



Extraction of total wheat (*Triticum aestivum*) protein fractions and cross-reactivity of wheat allergens with other cereals

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ABSTRACT

A one-step mild extraction of total wheat protein fractions was developed in this study, and the allergic cross-reactivity among dietary cereals were assessed by SDS-PAGE, western blotting, indirect ELISA, and inhibition ELISA using sera from 12 wheat allergic patients. The fractions of albumin, globulin, gliadin and glutenins in wheat flour can be obtained by a one-step extraction with Na₂CO₃-NaHCO₃ (20 mM, pH 9.6, 0.5 M NaCl, 40% ethanol, 1 mM PMSF) in comparison to sequential extractions. Results showed high cross-reactivity in wheat, barley and rye due to close resemblance and high sequence identity (>50%), whereas nearly negligible cross-reactivity among rice, buckwheat, and quinoa was observed. Our research findings suggest that people with wheat allergy should rely primarily on the use of rice, quinoa and non-grain buckwheat, which is an effective substitute for wheat, while those with hypersensitivity should avoid the use of barley and rye in their diet.

1. Introduction

Wheat (*Triticum aestivum*) is a staple food for individuals around the world and is an important source of dietary protein, but it can also trigger IgE-mediated food allergy (Quirce, Boyano-Martínez, & Díaz-Perales, 2016; Shewry, 2009). Due to the extensive applications of wheat and closely related cereals (rye, barley and oats) in our daily diet, cereal-associated allergies are particularly considered as a serious problem (Nakase, Usui, Alvarez-Nakase, Adachi, & Matsuda, 1998; Quirce, Boyano-Martínez, & Díaz-Perales, 2016). Wheat-based foods cause severe health problems in at least 0.5% of the pediatric population and 1% of the adult population due to allergen proteins derived from wheat, and might cause severe and a life-threatening symptoms in some allergic patients (Pourpak, Mansouri, Mesdaghi, Kazemnejad, & Farhoudi, 2004; Sander, Rozynek, Rihs, Van Kampen, Chew, Lee, et al., 2011; Sicherer, 2011).

All cereals, including wheat, barley, rye, oat, rice and maize, belong to the family Poaceae or Gramineae. Cereal protein can be classified into four different fractions according to Osborne (1924) classification, based on sequential extraction in water (albumins), dilute salt solutions (globulins), 40%-70% aqueous alcohol (gliadins), and dilute alkali or

acid (glutenins). Many researchers have identified both water/salt soluble proteins and prolamins (gliadins and glutenins) as the major allergens in patients with wheat allergies (Quirce, Boyano-Martínez, & Díaz-Perales, 2016; Sander, et al., 2011; Scherf, 2019; Tatham & Shewry, 2008). Wheat proteins in water/salt-soluble fraction, comprising primarily α -amylase/trypsin inhibitors, peroxidase, thio-redoxin, serpin and non-specific lipid transfer protein 1, were found to be significant allergens in patients with urticaria, baker's asthma and atopic dermatitis (Quirce, Boyano-Martínez, & Díaz-Perales, 2016; Sander, et al., 2011). Furthermore, prolamins accounted for 80% of all wheat proteins, such as α/β -, α -, and ω -gliadins, as well as LMW/HMW glutenin subunits and were considered to be the main allergens for wheat allergies (Inomata, 2009; Quirce, Boyano-Martínez, & Díaz-Perales, 2016). Wheat allergy occurs through various routes, such as ingestion, inhalation and skin contact, which is responsible for urticaria, baker's asthma, atopic dermatitis, celiac disease (CD) and wheat-dependent exercise-induced anaphylaxis (WDEIA) (Pastorello, Niguarda, & Maggiore, 2019; Quirce, Boyano-Martínez, & Díaz-Perales, 2016; Sander et al., 2011). Dietary allergy to wheat, typically represented as CD and WDEIA, is reported in various studies with less prevalence in the general population but in its severe form, it can lead to

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anaphylaxis shock and even death (Quirce, Boyano-Martínez, & Díaz-Perales, 2016; Tatham & Shewry, 2008). The adverse reaction of CD and WDEIA is primarily caused by wheat gluten with roughly equal quantities of gliadins, glutenins, wheat ω_5 -gliadins well as high molecular weight (HMW) glutenin subunits. (Inomata, 2009; Quirce, Boyano-Martínez, & Díaz-Perales, 2016; Scherf, 2019). Therefore, there is a demanding need to improve the diagnosis and treatment of wheat allergies further.

