Immunoglobulin E and immunoglobulin G cross-reactive allergens and epitopes between cow milk α_{S1} -casein and soybean proteins

YanJun Cong,^{1*} Ye Li,¹ and Linfeng Li²

¹Beijing Advanced Innovation Center for Food Nutrition and Human Health, College of Food and Health, Beijing Technology and Business University, Beijing 100048, P. R. China

²Department of Dermatology, Beijing Friendship Hospital, Beijing 100050, P. R. China

ABSTRACT

Some infants allergic to cow milk-based formula are also sensitive to soybean-based formula. This paper aimed to explore the association of IgE and IgG cross-reactivity between α_{S1} -casein in cow milk (CM) and soybean proteins. The IgE and IgG cross-reactive allergens and epitopes were identified using sera from infants allergic to CM or mice monoclonal antibodies. The AA sequence alignment was performed using bioinformatics software. Finally, the digestion and heating stability of the cross-reactive allergen were explored by sodium dodecyl sulfate (SDS)-PAGE and Western blotting. The results showed that the IgE and IgG cross-reactive allergen was α subunit of β -conglycinin named Gly m Bd 60K. The IgE and IgG epitopes were the sequences at AA 319–341 and AA 164–182. No intact Gly m Bd 60K allergen could be observed after 2 min in simulated gastric fluid by SDS-PAGE. Heating did not change IgE and IgG cross-reactivity by Western blotting. Therefore, the existence of cross-reactivity between CM α_{S1} -casein and soybean proteins possibly contributes to the frequently observed cosensitization for these allergens in cow milk-allergic patients. The same IgE- and IgG-binding epitopes of cross-reactive allergens may provide important information for elucidation of the association between IgG and IgE antibody generation.

Key words: α_{S1} -casein, soybean protein, cross-reactivity, epitope

INTRODUCTION

Patients who suffer from specific food allergies are often also allergic to other foods. For example, patients allergic to cow milk (CM) may be allergic to other milk

and patients allergic to peanuts may be allergic to other legumes. Significant associations were observed both in related and nonrelated allergens. However, associations were more frequent and significant among related than nonrelated foods; this represents a true allergy to the related food, defined as cross-reactivity (Kazatsky and Wood, 2016; Bexley et al., 2017; Eischeid and Stadig, 2018). The universality and complexity of food cross-reactive allergies increased not only the frequency of food allergies, but also the difficulties of establishing an accurate and efficient detection method for quantitative allergens and developing hypoallergenic foods. It is essential to study the mechanism of food cross-reactivity for allergen detection, diagnosis, and design of an allergen-desensitization vaccine.

Cow milk-based infant formula is an important substitute when exclusive breast-feeding is not possible during the first year of life. However, CM contains more than 30 types of proteins, all of which can potentially induce allergic reactions. As a milk substitute, soy-based formulas have been widely used in children intolerant to CM protein-based formulas (Bhatia and Greer, 2008). However, some infants with CM allergies develop a clinical intolerance to soy protein-based formulas during the restrictive treatment, which may be explained by cross-reactivity (Klemola et al., 2002). As a result, interests have been focused on identification of cross-reactive allergens between CM and soybean. Curciarello et al. (2014) found that Gly m 5 was a cross-reactive soy allergen and contained IgG and IgE immunodominant epitopes lying in the α -subunit fragment. In addition, soy P34 protein shared the similar AA sequence with CM casein (Candrea et al., 2015). Candrea et al. (2016) showed that P28 was recognized by IgE sera from CM-allergic patients and activated the degranulation of human peripheral basophils as well as splenocytes from sensitized animal-secreted interleukin-13 when treated with P28.

A major allergen in CM is α_{S1} -casein. We identified the B-cell epitopes and critical AA of α_{S1} -casein in previous studies (Cong et al., 2013). In this study, we

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*Corresponding author: congyj@th.btbu.edu.cn

aimed to explore the association of IgE and IgG cross-reactivity between CM α_{S1} -casein and soybean proteins by IgE sera from infants allergic to CM, IgG from α_{S1} -casein monoclonal antibodies, and bioinformatics software. In addition, the digestion and heating stability of cross-reactive allergens were explored by SDS-PAGE and Western blotting.

