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Development and validation of a mouse-based primary screening method for testing relative allergenicity of proteins from different wheat genotypes

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ABSTRACT

Background: Wheat allergy is a major food allergy that has reached significant levels of global public health concern. Potential variation in allergenicity among different wheat genotypes is not well studied at present largely due to the unavailability of validated methods. Here, we developed and validated a novel mouse-based primary screening method for this purpose.

Methods: Groups of Balb/c mice weaned on-to a plant protein-free diet were sensitized with salt-soluble protein (SSP) extracted from AABB genotype of wheat (durum, Carpio variety). After confirming clinical sensitization for anaphylaxis, mice were boosted 7 times over a 6-month period. Using a pooled-plasma mini bank, a wheat-specific IgE-inhibition (II)-ELISA was optimized. Then the relative allergenicity of SSPs from tetraploid (AABB), hexaploid (AABBDD) and diploid (DD) wheat genotypes were determined. The IC_{50}/IC_{75} values were estimated using IgE inhibition curves.

Results: The optimized II-ELISA with an inhibition time of 2.5 h had a co-efficient of variation of < 2%. Primary screening for relative allergenicity demonstrated that IgE binding to AABB-SSP was significantly abolished by the other two wheat genotypes. Compared to AABB, the relative allergenicity of SSPs of AABBDD and DD were significantly lower ($p < .01$). Furthermore, IgE inhibition curves showed significant differences in IC_{50} and IC_{75} values among the three wheat genotypes.

Conclusion: We report a novel mouse-based primary screening method of testing relative allergenicity of wheat proteins from three different wheat genotypes for the first time. This method is expected to have broad applications in wheat allergy research.

1. Introduction

Wheat allergies are a major type of food allergy that affect both children and adults in many countries around the world (Sicherer and Sampson, 2018; Renz et al., 2018; Cianferoni, 2016). They are increasingly recognized as a growing public health problem of global significance because they not only affect millions of wheat consumers, but also adversely affect the wheat industry and the global economy (Scherf et al., 2016; Cianferoni, 2016; Shewry, 2018). The overall prevalence of wheat allergy is estimated to be up to 1–3% in the USA and up to 0.9% at the global level (Cianferoni, 2016; Leonard and Vasagar, 2014; Venter et al., 2006a,b).

Wheat allergy, similar to other food allergies, is thought to develop in genetically susceptible individuals in two phases: a sensitization phase and a disease elicitation phase (Sicherer and Sampson, 2018). During the sensitization phase, IgE antibodies are produced against wheat allergens and these IgE antibodies bind to the mast cells via the high affinity IgE receptor (Sicherer and Sampson, 2018; Renz et al., 2018). Such subjects are deemed sensitized to wheat. In the second phase, exposure of sensitized subjects to wheat results in binding of allergens to the IgE on mast cells and basophils resulting in activation and release of mediators from these immune cells, causing clinical disease (Sicherer and Sampson, 2018; Renz et al., 2018).

Wheat allergy disease can manifest in at least three ways: 1) classical wheat food allergy with symptoms of vomiting, diarrhea, atopic dermatitis, or life-threatening anaphylaxis after consumption of wheat; 2) airways allergies (allergic rhinitis, baker's asthma) and eye allergy (allergic conjunctivitis) among wheat industry workers (e.g., bakery, pizzeria etc.,); and 3) wheat-induced exercise-dependent anaphylaxis, when sensitized subjects develop disease doing exercise immediately

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after eating wheat products (Cianferoni, 2016; Leonard and Vasagar, 2014). The binding of mast cell surface-attached IgE antibody to the wheat allergenic proteins is central to eliciting allergic disease (Cianferoni, 2016). Consequently, allergenicity of proteins is typically measured by their ability to bind to the IgE antibody in ELISA or Western blot methods (Pastorello et al., 2007; Nakamura et al., 2005; Mohan Kumar et al., 2017).

Wheat allergens belong to the three classical Osborne's wheat protein fractions: i) water/saline-soluble (albumins, globulins); 2) alcohol-soluble (gliadins) and 3) acid-soluble (glutenins) (Shewry, 2009). Although all these allergens have been implicated in wheat allergies, the first two groups are most common causes of wheat food allergies (Cianferoni, 2016).

There are five distinct wheat genotypes known to contribute to the genetic diversity of the wheat crop (Shewry, 2018). They are AA, BB (extinct today, SS is the closest relative available), DD, AABB, and AABBDD (Shewry, 2018). Among them, the last two genotypes are most commonly used to produce wheat-based food and animal feed. Using these genotypes, wheat breeders have successfully developed thousands of wheat varieties and wheat lines (Shewry, 2018; Mishra and Arora, 2017). Furthermore, efforts are also underway currently to genetically modify and produce engineered wheat lines (Mishra and Arora, 2017; Hellemans et al., 2018; Rey et al., 2015; Kohno et al., 2016). Thus, there is tremendous genetic diversity in the wheat crop currently cultivated for human and animal consumption.

It is noteworthy that despite this genetic diversity of wheat, the plausibility of differences in allergenicity among genetically distinct wheat is not well studied at present (Larre et al., 2011; Nakamura et al., 2005; Mohan Kumar et al., 2017). The major reason appears to be the unavailability of a validated primary screening method to compare the relative allergenicity of wheat proteins obtained from different genotypes of wheat. Such a method is urgently needed because it will help to identify not only the historical changes in the allergenicity of different wheat genotypes–if that has at all happened, but also to monitor potential future changes in wheat allergenicity due to ongoing breeding/selection and genetic engineering of wheat (Larre et al., 2011; Mishra and Arora, 2017; Kohno et al., 2016). Furthermore, food-processing methods have been shown to alter food allergenicity including wheat allergenicity (Phromraksa et al., 2008; Vanga et al., 2017; Verhoeckx et al., 2015; Maleki and Hurlburt, 2004). A validated method will also be useful to assess the changes in allergenicity of differently processed wheat products.

In order to address this critical need in this area of cereal science, we developed and validated a novel mouse-based primary screening method to determine relative allergenicity of wheat proteins obtained from three wheat genotypes—AABB, AABBDD, and DD.

2. Materials and methods

2.1. Chemicals and reagents

The following chemicals and reagents were obtained from the sources indicated in parenthesis: Biotin conjugated rat anti-mouse IgE paired antibodies and isotype standards (BD BioSciences, San Jose, CA); para-Nitrophenylphosphate (Sigma, St Louis, MO); streptavidin alkaline phosphatase (Jackson ImmunoResearch, West Grove, PA); protein estimation reagents: bovine serum albumin standard and reagents A and B (Sigma, St Louis, MO).