Wheat allergy diagnosis has been historically hampered by the low positive predictive value of the available ImmunoCAP wheat IgE test and a great deal of cross-reactivity among the different cereals (Jones, Magnolfi, Cooke, & Sampson, 1995; Palosuo, Alenius, Varjonen, Kalkkinen, & Reunala, 2001; Pourpak, Mesdaghi, Mansouri, Kazemnejad, Toosi, & Farhoudi, 2005; Tatham & Shewry, 2008). Cross-sensitization in patients with wheat allergies without prior use makes it extremely difficult to evaluate the main sensitizing agent. Palosuo, Alenius, Varjonen, Kalkkinen, and Reunala (2001) reported that wheat, rye and barley may elicit symptoms in patients with WDEIA. Also, the cross-reaction between wheat, barley, and oat has been seen in radioallergo-sorbent (RAST) inhibition assay (Block et al., 1984).

Currently, serum tests for clinical wheat allergy patients are laborious and time-consuming, which need to be detected individually using wheat water-soluble protein, gliadin and gluten components. Clinical serum tests are significantly precluded by high-purity allergen extracts of individual protein components due to varying solubilities of cereal protein fractions, thereby causing poor detection stability and high false positives. A sequential technique using denaturants such as SDS, DTT or β -mercaptoethanol (β -ME) and urea are currently available for extraction of wheat allergens based on Osborne methods (Osborne, 1924) or with a slight adjustment to obtain water-insoluble protein (Akagawa, Handoyo, Ishii, Kumazawa, Morita, & Suyama, 2007; DuPont, Chan, Lopez, & Vensel, 2005; Osborne, 1924). Nevertheless, the sequential process of wheat protein is labor-intensive and time-consuming. Thus, selecting an extraction buffer that is more effective in isolating whole wheat protein fractions is of prime importance so that it can be diagnosed effectively and easily by detection methods.

In the present study, we developed and optimized a one-step extraction of total wheat protein fractions including the major wheat allergens with mild solutions. Analysis of this cross-reactivity among different cereals protein will contribute to finding the origin of strong IgE mediated responses, more specific reagents for allergen detection, allergy diagnosis and more focused on dietary restrictions. The allergenic cross-reactivity among dietary cereals (wheat, barley, rye, oat, rice, and maize), buckwheat, and quinoa were examined by western blotting (WB), indirect ELISA, and inhibition ELISA, using 12 wheat-allergic serum samples. Additionally, we compared the sequence of known allergens by *in silico* analysis.

2. Materials and methods

2.1. Materials

Wheat, barley, rye oat buckwheat, quinoa, rice and maize cultivated in Jilin Province of China were obtained from a local market (Tonghua, Jilin, China). Horseradish peroxidase (HRP) conjugated goat anti-human IgE antibody was obtained from Zhongshan Jinqiao Co. (Beijing, China). The iBind™ Solution Kit and enhanced chemiluminescent (ECL) kit for western blot analysis was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). All other chemicals used in the study were of analytical grade.

2.2. Wheat allergic patients

This study was approved by the Ethics Committee of the Affiliated Hospital of Qingdao University (Qingdao, Shandong, China). Each of the studied participants had received signed permission to use his/her

Table 1

Clinical and serological characterization of wheat-allergic patients.

Serum No.	Gender	Age	Symptoms	Total IgE (kU/L)
P1	F	28	WDEIA, syncope, vomit	856
P2	F	33	WDEIA, syncope, urticaria	1164
P3	M	32	Urticaria, edema	568
P4	M	37	Syncope, dyspnea, hypotension	115
P5	F	44	Syncope, dysphagia, pruritus	176
P6	M	57	WDEIA, allergic rhinitis	800
P7	F	25	Syncope, dyspnea, hypotension	850
P8	F	36	Urticaria,	544
P9	M	62	Urticaria, syncope	82.5
P10	F	46	Syncope, fatigued	48.8
P11	F	53	Syncope, fatigued, hypotension	151
P12	F	53	WDEIA, Syncope, vomit	235
N1	F	28	–	≤0.35
N2	M	56	–	≤0.35

WDEIA: wheat-dependant exercise-induced anaphylaxis.

serum in this study. Sera was obtained from individuals with wheat allergy (positive for wheat-specific IgE by ImmunoCAP [>0.35 UA/mL] and history of immediate reaction to wheat or positive for wheat food challenge; $n = 12$) or without wheat allergy (negative for wheat-specific IgE; $n = 2$) (Table 1).