MATERIALS AND METHODS

Sera from Infants with CM Allergy

Eight sera from 45 clinically examined infants with cow milk allergies were selected by immunoblotting and ELISA. They were sensitive to α_{S1} -casein, but not allergic to soybean proteins. The median age of these 8 infants was 1 yr, ranging from 6 mo to 3 yr. The CM α_{S1} -casein-specific IgE antibodies ranged from 25 to 100 kU/L, measured by the ELISA assay. The clinical symptoms of these 8 infants were all atopic dermatitis. Additionally, sera from 5 children with no milk allergies (aged 3–6 yr) were used as negative controls. Informed consent was obtained from the patients and their parents, and the study was approved by Peking Friendship Hospital (Beijing, P. R. China).

Preparation of Anti- α_{S1} -Casein Monoclonal Antibodies

Anti- α_{S1} -casein monoclonal antibodies were prepared according to the methods of Cao et al. (2017) and Restani et al. (1999) with some modifications. Briefly, Balb/c mice were injected intraperitoneally with 50 mg of total α_{S1} -casein (purity $\geq 85\%$; purification of α_{S1} -casein from raw CM in our laboratory according to the method of Hodgkinson et al., 2012) solution mixed with Freund's adjuvant. The spleen cells were hybridized with X63-Ag8 myeloma cells in the presence of 50% (w:w) polyethylene glycol, and then the fusion mixture was diluted with aminopterin, hypoxanthine, and thymidine medium. The cloned cells ($5\text{--}19 \times 10^5$ cells) were injected in Balb/c mice 1 wk after pretreatment with 0.5 mL of pristane. The monoclonal antibodies harvested from the peritoneal fluid were purified for this study. The subtypes of monoclonal antibodies were determined by an ELISA kit. The titers of monoclonal antibodies were detected with indirect ELISA.

IgE and IgG Cross-Reactivity Between CM α_{S1} -Casein and Soybean Proteins

The cross-reactive allergens were identified using Western blotting (Restani et al., 1999; Cong et al., 2007, 2008). The SDS-PAGE was first performed as

previously described (Curciarello et al., 2008). Raw CM (Sanyuan Food Co. Ltd., Beijing, China), soy proteins (Beijing Solarbio Science and Technology Co. Ltd., Beijing, China), α -casein (Sigma-Aldrich, St Louis, MO), and α_{S1} -casein were used. The proteins were first transferred to a polyvinylidene fluoride (PVDF) membrane (pore size 0.45 μm ; Sartorius, Göttingen, Germany) by blotting for 4.5 h in 25 mM Tris buffer containing 20% methanol and 192 mM glycine (pH 8.3) after separation by SDS-PAGE. The PVDF membrane was then cut into halves, 1 for cyanamid (0.2% in acetic acid) staining, and 1 for Western blotting. The membrane was washed with wash buffer (10 mM Tris, containing 0.05% (w:v) Tween 20 and 0.8% NaCl; pH 7.4) thoroughly 4 times at 15 min per wash. The membrane was then blocked for 1 h with 1% BSA (Sigma-Aldrich) in wash buffer. Membrane was then immersed in a pool of serum from allergic children or monoclonal antibodies diluted (1:10 or 1:3,500) in a solution containing Tris-NaCl (10 mM Tris-HCl and 500 mM NaCl; pH 7.5) and 1% BSA for 2 h at 37°C. A pool of serum was composed of 8 individual serum of equal volume. Antigen-IgE complex was detected at 37°C using goat-anti-human IgE antibodies (Sigma-Aldrich), and antigen-IgG complex was detected using goat-anti-mouse IgG antibodies (Sigma-Aldrich). Both were labeled with horseradish peroxidase and subsequently stained for peroxidase activity with 4-chloro-1-naphthol. The blots were scanned with a gel image analysis system (Gel-Pro, Media Cybernetics, Rockville, MD; He et al., 2016).

To exclude nonspecific IgE binding to soy proteins, an immunoinhibition test was carried out. We diluted 300 μL of negative control sera with 1% BSA in wash buffer, and preincubated them with 25 μg of soybean proteins. Afterward, immunoblotting was performed as described above.

Amino Acid Sequence Alignment Between CM α_{S1} -Casein and Soybean Gly m Bd 60K Protein

First, the gene sequences of α_{S1} -casein and Gly m Bd 60K protein were retrieved by Gen Bank (<https://www.ncbi.nlm.nih.gov/genbank/>), then the AA sequences of α_{S1} -casein and Gly m Bd 60K protein were derived by DNASTar software (version 8.0; <https://www.dnastar.com/>). Amino acid sequence alignment was carried out by Blastp software at NCBI web (Li et al., 2017; Foroutan et al., 2018).