2.2. Mice

Balb/cJ mice (female) weaned on-to a plant protein-free diet (AIN-93 M) were purchased from the Jackson Laboratory (Bar Harbor, ME). All mice were housed in the animal facility of the Trout Food Science and Human Nutrition Building at the Michigan State University. Mice were maintained on the plant protein-free diet (AIN-93 M). All mice used in this study were 4–6 weeks old at onset of the studies. All animal procedures used were in accordance with Michigan State University policies.

2.3. Preparation of salt-soluble wheat protein extract

the following wheat genotypes were used in this study: AABB (Durum, variety Carpio), AABBDD (variety Ambassador) and, DD (*Aegilops tauschii)*. Protein extraction was conducted following the standard published method (Jin et al., 2017; Tatham et al., 2000). Briefly, ten grams of flour in 100 mL of 0.5 M NaCl was stirred continuously for 2 h at 20 °C followed by centrifugation (5000 x g, 10 min) at 20 °C. The supernatant was frozen overnight at −16 °C and then freeze-dried. Prior to use, the protein samples was reconstituted with saline. The protein contents of the reconstituted samples were quantified according to Bradford dye-binding method (Bradford, 1976).

2.4. Sensitization and quantitation of systemic anaphylaxis and mucosal mast cell degranulation responses

Mice were sensitized with the salt-soluble protein (SSP) extract obtained from AABB wheat (durum wheat, Carpio) as described before (Jin et al., 2017). Briefly, groups of mice (n = 5/group, 4 groups, 20 mice in total) were injected by intraperitoneal (IP) route four times (days 0, 10, 24, 40) with the SSP (0.01 mg/mouse/injection) plus alum (1 mg/mouse). Blood was collected from the saphenous vein on days 26 and 46 after the first injection and used to measure wheat-specific IgE antibody. Clinical sensitization was confirmed by IP challenge with SSP (0.5 mg/mouse) and hypothermia shock responses were quantified. Mucosal mast cell degranulation responses were quantified by measuring murine mucosal mast cell protease (MMCP)-1 protein elevation in the plasma after the challenge as described earlier (Jin et al., 2017).

2.5. Generation and characterization of a wheat-specific hyper-IgE plasma pool (AABB mini-plasma bank)

The overall approach used to generate the mini-plasma bank is shown in Fig. 1. After confirmation of the clinical sensitization for anaphylaxis, mice were boosted 7 times over a 6-month period. Blood was collected at bi-weekly intervals. Plasma from different mice at the same time point was pooled to create the hyper-IgE plasma pool that we have named as 'AABB mini-plasma bank'. Aliquots of plasma were stored at −70 °C in Eppendorf tubes.

The wheat-specific IgE antibody titer of the plasma bank was determined using an optimized wheat-specific IgE ELISA (Jin et al., 2017; Birmingham et al., 2003). Briefly, ELISA plates were coated with SSP (5 mg/mL) in coating buffer (sodium bicarbonate buffer, pH 9.6) at 4 °C for 18 h followed by blocking with 5% gelatin in phosphate buffered saline (PBS), pH 7.4, at 37 °C for 3 h. After washing three times with washing buffer (0.05% Tween 20 in PBS), plasma samples were added in triplicates and a two-fold dilution was conducted in dilution buffer (0.085% BSA in 0.05% Tween 20 in PBS, pH 7.4) and incubated at 4 °C for 18 h. After washing the plates three times, a biotin-labelled anti-mouse IgE antibody (BD Biosciences) was added and incubated at 4 °C for 1.5 h. Plates were washed three times and then streptavidin alkaline phosphatase conjugate was added and incubated at 37 °C for 0.5 h. After washing three times, the para-nitro phenyl phosphate substrate was added and the plates were allowed to develop for 6 and 21 h. The Optical Density (OD) was read at 405–690 nm using a spectrophotometer (BioTek, Synergy HT). The IgE antibody titer was defined as the reciprocal of the highest plasma dilution that shows positive signal at or above the background ± 3 SD (Birmingham et al., 2003).

The intra-assay variation of the wheat-specific IgE ELISA was determined by conducting the ELISA with an identical sample in quadruplicates. Variation in the OD among replicates was measured and used in the estimation of intra-assay coefficient of variation. The inter-