2.3. Wheat protein extraction and sample preparation

Wheat was milled to pass through 80-mesh sieves and the flour was treated with petroleum ether (1:10, w/v) for degreasing. Wheat proteins were extracted according to Osborne's (Osborne, 1924) sequential procedure, with slight modifications as shown in Fig. 1. Water/salt-soluble proteins (albumins/globulins) were extracted with Tris-HCl (20 mM, pH 8.6) plus 0.5 M NaCl for 2 h at 4 °C with constant stirring from 10.0 g of defatted wheat meal. After centrifugation at 9000 rpm at 4 °C for 30 min, the supernatant (water/salt-soluble protein) and sediment were collected, respectively. The supernatant fractions were dialyzed extensively with distilled water at 4 °C to obtain water-soluble protein fraction and salt-soluble protein fraction respectively. The sediment was applied in the extraction of water/salt-insoluble protein fractions (ethanol-soluble protein and glutenin). The sediment was then dissolved in 70% ethanol, and centrifuged as above to obtain ethanol-soluble protein fractions (supernatant), followed by Tris-HCl (20 mM, pH 8.6) extraction plus 50% 1-propanol, 0.5 percent DTT, 1% SDS and 1 percent acetic acid to obtain glutenin fractions.

In order to obtain total wheat fraction extracts for cross-allergenicity analysis, defatted wheat meal was suspended in 10-fold Na_2CO_3 - NaHCO_3 buffer (20 mM, pH 9.6, 0.5 M NaCl, 1 mM PMSF) containing 10%, 20%, 30%, 40%, 50%, 60% and 70% ethanol, respectively, and stirred at 4 °C for 10 h to optimize the extraction condition. The slurry was centrifuged for 30 min at 9000 rpm, and the supernatants were then used by SDS-PAGE for further analysis. Other cereals, including barley, rye, oat, buckwheat, quinoa, rice and maize extracts, have been prepared according to our optimized methods.

Protein recovery in defat wheat flour samples using different extraction methods as determined by the Bradford reagent assay kit (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). Protein recovery were expressed as mg protein/g wheat flour.

2.4. SDS-PAGE analysis

Electrophoresis was conducted with 5% concentration gel and 12% resolving gel, using a Bio-Rad electrophoresis method (Powerpac Universal, Bio-Rad Laboratories, Inc., USA). Each sample was boiled with 2 $\times$ loading buffer containing SDS and β -ME at a ratio of 1:1 (v/v) for 7 min. Five micrograms (5 μg) of each sample were loaded into each well,