Synthesis and Purification of Predictive Epitopes

Based on the results predicted by the bioinformatics method, individual AA sequence was synthesized using the multiple method according to the Fmoc (9

fluorenylmethoxy carbonyl) strategy (Ruiter et al., 2006). Briefly, the peptides were synthesized using an Fmoc-AA-Wang-resin containing free hydroxyl groups. All AA residues were blocked by acetylation and then Fmoc protective groups were removed by addition of piperidine to render nascent peptides reactive. Each additional Fmoc-AA was esterified to the previous one by this procedure. The last AA side chains de-protected and the resin was cleaved using a mixture of 1:1:0.05 (vol:vol:vol) trifluoroacetic acid:dichloromethane:triisobutylsilane, followed by washing with methanol and dichloromethane. Cleavage reagents were removed in vacuo after filtration, and the crude peptides were precipitated with ice-cold tert-butylmethyl ether.

The crude peptides were purified by C₁₈ reverse-phase HPLC (Macherey-Nagel GmbH and Co. KG, Düren, Germany) at a flow rate of 12.0 mL/min and a gradient of 1% acetonitrile per minute. Acetonitrile in water with 0.1% trifluoroacetic acid was used as the eluting solvent and 50 mg/mL synthetic peptides were loaded.

Identification of IgE and IgG Epitopes

The ELISA was performed as described by Cocco et al. (2003). Before immunological detection, the purified peptides were coupled with BSA. Then, the peptides that reacted with BSA were added to 96-well plates. Peptides were incubated with the serum pool from patients with a CM allergy or monoclonal antibodies diluted (1:10 or 1:3,500) in a solution containing Tris-NaCl (10 mM Tris-HCl and 500 mM NaCl; pH 7.5) and 1% BSA for 2 h at 37°C. The second antibody was detected using monoclonal mouse-anti-human IgE (1:3,000; Pharmacia and Upjohn Diagnostics, Uppsala, Sweden) or monoclonal goat-anti-mouse IgG (1:2000; Pharmacia and Upjohn Diagnostics, Uppsala, Sweden) conjugated to horseradish peroxidase, subsequently stained for peroxidase activity with 4-chloro-1-naphthol staining, and then expressed as the optical density at a wavelength of 450 nm. Between each step, plates were washed 5 times with tris-buffered saline containing 0.1% Tween 20. Nonspecific binding was studied using sera from nonallergic individuals or mice considered as negative controls.

Gly m Bd 60K Digestion in Simulated Gastric Fluid

Simulated gastric fluid (SGF) digestion of Gly m Bd 60K allergen was performed using the methods described by Minekus et al. (2014) and Faisal et al. (2019) with slight modifications. Briefly, 5 g of Gly m Bd 60K was blended with 5 mL of simulated salivary fluid electrolyte stock solution for 2 min. The protein

slurry was further mixed with 7.5 mL of SGF, 1.25 mL of pepsin (10,000 U mL⁻¹, Sigma-Aldrich) stock solution and 5 µL of CaCl₂ (0.3 M). The pH of the mixture was adjusted to 3.0 and digested at 37°C in a rotary shaker (Mixel Co. Ltd., Beijing, China) at 100 horizontal strokes per minute for 2.5 h. The Na₂CO₃ (0.2 M) was used to inactivate the enzymes after complete digestion. Ovalbumin (OVA; Sigma-Aldrich) and soybean trypsin inhibitor (Sigma-Aldrich), which were recognized as allergens, were used as positive controls, and potato acid phosphatase (PAP; Sigma-Aldrich) was used as a negative control.

Effect of Heating Processing on Gly m Bd 60K

The cross-reactive allergen of Gly m Bd 60K was separated and purified from soybean proteins (Liu et al., 2007). Then, Gly m Bd 60K proteins were heated for 10, 20, 30, 40, 50, and 60 min at 70°C or 100°C. The α_{S1}-casein monoclonal antibodies and patients' sera were used to evaluate the cross-reactivity of allergens by Western blotting.

Statistical Analysis

Statistical analysis was performed using SPSS software (version 17.0; SPSS Inc., Chicago, IL). All determinations were carried out in triplicate. Significant differences between data were assessed after ANOVA using the Kruskal–Wallis test at a confidence level of 95%.