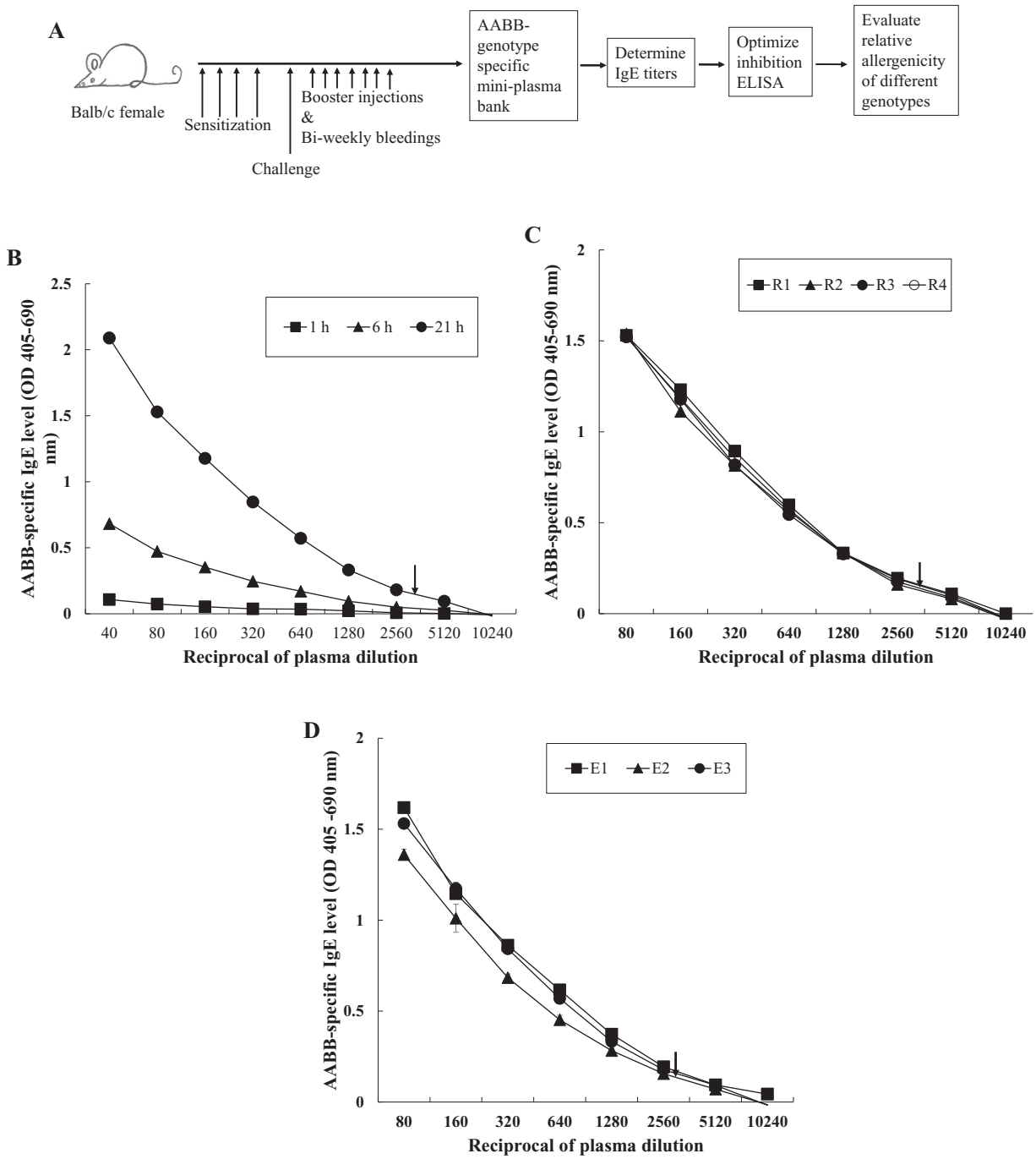

Fig. 1. (A-D). Construction and characterization of 'AABB mini-plasma bank'. Fig. 1A: Groups of Balb/c mice (n = 5/group, 4 experiments) were injected with salt-soluble wheat protein extract (AABB durum, Carpio) and clinical sensitization was confirmed by anaphylaxis and mast cell degranulation response upon IP challenge. Animals then received 7 booster injections over a 6-month period. Hyper-IgE plasma was pooled to create a 'AABB mini-plasma bank'. Then, IgE inhibition (II)-ELISA was optimized to determine the relative allergenicity of proteins from different wheat genotypes. Fig. 1B shows determination of wheat-specific IgE antibody titer using an ELISA-based method as described in methods. The vertical downward arrow corresponds to the titer of IgE antibody; n = 3; SEM is too small to be visible. Fig. 1C shows intra-assay variation. Figures show variation in the OD at the 21-h reading; n = 3. Fig. 1D shows inter-assay variation; ELISA was conducted on different days using the same plasma sample. Figures show variation in the OD at the 21-h reading from 3 independent experiments (E1, E2, and E3); n = 3; in many cases, SEM is too small to be visible.

assay variation of the wheat specific IgE ELISA was determined by conducting three identical experiments on three different days by the same individual. Variation in the OD between the three independent experiments were measured and used in estimation of the inter-assay coefficient of variation.

2.6. Optimization and validation of an IgE inhibition (II)-ELISA for wheat protein

The ELISA plates were coated with 5 mg/mL of AABB salt-soluble protein (SSP) in coating buffer (sodium bicarbonate buffer, pH 9.6) at 4 °C for 18 h. Blocking was done at 37 °C for 3 h with 5% gelatin. After washing three times (0.05% Tween 20 in PBS), plasma sample was

added in various dilutions starting from 1/80 to obtain the titration curve. Parallel plasma samples were mixed with AABB protein (at 0.5 mg/mL) and incubated for 0.5, 1.0 and 2.5 h for IgE binding. At the end of incubation time, samples were centrifuged at 5000 ×*g* for 20 s. Supernatant was collected and added to the already blocked ELISA wells at the final dilution of 1/80. Control plasma samples without pre-incubation with the AABB protein were added to the control wells. The ELISA plate was incubated at 4 °C for 18 h. After washing, anti-mouse IgE antibody labelled with biotin was added and incubated at 37 °C for 1.5 h. After washing, streptavidin alkaline phosphatase conjugate was added and incubated at 37 °C for 0.5 h. After washing, the para-nitro phenyl phosphate substrate was added. Initial experiments showed that the wheat-specific IgE signals at 1, 2, 3, and 5 h were weaker. Therefore, plates were allowed to develop for 6 and 21 h to get stronger signals relative to the background activity. The optical density (OD) was read at 405–690 nm (Bio-Tek Synergy HT).

The IgE inhibition results were expressed as % B/B_0, where B corresponds to the specific IgE-binding to immobilized AABB-derived salt-soluble protein when a known concentration of the inhibitor protein is present, and B_0 corresponds to the binding in the absence of inhibitor.

For determination of intra-assay variation of IgE inhibition (II)-ELISA, samples were run in triplicates in II-ELISA. Variations in OD (405–690 nm) at 6- and 21-h readings were measured and used in calculation of intra-assay coefficient of variation. To determine inter-assay variation, two experiments were conducted by the same person on two different days. The variation in OD (405–690 nm) at 6- and 21-h readings were measured and used in calculations of inter-assay coefficients of variation.

2.7. Determination of relative allergenicity among the three wheat genotypes

The percent IgE inhibition obtained using AABB as the inhibitor was used as the 100% allergenicity control, and allergenicity of other genotypes relative to this control were determined. The SSP extracted from variety Ambassador (a soft-wheat) was used as a model for AABBDD hexaploid genotype for allergenicity determination in the II-ELISA. The SSP extracted from *A. tauschii* was used as a model for DD diploid genotype for allergenicity determination in the II-ELISA. In these experiments, SSPs from these genotypes were pre-incubated at 5 and 1 mg/mL with the AABB-specific plasma (at 1/80) and the IgE inhibition was determined. The relative IgE inhibition percents were calculated using the AABB data as the 100% allergenicity control.

2.8. Determination of inhibition concentrations of three wheat genotypes using IgE inhibition curves

Using various concentrations of AABB-, AABBDD- and DD-derived SSPs as IgE inhibitors, II-ELISA evaluations were conducted. The % B/B_0 values were used to construct the IgE inhibition curves. The concentration of inhibitor that inhibits 50% (IC_{50}) and that inhibits 75% (IC_{75}) of the IgE binding to the immobilized AABB-SSP was determined for each. In this analysis, an increase in IC_{50} and IC_{75} values correlates with reduced allergenicity of the protein used as inhibitor.

2.9. Statistical analysis

Comparison of two groups for significance was done using Student's *t*-test. For multiple group comparisons, Tukey's test was used. Online software service was used in these analyses (http://www.socscistatistics.com/tests/pearson/). The statistical significance level was set at 0.05.