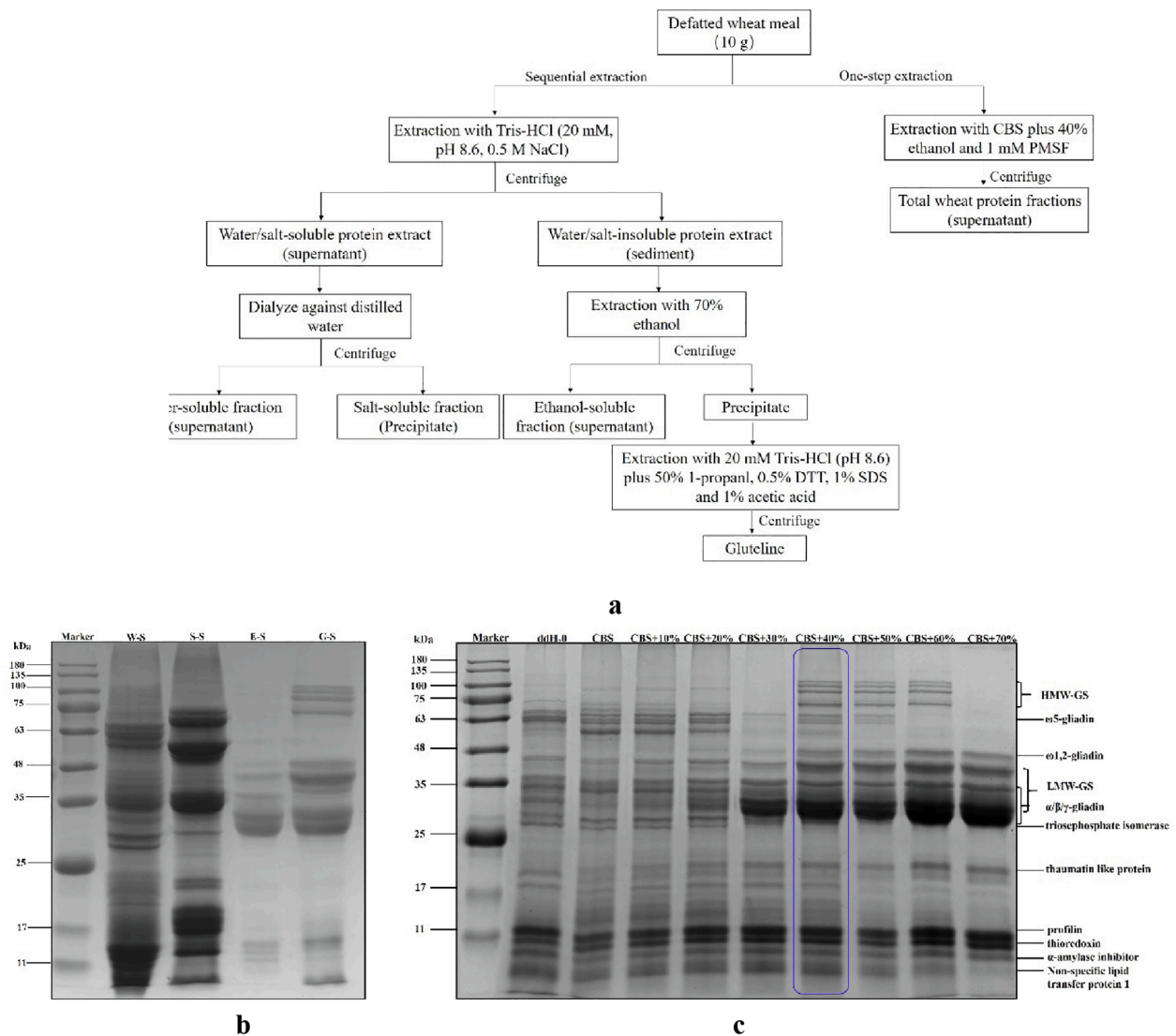


Fig. 1. Wheat protein extraction schematic diagram and SDS-PAGE analysis. (a) Schematic diagram of the wheat protein extraction using sequential extraction and one step extraction, CBS represents $\text{Na}_2\text{CO}_3\text{-NaHCO}_3$ (20 mM, pH 9.6, 0.5 M NaCl, 1 mM PMSF). (b) SDS-PAGE analysis of wheat flour protein fractions using sequential extraction, W-S represents water-soluble proteins fractions, S-S represents salt-soluble proteins fractions, E-S represents ethanol-soluble proteins fractions, G-S represents glutenins fractions, (c) SDS-PAGE analysis of total wheat flour extracts.

ran at 80 V for 45 min, then ran at 120 V for about 1 h under tris-glycine-SDS buffer condition. The gel was stained with Coomassie Brilliant Blue stain solution (0.25% Coomassie Brilliant Blue R-250, 45% methanol, and 45% acetic acid) for 10 min and destained until the separate protein bands were clear and then photographed using a BIO RAD Universal Hood II gel imaging system (Bio-Rad Laboratories, Inc., CA, USA).

2.5. Western blotting analysis

Western blotting was done according to the previous method to evaluate the IgE binding and cross-allergenicity of different cereal allergens (Xu et al., 2020). The separating gel with resolved proteins was washed using ddH_2O , and the gel was well-adjusted with 20% ethanol for 7 min before allocation, then the proteins were transferred to a PVDF membrane using iBlotTM gel transfer stacks (Thermo Fisher Scientific, Massachusetts, USA). Subsequently, the membrane was incubated with wheat allergy sera (diluted 1:2 with ibind solution) and HRP-labeled goat anti-human IgE (1:2000 with ibind solution) at room temperature for 6 h. Following washing with PBST (10 mM PBS, 0.15 M NaCl, 0.05%

Tween 20, pH 7.4) for 30 min, the results were obtained using multiple exposure time ECL solution to obtain an optimal signal from the BIO RAD Universal Hood II gel imaging system (Bio-Rad Laboratories, Inc., CA, USA).