RESULTS

IgE Cross-Reactivity to Soy Proteins by Sera from CM-Allergic Infants

As shown in Figure 1A, there were several bands in lane 3; the first 3 bands are α' (~67–72 kDa), α (~63 kDa), and β subunits (~47 kDa) of β-conglycinin. Glycinin is composed of 2 subunits, the acidic subunit A (~29–33 kDa) and the basic subunit B (~22 kDa). Lane 4 corresponded with CM proteins based on the molecular weight standards; the protein components were identified as lactoferrin, serum albumin, immunoglobulin heavy chain, α-casein, β-casein, κ-casein, β-lactoglobulin, and α-lactalbumin. Then, the proteins were transferred to PVDF membranes and reacted with IgE from CM-allergic infants' pooled sera. As shown in Figure 1B, 1 protein band was visible in lane 1, 2, or 4, and was identified as α_{S1}-casein by molecular weight. Two proteins were showed in lane 3 in Figure 1B and were characterized as Gly m Bd 60K and 11S basic sub-

Figure 1. Identification of cross-reactive allergens between α_{S1} -casein and soybean proteins with IgE from cow milk (CM) patients' sera. Lane 1 corresponds with α_{S1} -casein, 2 corresponds with α -casein, 3 with corresponds soybean proteins, 4 corresponds with cow milk proteins, and M corresponds with protein marker at 97.4, 66.2, 43.0, 31.0, 20.1, and 14.4 kDa. (A) corresponds with the SDS-PAGE pattern, (B) corresponds with the Western blotting results identified with IgE from CM patients' sera, and (C) corresponds with Western blotting results identified with sera from children with no milk allergies.

unit by molecular weight. To exclude a false-positive reaction, the proteins transferred to PVDF membrane were also reacted with negative pooled serum from children with no milk allergies. The results were shown in Figure 1C where no proteins were identified in lanes 1, 2, or 4; however, the 11S basic subunit was observed to react with negative sera in lane 3. This result indicated that the band of 60 kDa corresponded to the only cross-reactive allergen. Thus, the IgE cross-reactive allergen was Gly m Bd 60K between α_{S1} -casein and soybean proteins, which was α subunit of β -conglycinin.

IgG Cross-Reactivity to Soy Proteins by Monoclonal Antibodies Against α_{S1} -Casein

The subtype of α_{S1} -casein monoclonal antibody was IgG₁ identified by ELISA, and the titer value of monoclonal antibody was 1:320,000. After electrophoresis by SDS-PAGE, the proteins were transferred to PVDF membranes, then cross-reactive allergens were identified by Western blotting with IgG from monoclonal antibodies. As shown in Figure 2A, α_{S1} -casein and α -casein were both identified by IgG in lanes 1 and 2. Two bands were observed in lane 3, and by molecular weight, these 2 bands were α subunit of β -conglycinin (Gly m Bd 60K) and 11S basic subunit, respectively. To exclude nonspecific reactions, negative control sera from mice were used to carry out the Western blot inhibition test. The results were shown in Figure 2B where there was no reaction between negative sera and α_{S1} -casein or α -casein in lanes 1 or 2; however, 1 band was

observed to react with negative sera in lane 3, which was 11S basic subunit based on its molecular weight of 21 kDa. Therefore, there was a specific immunoreaction between monoclonal antibody and Gly m Bd 60K, and the IgG cross-reactive allergen was successfully identified.

Figure 2. Identification of cross-reactive allergens between α_{S1} -casein and soybean proteins with IgG from α_{S1} -casein monoclonal antibodies. Lane 1 corresponds with α_{S1} -casein, 2 corresponds with α -casein, and 3 corresponds with soybean proteins. (A) corresponds with Western blotting results identified with monoclonal antibodies, and (B) corresponds with Western blotting results identified with mice negative control sera.