3. Results

3.1. Development of a wheat-specific hyper-IgE plasma pool

We used a previously published mouse model of wheat allergy to generate a colony of wheat allergic mice ($n = 20$). All mice were tested and confirmed for elevation of wheat-specific IgE antibodies. Clinical sensitization for systemic anaphylaxis was confirmed by intraperitoneal challenge with SSP (0.5 mg/mL) and by measuring plasma MMCP-1 protein as described (Jin et al., 2017). The overall method used is shown in Fig. 1A. These mice then received repeated booster injections (7) without alum adjuvant. By repeated bi-weekly bleedings and pooling of the plasma, a hyper-IgE plasma pool, that we have named as 'AABB mini-plasma bank', was established.

3.2. Characterization of the AABB mini-plasma bank

Using a previously published wheat-specific IgE ELISA method, the IgE antibody titer of the pooled plasma was determined (Birmingham et al., 2003). As evident, the repeated analysis demonstrated IgE antibody titer of the mini-plasma bank to be 2560 (Fig. 1B). We then determined the inter- and intra-assay variation of this analysis. The coefficients of variation (CV) for intra-assay and inter-assay analyses were 2.2% and 8.7% respectively (Fig. 1C, D).

3.3. Optimization of an IgE inhibition ELISA for wheat protein

Based on the wheat-specific IgE ELISA, a plasma dilution of 1/80 provided a robust signal within the linear portion of the titration curve, and was identified as the appropriate plasma dilution to use in the optimization of an IgE inhibition (II)-ELISA (Fig. 1B–D). First, we determined the optimal pre-incubation inhibition time by pre-treatment of plasma with AABB-SSP at two different concentrations (0.5 and 1.0 mg/mL) for 0.5, 1.0 and 2.5 h. Results of residual IgE binding are shown at both the 6-h reading time (Fig. 2A, B) and the 21-h reading time (Fig. 2C, D). We then calculated the % B/B_0 using these data (Fig. 2E–H). As shown, the residual IgE binding significantly decreased ($p < .05$) as inhibition time increased from 0.5 to 1.0 h; there was no significant change between 1 and 2.5 h. The same conclusions were drawn based on the % B/B_0 analysis (Fig. 2E–H). In summary, the optimized II-ELISA had an inhibition time of 2.5 h, inhibitor concentrations of 0.5, and 1 mg/mL and plasma dilution of 1/80.

3.4. Inter-assay and intra-assay variation of the IgE inhibition ELISA for wheat protein

The intra-assay and inter-assay co-efficients of variation (CV) for the II-ELSIA method were determined (Fig. 3A, B). Variations observed among the replicates within an experiment conducted on a single day by an individual (intra-assay variation) for the optimized II-ELISA was acceptable, with a CV of 1.7%. Variations observed between the experiments conducted on different days by an individual (inter-assay variation) also was acceptable, with an inter-assay CV of 1.5%.

3.5. Determination of % B/B_0 for AABB, AABBDD and DD wheat genotypes

Using the II-ELISA, the relative ability of the SSPs obtained from three wheat genotypes to inhibit IgE binding to AABB was quantified as described above. Using the method described earlier in the methods section, the $\%B/B_0$ values were determined. As evident, $\%B/B_0$ values were significantly different among the three wheat genotypes at both inhibitor concentrations (5 and 1 mg/mL) and at both reading time points (6 and 21 h) ($p < .01$, all comparisons) (Fig. 4A-D).