2.6. Indirect ELISA analysis

In order to determine cross-allergenicity of wheat allergens, wheat, barley, rye, oat, buckwheat, quinoa, rice and maize extract with 50 mM $\text{Na}_2\text{CO}_3\text{-NaHCO}_3$ buffer (pH 9.6) were diluted to 5 $\mu\text{g}/\text{mL}$ and coated in a 96-well microplate (Greiner Bio-One, Kremsmünster, Austria) with 100 μL per well at 4 °C overnight. The plate was washed three times with PBST and then blocked with 1% (w/v) BSA (200 $\mu\text{L}/\text{well}$) at 37 °C for 2 h. After that, the plate was again washed with PBST three times and incubated with 100 μL of wheat-allergic human sera (1:10 dilution) at 37 °C for 1.5 h. Subsequently, the plate was washed and incubated with HRP-labelled goat anti-human IgE (1:4000 dilution) at 37 °C for 1.5 h and washed five times with PBST. Until detection, the chromogenic reaction was developed in the dark using TMB for 10 min and stopped with 2 M sulphuric acid, then the optical density (OD) was obtained at

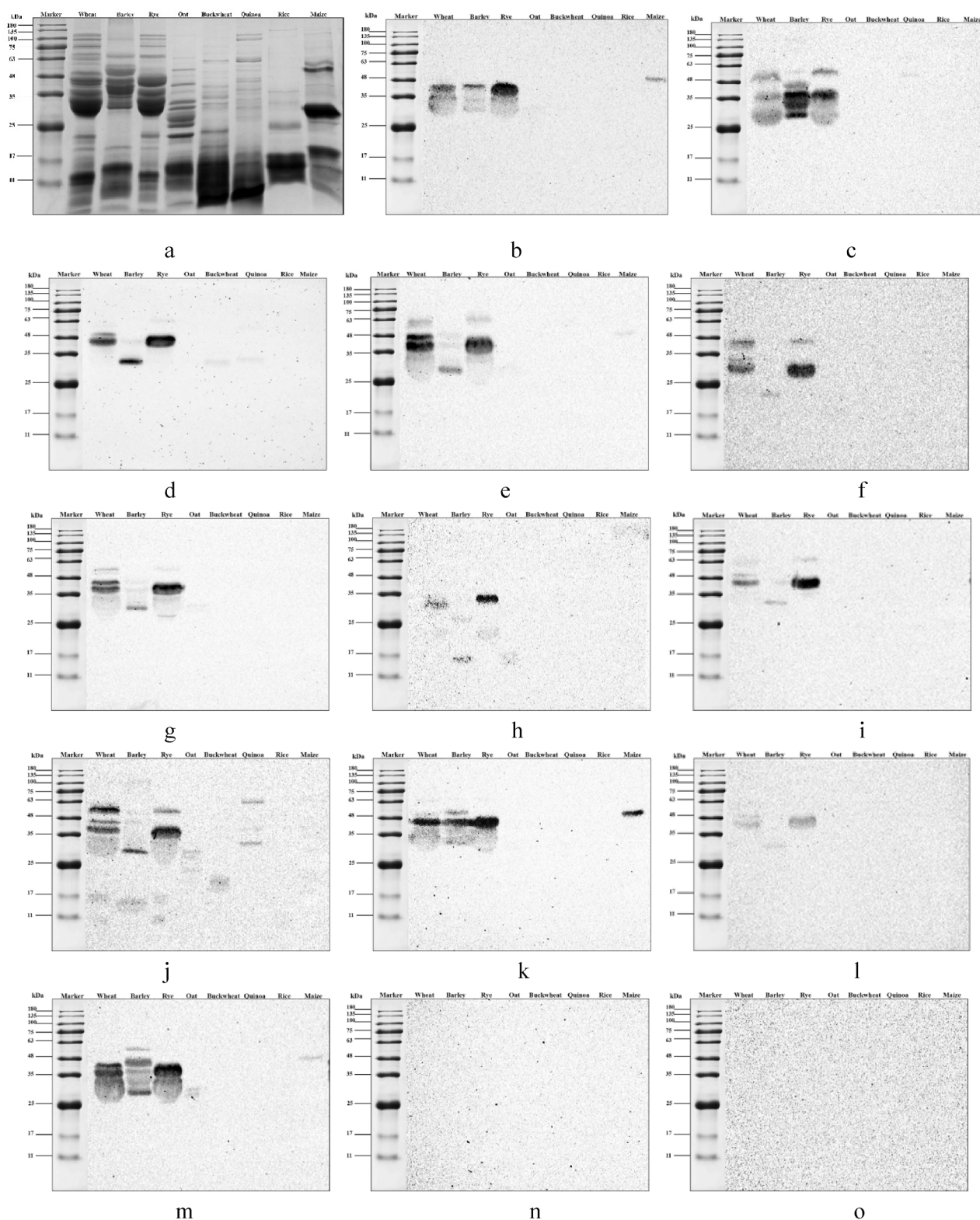


Fig. 2. Western blot analysis of patient IgE reactivity to different protein extracts from wheat, barley, rye, oat, buckwheat, quinoa, rice and maize. (a) SDS – PAGE pattern of different protein extracts. Western blot analysis of different protein extracts using 12 wheat allergic sera (b-m, represent different wheat patients' serum P1-P12) and 2 non-allergic patients (n and o represent 2 non-allergic patients).