Table 1. Similar AA sequences between α_{S1} -casein and Gly m Bd 60K predicted by Blastp software (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>)

Allergen	Similar AA sequences		Descriptions				
	Group I	Group II	Max score	Total score	Query cover	E-value	Percent identities
α_{S1} -Casein Gly m Bd 60K	K ₄₉ EKVNELSKDIGSESTEDQAMED ₇₁ K ₃₁₉ EQIRALSKRAKSSSRKTISED ₃₄₁	Q ₁₁₂ LLRLKKYKVPQLEIVPNS ₁₃₀ Q ₁₆₄ LQNLRDYRILEFNSKPNT ₁₈₂	17.3	33.9	19%	0.5	39%

Amino Acid Sequence Alignment Between Cow Milk α_{S1} -Casein and Soybean Gly m Bd 60K

The registered number of α_{S1} -casein was AAA30429.1 in Gen Bank. The registered number of Gly m Bd 60K was BAA23360.2 in Gen Bank. In addition, the AA sequences of α_{S1} -casein or Gly m Bd 60K were derived using DNASTar software. Amino acid sequence alignment was compared between CM α_{S1} -casein and Gly m Bd 60K by Blastp software. The sequence similarity of the 2 allergens was 39%. Similar AA sequences and their descriptions are shown in Table 1. The E-value was 0.5.

Effect of Heating Processing on Gly m Bd 60K

Figure 5A and C show that Gly m Bd 60K was still observed in PVDF membranes with monoclonal antibodies by Western blotting after heating at 70°C or 100°C for different times. In addition, there was no reaction between Gly m Bd 60K and mice negative control sera, as shown in Figure 5B and D, which means a false-positive reaction had not happened. Thus, heat processing did not affect IgG cross-reactivity of Gly m Bd 60K. The Western blotting test was also carried out with IgE from patients' sera and the result was the same as that of the monoclonal antibody.

IgE- and IgG-Binding Epitopes of Gly m Bd 60K

Based on similar AA sequences by Blastp software, the sequences at AA319–341 and AA 164–182 were synthesized by Fmoc synthesis method. The purity of these 2 sequences was above 90% as shown in Figure 3A and B. The 2 sequences were respectively probed with IgE from CM patients' sera or IgG from monoclonal antibodies shown in Figure 3C. The optical density values of these 2 peptides were both significantly higher than negative controls ($P < 0.05$), which indicated the sequences at AA319–341 and AA 164–182 were IgE- and IgG-binding epitopes.

Gly m Bd 60K Digestion in Simulated Gastric Fluid

Figure 4 shows SDS-PAGE protein profiles of soybean trypsin inhibitor, OVA, PAP, Gly m Bd 60K, and α_{S1} -casein subjected to SGF digestion. The protein band of approximately 39 kDa was the pepsin enzyme using for digestion. Soybean trypsin inhibitor was a strong allergen and was still observed by SDS-PAGE after digestion for 60 min. The OVA was also considered as a strong allergen, which was not visible after digestion for 30 min. However, PAP was not an allergen, and was not visible at 15 s. The bands of Gly m Bd 60K and α_{S1} -casein were both not observed at 2 min by SDS-PAGE.

DISCUSSION

Although there are concerns about possible cross-reactivity among food allergens resulting from similar linear or conformational epitopes, it has also been reported that conformational epitopes played no important role (Curin et al., 2018). The cross-reactive allergens for novel food proteins can be predicted based on percent AA identity over local alignments between a new protein sequence and known allergen sequences. In addition, a threshold of >35% identity over the sequence of ≥ 80 AA is considered a cross-reactive risk under Codex guidance (Codex Alimentarius Commission, 2007). However, the high false-positive rates often occurred by the Codex approach (Silvanovich et al., 2009; Song et al., 2014). Therefore, most researchers have suggested that similarity, as reflected using statistical measures such as E-values, in combination with local alignment algorithms such as FASTA, is more selective than the Codex criterion for predicting cross-reactive allergens (Herman et al., 2015). The preceding studies indicate that IgE identification of allergic patients' sera toward similar homologous peptides to the allergen epitopes is the basis of the molecular cross-reactivity (Huang et al., 2014). Moreover, high homology in the protein primary, secondary, or even tertiary structure does not necessarily give rise to IgE epitope cross-reactivity (Nilsson et al., 2014). However, we found that the epitopes predict-

Figure 3. Synthesis, purification, and identification of IgE- and IgG-binding epitopes of Gly m Bd 60K. (A) corresponds with the purification map of the sequence at AA319–341, (B) corresponds with the purification map of the sequence at AA164–182, and (C) corresponds with identification of IgE- and IgG-binding epitopes with cow milk patients' sera or monoclonal antibodies using the ELISA method.

ed by the bioinformatics analysis were consistent with real IgE and IgG epitopes identified by the serologic method. First, the similar sequences of α_{S1} -casein and Gly m Bd 60K were predicted by the bioinformatics method and then the sequences at AA319–341 and AA 164–182 had be probed to verify IgE and IgG epitopes with sera from CM patients and monoclonal antibodies. More accurate and efficient ways for studying the cross-

reactive epitopes are needed because identification of IgE- and IgG-binding epitopes is important for better understanding the mechanisms of food cross-reactive allergies.