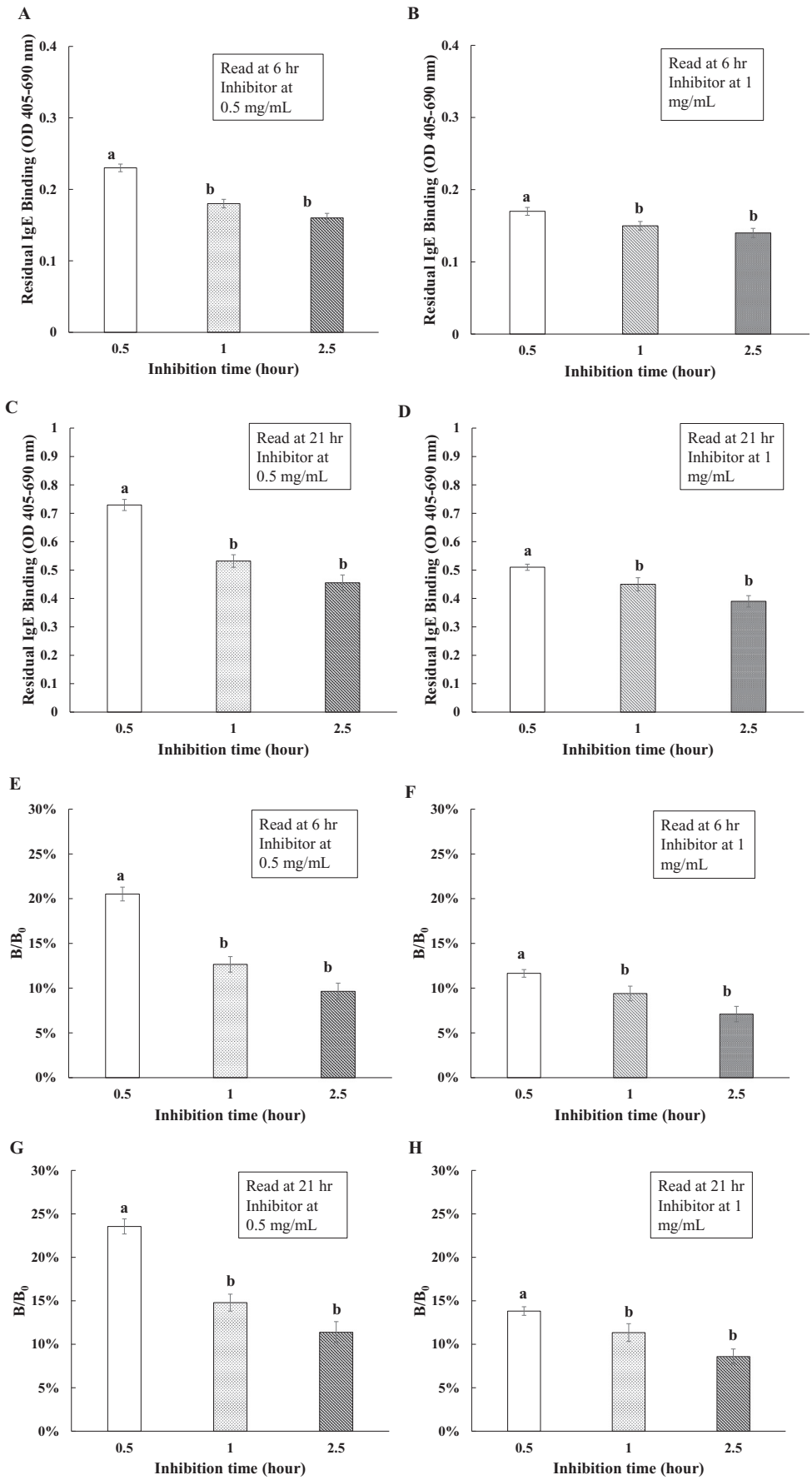

Fig. 2. (A–H). Optimization of wheat-specific IgE inhibition ELISA. To determine optimal inhibition time, plasma was mixed with 0.5 mg/ml or 1 mg/mL of AABB-SSP and incubated for the indicated time, then centrifuged. Supernatant was collected and used in ELISA coated with AABB- SSP. Fig. 2A–B show residual IgE binding after inhibition times (0.5, 1.0, and 2 h), but all at the 6-hour time point of plate reading; $n = 3$. Fig C–D show residual IgE binding after inhibition times (0.5, 1.0, and 2 h), but all at the 21-h time point of reading. Y-axis in figures A–D shows residual IgE binding as OD at 405–690 nm; n = 3. Fig 2E–F show $\%B/B_0$ after inhibition time (0.5, 1.0, and 2 h) at the 6-h time point of plate reading; n = 3. Fig 2G–H show $\%B/B_0$ after inhibition time (0.5, 1.0, and 2 h) at 21-h time point of reading; n = 3.

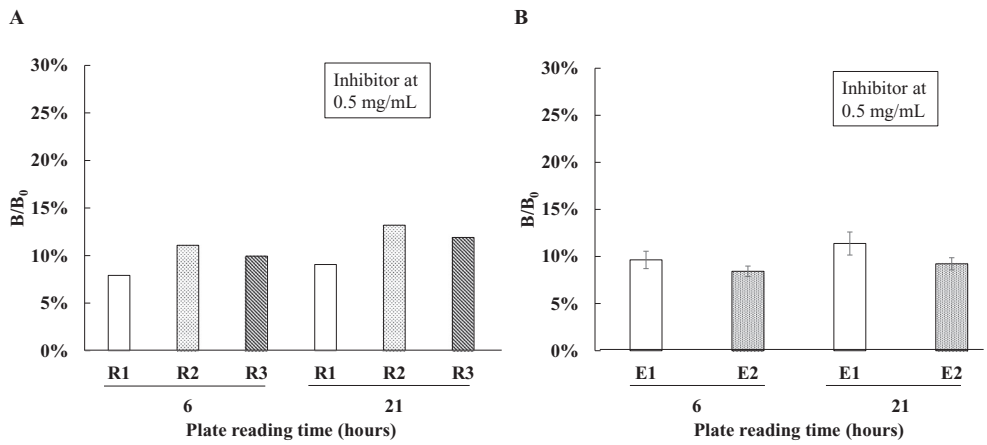

Fig. 3. (A-B). Determination of intra-assay and inter-assay variation of IgE inhibition ELISA. To determine intra-assay variation, plasma was used in various dilutions as shown. Fig. 3A shows variation among 3 replicates (R1, R2, R3) in the percent B/B_0 at 6- and 21-h readings; n = 3. To determine inter-assay variation, ELISA was conducted on different days using the plasma sample at indicated dilutions. Fig. 3B shows variation in the percent B/B_0 among 3 replicates at 6- and 21-h readings from two independent experiments (E1 and E2); n = 3.

3.6. Determination of relative allergenicity of AABB vs. AABBDD and DD genotypes using the optimized II-ELISA

Using the homologous IgE inhibition by AABB as 100% reference level for allergenicity, the relative allergenicity of AABBDD and DD genotypes were determined. As evident, relative to homologous IgE inhibition by AABB-SSP at 100%, the AABBDD-SSP abolished IgE binding by 92.2% ± 0.7% at 6 h and by 91.8% ± 0.9% at 21 h with inhibitor concentration of 1 mg/mL and by 94.4% ± 0.1% at 6 h and by 93.7% ± 0.1% at 21 h with inhibitor concentration of 5 mg/mL; overall, the average IgE inhibition was ~93.1% (Fig. 5A-D). Similarly, compared to the homologous IgE inhibition by AABB-SSP at 100%, the DD-SSP abolished IgE binding by only 82.6% ± 1% at 6 h and by 79.8% ± 1.1% at 21 h, with inhibitor concentration of 1 mg/mL and by 85.2% ± 0.8% at 6 h and by 83.3% ± 0.7% at 21 h, with inhibitor concentration of 5 mg/mL; overall, the average IgE inhibition was ~82.8% (Fig. 5A-D). Thus, on average, compared to AABB, the relative allergenicity of AABBDD was lower by ~7% and the relative allergenicity of DD was lower by ~17%.

3.7. Establishment of IgE inhibition curves and estimation of IC_{50} and IC_{75} values for the three wheat genotypes

We established dose-response IgE inhibition curves using the II-ELISA method for all three wheat genotypes (Fig. 6A–C). Using these curves, IC_{50} and IC_{75} values were estimated and compared. As evident (Table 1), there were significant differences in both IC_{50} and IC_{75} values among the three wheat genotypes ($p < .01$ all comparisons).