450 nm wavelength using a MultiscanMK3 microplate reader (Thermo Fisher Scientific Inc., Waltham, MA, USA). Each sample was repeated three times.

2.7. Inhibition ELISA analysis

The cross-allergenicity of different cereal allergens was assessed by using 5 selected serum samples (P1, P2, P6, P7 and P9) by inhibition ELISA method as described in previously reported work (Lv, Lin, Li,

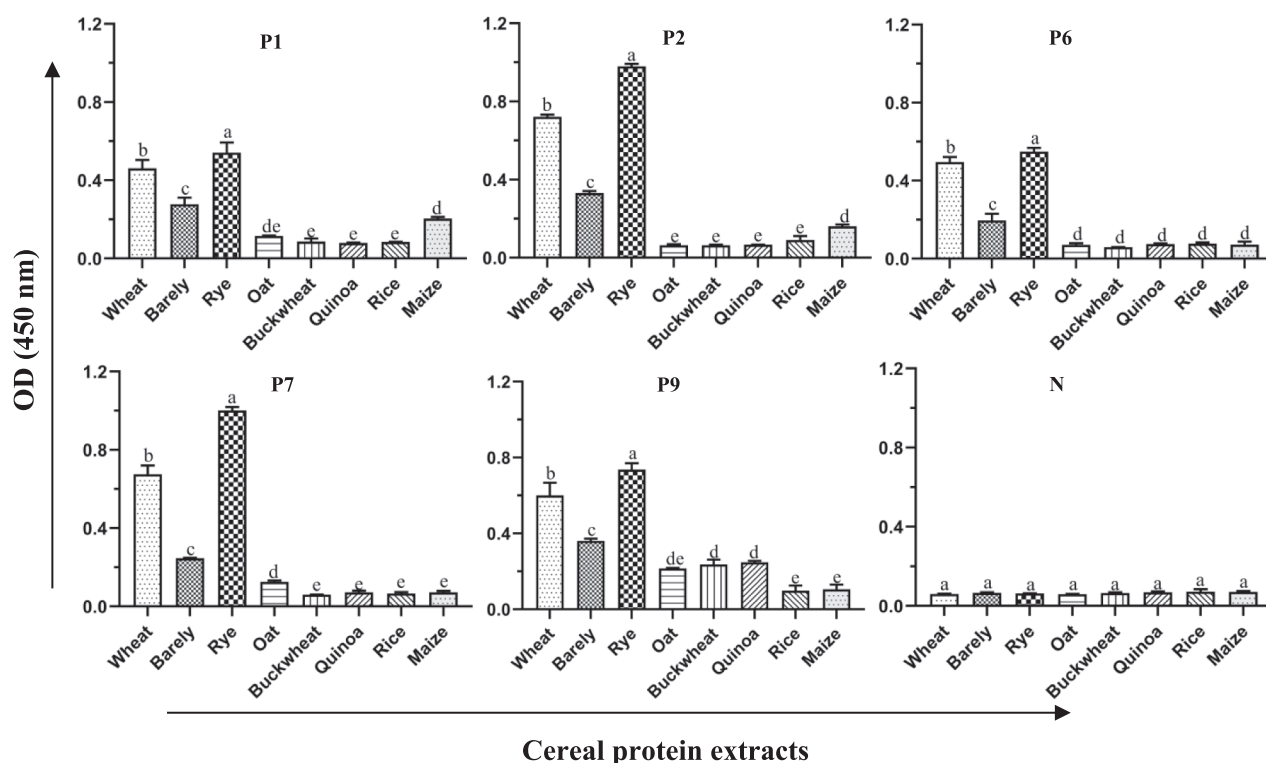


Fig. 3. Indirect ELISA for wheat patient serum IgE binding to different protein extracts from wheat, barley, rye, oat, buckwheat, quinoa, rice and maize. Different protein extracts (0.5 μ g) was coated in ELISA plate and was incubated with five typical wheat allergic patients' sera (P1, P2, P6, P7 and P9) and a negative pool serum (N) from two non-allergic human sera at a dilution of 1:10. The OD values (450 nm) in control wells without antigen using equal amounts of PBST was subtracted from antigen-coated wells. The data is presented as the mean values for triplicates and the standard deviation is indicated by error bars. Different lowercase letters in column mean significant differences ($P < 0.05$) among different cereal protein extracts.