The incidence of CM allergy is on the rise; more than 8% children suffer from this. Candreva et al. (2017) characterized 4 peptides on α -casein and 3 peptides on Gly m 5 with a common core motif by MALDI-TOF-

Figure 4. The SDS-PAGE profile of Gly m Bd 60K digested in simulated gastric fluid (SGF; pepsin). (A) corresponds with SGF-digested soybean trypsin inhibitor (STI), (B) corresponds with SGF-digested ovalbumin (OVA), (C) corresponds with SGF-digested potato acid phosphatase (PAP), (D) corresponds with SGF-digested α_{S1} -casein (α_{S1} -CN), (E) corresponds with SGF-digested Gly m Bd 60K (60K), and M corresponds with protein marker at 97.4, 66.2, 43.0, 31.0, 20.1, and 14.4 kDa.

Figure 5. Western blotting map of Gly m Bd 60K heated at 70°C or 100°C for different times. Lanes 5 through 10 correspond with Gly m Bd 60K heated for 10, 20, 30, 40, 50, and 60min, respectively. Control corresponds with Gly m Bd 60K without denaturation; (A) and (B) correspond with Western blotting maps of Gly m Bd 60K heated at 70°C with monoclonal antibodies or mice negative control sera, respectively; (C) and (D) correspond with Western blotting maps of Gly m Bd 60K heated at 100°C with monoclonal antibodies or mice negative control sera, respectively.

MS. Some CM allergies were mediated with IgG antibody; moreover, IgG may compete with IgE for common epitopes on allergens (James and Till, 2016; Zhao et al., 2016). The IgG-blocking antibody has played an important role in patients undergoing allergen-specific immunotherapy for peanut allergies (Vickery et al., 2013). The present study revealed that IgG and IgE shared the same epitopes of Gly m Bd 60K, which was the cross-reactive allergen between CM α_{S1} -casein and soy proteins. Immunotherapeutic approaches using IgG epitopes may be able to shift the balance of antigen-specific antibody production from IgE to IgG. We are currently identifying which IgE-binding epitopes also bind IgG to find a feasible immunotherapeutic strategy for patients with CM or soybean allergies.

Heating and digestion may affect IgE reactivity of allergens by chemically altering protein structures. Oyster extracts showed highest IgE cross-reactivity with other mollusks after cooking (Rolland et al., 2018). In this study, heat treatment did not affect the immunoreactivity of α_{S1} -casein monoclonal antibodies or patients' sera and Gly m Bd 60K at 70°C or 100°C. Although Gly m Bd 60K was not visible at 2 min in SGF by SDS-PAGE, further research needs to be done, including whether the residue allergens or immunoreactive epitopes could be detected and whether it could be degraded in simulated intestinal fluid after SGF digestion.

It is important that the clinician recognize these patterns of food allergen cross-reactivity to advise patients on foods that should be avoided and to make sure that

necessary foods are restricted from the diet. Further research needs to be done in vivo to reveal detailed mechanism of immunopathology in food allergen cross-reactivity.

CONCLUSIONS

Our original data showed that the IgE and IgG cross-reactive allergen was Gly m Bd 60K between α_{S1} -casein and soybean proteins by a serologic method and a bioinformatics method, and 2 epitopes at AA319–341 and AA 164–182 were identified. The same IgE- and IgG-binding epitopes of the cross-reactive allergen may provide important information for elucidation of the association between IgG and IgE antibody generation. In this study, heating did not change IgE and IgG cross-reactivity with Gly m Bd 60K by Western blotting, and further studies need to be done to evaluate thermal stability of Gly m Bd 60K. Gly m Bd 60K may be completely degraded in SGF digestion, and more accurate and efficient ways for studying the digestive hydrolysates and epitopes are needed.

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ORCIDS

YanJun Cong <https://orcid.org/0000-0003-1473-2440>

Ye Li <https://orcid.org/0000-0002-1206-4998>

LinFeng Li <https://orcid.org/0000-0002-8736-6464>