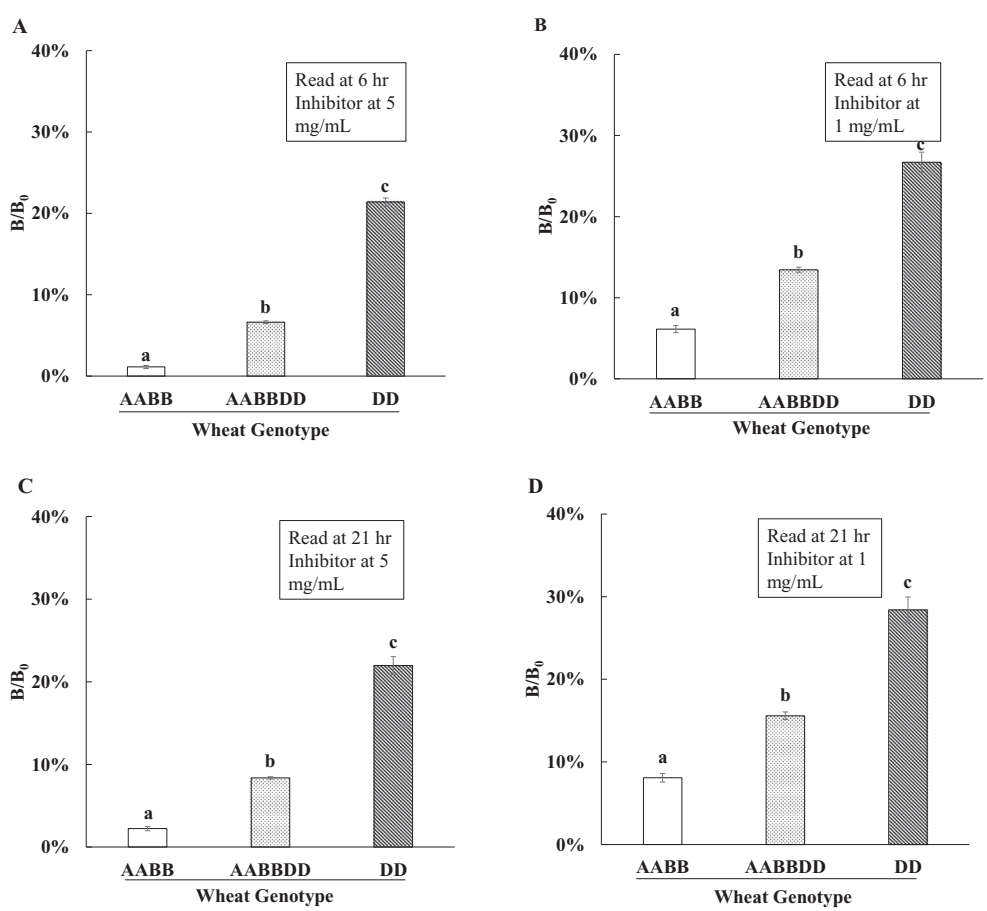

Fig. 4. (A-D). Determination of $\%B/B_0$ values for AABB, AABBDD, and DD genotypes based on II-ELISA. The IgE inhibition ELISA was conducted using AABB-SSP as coating antigen. The SSPs from AABB, AABBDD and DD wheat genotypes were used as inhibitors. Fig. 4A–B show percent B/B_0 at inhibitor concentrations of 5 mg/mL and 1 mg/mL, respectively, using 6-h readings; n = 3. Fig. 4C–D show percent B/B_0 at inhibitor concentrations of 5 mg/mL and 1 mg/mL, respectively, using 21-h readings; n = 3.

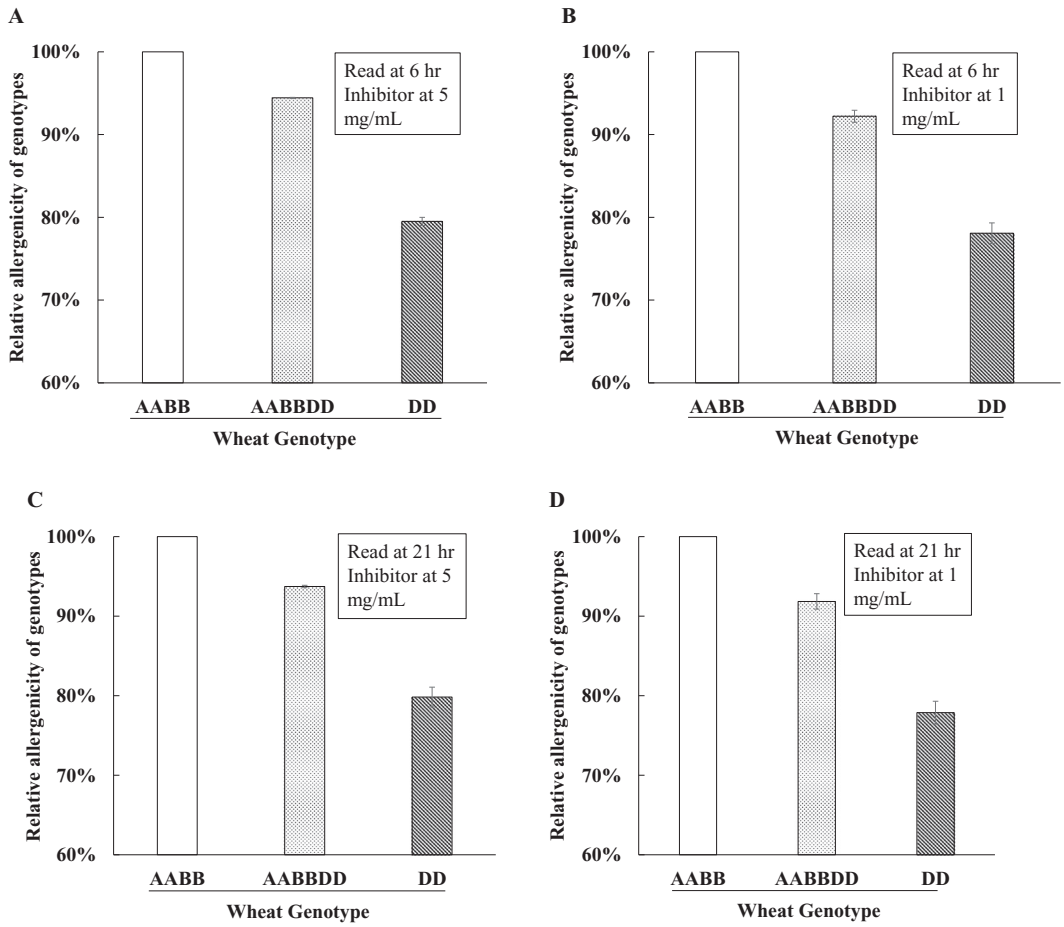

Fig. 5. (A-D). Determination of allergenicity of AABBDD, and DD genotypes relative to AABB genotype. The IgE inhibition ELISAs were conducted using AABB-SSP as coating antigen. The SSPs from AABB, AABBDD and DD wheat genotypes were used as inhibitors. Using allergenicity measurements obtained for AABB genotype as 100% reference allergenicity values, the relative allergenicity of AABB and DD genotypes were calculated. Fig. 5A–B show relative allergenicity of the genotypes at inhibitor concentrations of 5 mg/mL and 1 mg/mL, respectively, using 6-h readings; n = 3. Fig. 5C–D show relative allergenicity at inhibitor concentrations of 5 mg/mL and 1 mg/mL, respectively, using 21-h readings. n = 3.