Yuan, Gao, & Ma, 2016). The wheat allergen extracts (0.5 μ g) were coated in a 96-well microplate at 4 °C overnight and blocked with 1% (w/v) BSA (200 μ L/well) followed by three washes with PBST. In addition, the wheat-allergic patients serum diluted 10 times were incubated at a final concentration of 0.2, 1.0, 5.0, 25.0 and 125.0 μ g/mL for 2 h at a temperature of 37 °C with protein extracts (wheat, barley, rye, oats, buckwheat, quinoa, rice or maize). The inhibition mixtures (with no positive control inhibitor) were then applied to each well (100 μ L/well) and incubated at 37 °C for 1.5 h. The assay was then performed as described above and the percentage inhibition was calculated using the formula below:

$$\% \text{Inhibition} = 100 - \text{Inhibition} = 100 - \left(\frac{\text{OD}_{450} \text{ of serum with inhibitor}}{\text{OD}_{450} \text{ of serum without inhibitor}} \times 100 \right)$$

2.8. Statistical analysis

All assays were conducted in triplicates, and the values observed were represented as mean $\pm$ standard deviation. The statistical analysis was carried out using the software SPSS, version 11.5 and GraphPad Prism 8.01. The statistical difference when $P < 0.05$ was considered significant.

3. Results

3.1. Extraction of wheat flour protein

Wheat protein was first extracted using sequential extraction to assess our methods of total wheat flour protein fractions. The molecular

weight profile of different fractions of wheat protein was analyzed by SDS-PAGE (Fig. 1b). The fractions of water-soluble protein (W-S), salt-soluble protein (S-S), ethanol-soluble protein (E-S) and glutenins (G-S) have different polypeptide composition profiles. The W-S fraction had the highest proportion of 14 kDa with 6 major polypeptides (65, 60, 58, 30, 28 and 14 kDa). S-S fractions ranged from 14 kDa to 75 kDa in molecular weight with five main polypeptides (68, 58, 35 and 17 kDa). E-S fractions achieved with 70% ethanol showed a narrow range of molecular weight between 50 kDa and 25 kDa. While the G-S fraction exhibited a wider range of molecular weights, the protein profile corresponds to previous studies (DuPont, Chan, Lopez, & Vensel, 2005; Weiss, Vogelmeier, & Görg, 1993).

Fig. 1c reveals SDS-PAGE patterns of total protein extracts from defatted wheat flour in ddH₂O, CBS (Na₂CO₃-NaHCO₃, 20 mM, pH 9.6, 0.5 M NaCl, 1 mM PMSF) and CBS with varying ethanol content (10%-70%). The obtained protein band patterns showed a considerable difference under varied extraction conditions. Most bands were present in the region 7 kDa to 130 kDa, and CBS with 40% ethanol extraction solution with a significant number of bands was found in the region of 7 kDa to 130 kDa with the broadest range of molecular mass. In short, in lane ddH₂O, CBS, CBS + 10%, CBS + 20%, CBS + 30% and CBS + 70% the large molecular fractions (>75 kDa) were almost invisible. However, the lane CBS + 40%, CBS + 50% and CBS + 60% showed a similar profile, and 63 kDa was not clear.

In addition, the extractability of different extraction methods was determined using the Bradford assay (S Table 1). Recovery of defatted wheat flour using sequential extraction reach up to 107.90 mg protein/g wheat powder, while the recovery from single step extraction ($\leq 40.73 \pm 0.24$ mg protein/g wheat powder) is much lower than the sequential extraction. The protein recovery decreased from 40.73 ± 0.24 mg protein/g wheat powder (ddH₂O) to 16.47 ± 0.21 mg protein/g wheat powder (CBS + 50%) with increasing ethanol contents.

