-binding epitopes through the gastrointestinal mucosa, and contributed to its overall allergenicity. Our results provided the evidence that three-dimensional structure played an important role in the allergenicity of soybean allergens.

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