4. Discussion

In this study, we sought to develop and validate a novel mouse-based primary screening method for testing relative allergenicity of wheat proteins from different genotypes. Our data collectively demonstrate for the first time that it is possible to use the mouse system to accomplish this challenge. Furthermore, the results demonstrated that: (i) compared to the allergenicity of SSP from AABB, SSP from AABBD and DD are relatively less allergenic; (ii) the genetic differences among these three genotypes (tetraploid vs. hexaploid, vs. diploid) indeed translates to measurable variations in allergenicity by this method; and (iii) AABBDD, which is a hybrid between AABB and DD genotypes, is intermediate in allergenicity as measured by this method.

Wheat is a major allergenic food per food regulators in the US, Canada, Europe, Australia, Japan and New Zealand (US-FDA, 2018; Sicherer and Sampson, 2018; Gonipeta et al., 2015a,b). For regulation purposes all types of wheat are regarded as a single regulatory entity because they all are considered to be equally allergenic at present (US-FDA, 2018). However, there are five different wheat genotypes that are genetically distinct (Shewry, 2018), therefore, it is reasonable to hypothesize that genetically distinct wheats might differ in their allergenicity potential. However, this hypothesis has not been thoroughly investigated so far.

Here we developed and validated the approach of using a mouse-based system for evaluating potential differences in allergenicity of genetically different wheat genotypes. Our results confirmed that the IgE antibodies elicited in the mice cause disease (i.e., are functional) by challenge studies for systemic anaphylaxis and mast cell degranulation. This approach has several advantages over using wheat-allergic subjects for this type of analysis including: i) exposure to specific wheat genotypes for sensitization can be completely controlled in animal based system such as mouse models but not in humans; and ii) exposure to cross-reacting plant proteins can also be completely controlled in mouse models but not in humans. Thus, in this study, mice were maintained on a plant-protein free diet and they were exclusively sensitized to one wheat genotype (e.g., AABB) only. Consequently, interpretation of the data is straightforward when using the animal-based method to assess the relative allergenicity of different wheat genotypes. As a proof-of-concept, we used AABB-exposed mice in this study. However, a similar approach could be essentially used to generate other genotype-specific mini-plasma banks by exposing mice to other genotypes of wheat.

A major challenge in using ELISA based method to detect food-specific IgE antibodies is the presence of food-specific IgG1 antibodies in the plasma (Birmingham et al., 2003). In order to overcome this issue, we have previously reported a novel ELISA method that enables the detection of food-specific IgE antibodies even when food-specific IgG1 antibodies are present in the plasma (Birmingham et al., 2003). In this study, using the same method but using wheat as antigen, we developed wheat-specific IgE inhibition ELISA. Using the optimized method described here, we show that it is possible to detect wheat-specific IgE antibody binding using II-ELISA despite the presence of wheat-specific IgG antibodies in the plasma.

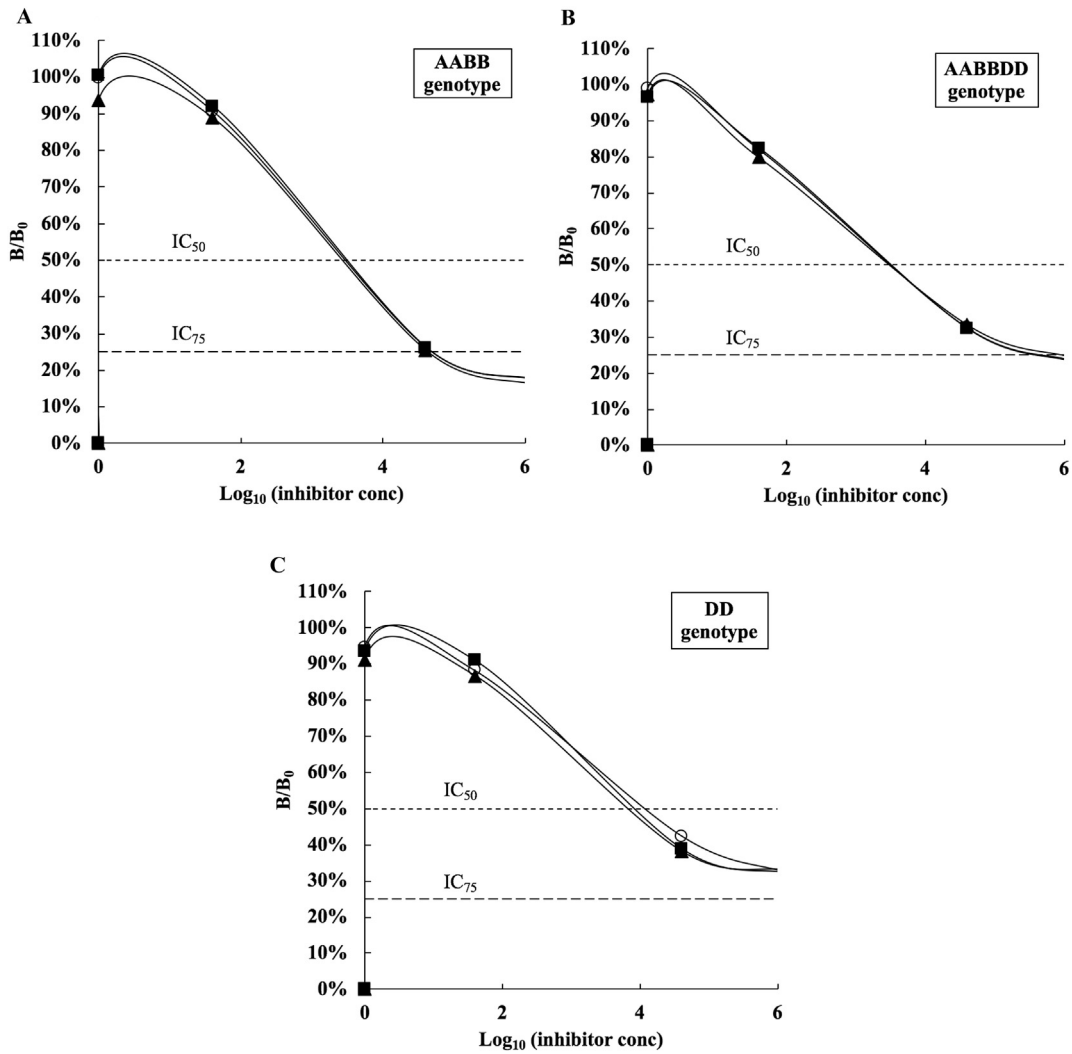

Fig. 6. (A–C). Determination of IC_{50} and IC_{75} values using IgE inhibition curves for three wheat genotypes. The IgE inhibition ELISA was conducted using AABB-SSP as coating antigen. The SSPs from AABB, AABBDD, and DD were used as inhibitors. Fig. 6A–C show inhibition curves for AABB, AABBDD, and DD genotypes, respectively. Figures show percent B/B_0 on the y-axis and concentrations of the inhibitors on the x-axis. The broken horizontal lines indicate the IC_{50} and IC_{75} values; n = 3.

Table 1
Comparison of estimated IC_{50} and IC_{75} values of three wheat genotypes using the optimized IgE inhibition ELISA method.

Wheat genotype	IC_{50} value (μg/mL)	IC_{75} value (μg/mL)
AABB	45.05 ± 0.65	87.80 ± 0.87
AABBDD	54.50 ± 0.16	177.21 ± 2.15
DD	62.12 ± 2.33	> 500

IgE Inhibition ELISAs were conducted using different wheat genotype derived salt-soluble proteins at various inhibitor concentrations as detailed in the text. Data shows estimated inhibition concentration values from triplicate analyses. ANOVA with post-hoc Tukey honestly significant difference (HSD) test results: AABB vs AABBDD, AABB vs DD, AABBDD vs DD all $p < .01$ for both IC_{50} and IC_{75} comparisons.

As a proof-of-concept, here we used saline-soluble wheat proteins because we had previously established a mouse model using this type of protein (Jin et al., 2017). Successful development and validation of this mouse-based method here, suggest that a similar approach could be used to develop and validate methods for allergenicity assessment of alcohol-soluble and acid-soluble proteins from wheat or any other allergenic food. For example, there are five useful mouse models that already have been developed using alcohol-soluble gluten proteins (gliadins) earlier (Bodinier et al., 2009; Denery-Papini et al., 2011; Gourbeyre et al., 2012; Abe et al., 2014; Adachi et al., 2012). These animal model protocols may be employed for developing and validating II-ELISA using the same approach that is described here in this paper.

We are not aware of an IgE inhibition ELISA (II-ELISA) method reported for wheat allergenicity testing in any animal models in the literature so far. There are two studies that used serum from wheat allergic patients and examined the binding of IgE to proteins obtained from various wheat varieties (Nakamura et al., 2005; Mohan Kumar et al., 2017). These studies used a direct IgE ELISA to study IgE binding to wheat proteins. They reported that there is variation in IgE binding among wheat varieties suggesting that there may be natural variation in the allergenicity among wheat varieties. However, interpretation of the data from these studies is difficult for two reasons: i) the observed differences in IgE binding to various wheat varieties could be simply due to differences in exposure of allergic subjects to different wheat varieties or different exposures to cross-reacting non-wheat plant proteins; and ii) the use of direct ELISA to study IgE binding to different proteins assumes that the proteins obtained from various wheat types will bind to ELISA plates to the same extent. If there were to be differences in the ability of wheat proteins to bind to ELISA plates, then that would translate into different levels of reactivity to IgE antibodies.

Nevertheless, these papers provide the first preliminary data and scientific premise to further test the hypothesis that genetically different types of wheat might differ in their relative allergenicity.

One limitation of the approach reported here includes the volume of the mini-plasma bank that one can establish using mice. Although mice provide little volume of blood, here we demonstrate successfully that indeed a mini-plasma bank (total volume of ~12 mL from 20 mice colony, over 8 months) can be created using the approach we developed. Nevertheless, should there be a need for larger size plasma bank, one might consider a similar approach using a larger colony of mice.

We used Balb/c mice because they are genetically prone to develop Th2-dominated immune responses to food antigens (Gonipeta et al., 2015a,b). Previous work showed that compared to other strains (C3H/Hej and B10.A), Balb/c are better suited for studying allergic responses to wheat proteins (Bodinier et al., 2009). It is reported that IgE responses to wheat in Balb/c are remarkably similar to IgE responses of human wheat allergic subjects (Denery-Papini et al., 2011). Nevertheless, mouse models, although show several characteristics similar to human disease, there are some limitations (Gonipeta et al., 2015a,b). For example, as opposed to human wheat allergy that start early in infancy or childhood, mouse models use adult mice due to technical difficulties in handling and management of mice < 4 weeks age (Gonipeta et al., 2015a,b).

Previous study showed that pollen allergens share epitope homologues with cereal grains (Astwood et al., 1995). Therefore, it is possible that our method may have broader application to study the cross-reacting IgE epitopes that are common between pollen allergens and cereal food allergens.

Currently, there are no approved genetically engineered (GE) wheat crops for human or animal consumption (Mishra and Arora, 2017; Hellemans et al., 2018; Rey et al., 2015; Kohno et al., 2016; Shewry, 2018). However, there are ongoing efforts to develop GMO wheat (Mishra and Arora, 2017; Shewry and Tatham, 2016). It will be important to demonstrate whether such GE wheat is similar to or different from native wheat in terms of allergenicity potential (Ekmay et al., 2017; Selgrade et al., 2009; Ladics and Selgrade, 2009). The method described here will be a useful as a primary screening tool for such GE wheat products.

There are numerous studies showing that food processing can affect allergenicity of food proteins, including wheat, due to the effect of processing on the IgE binding epitope structure on protein allergens (Verhoeckx et al., 2015). Studies so far have typically used SDS-PAGE, and western blot analysis to study these changes (Phromraksa et al., 2008; Vanga et al., 2017; Verhoeckx et al., 2015). The II-ELISA established in this study can be used in the future to study changes in the IgE binding epitope structure of wheat proteins that have been subjected to different types of food processing. For example, it is possible to study how non-thermal vs. thermal processing of wheat products can alter IgE binding epitope structures and consequently allergenicity. No such studies have been reported in the literature at this point, largely because of the absence of a validated II-ELISA for wheat proteins so far.

Another application of the method described here is in the development of novel hypo/non-allergenic wheat proteins. Hypo/non-allergenic wheat proteins are highly desired ingredients for human and animal consumption (Lombardo et al., 2015; Kohno et al., 2016; Tanabe, 2008; Phromraksa et al., 2008). Therefore, pre-clinical and clinical testing of novel wheat proteins that are being considered for hypo/non-allergenicity applications is vital. The method optimized here can also be used for primary screening of such novel products.

5. Conclusions

Here we developed and validated a novel mouse-based primary screening method for testing allergenicity of wheat proteins from three different wheat genotypes. This method will be a useful tool to assess the changes in allergenicity of novel types of wheat or wheat products, developed by genetic engineering, traditional breeding, or by different food processing techniques or any other newer technology in the future.

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Conflict of interest statement

Authors do not have any conflicts of interest to declare.

Ethical statement

An institutional review board approved animal procedures used in this study.

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