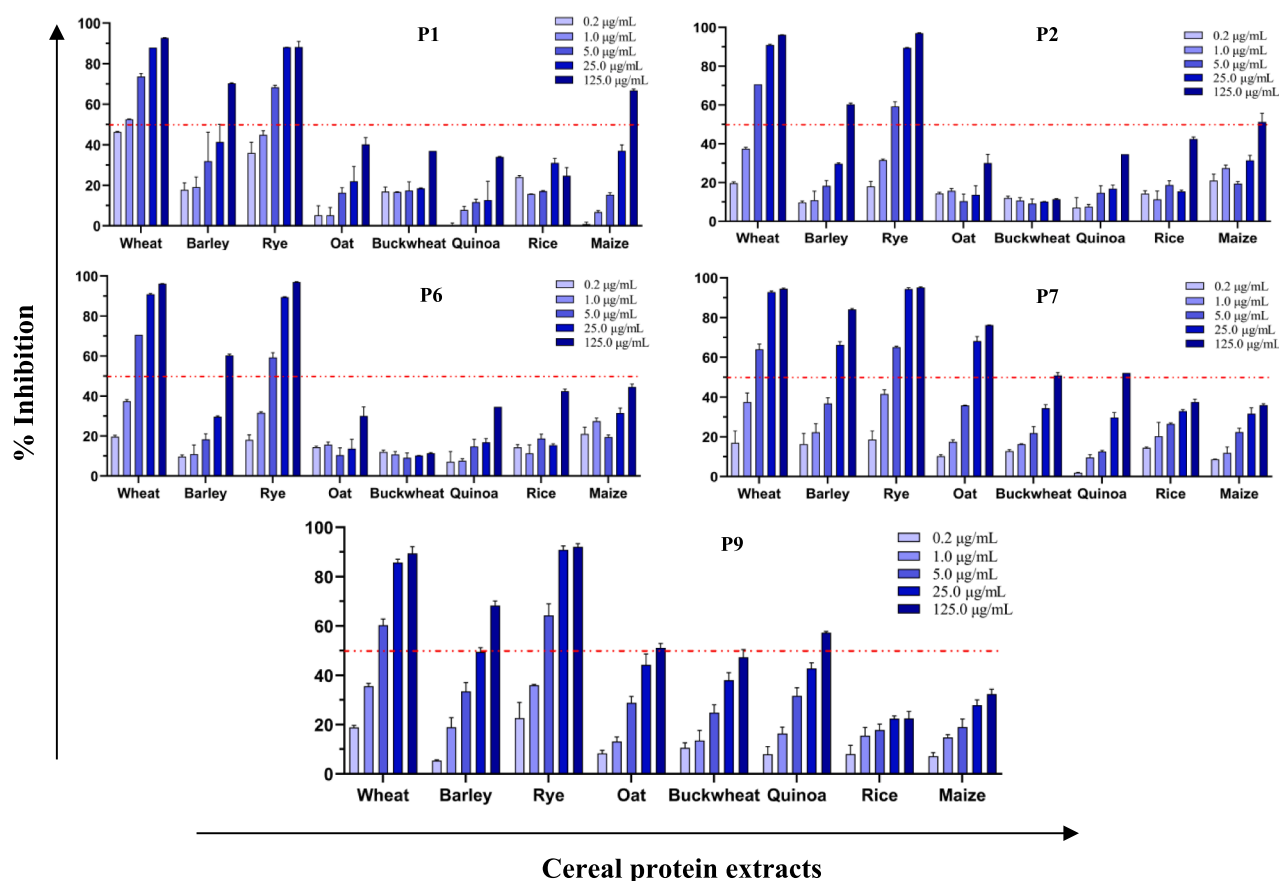


Fig. 4. Inhibition ELISA for wheat patient serum IgE binding to different cereal protein extracts. Wheat, barley, rye, oat, buckwheat, quinoa, rice and maize protein extracts (0.5 µg) was coated in ELISA plate, respectively. Five wheat allergic patients' serum (P1, P2, P6, P7 and P9) were inhibited by wheat, barley, rye, oat, buckwheat, quinoa, rice and maize protein extracts. The data is presented as the mean values for triplicates and the standard deviation is indicated by error bars. Red dotted line represents 50% inhibition of IgE binding. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Western blot

The pattern of SDS-PAGE for different protein composition of grains is shown in Fig. 2a. A similar profile of wheat and rye was observed, while other grains display obvious differences in protein profile. In accordance with previous studies, the fractions of barley, rye, oat, buckwheat, quinoa, rice and maize consist of polypeptides in the estimated molecular weight range (Amagliani, O'Regan, Kelly, & O'Mahony, 2017; DuPont, Chan, Lopez, & Vensel, 2005).

To analyze the cross-reactivity among wheat, barley, rye, oat, buckwheat, quinoa, rice and maize and to determine specific peptides responsible for IgE reactivity, immunoblotting analysis of 12 patients' sera including two non-wheat allergic sera incubated with cereal protein extracts was conducted (Fig. 2). Clinical data for patients with wheat allergy is summarized in Table 1. All serum of wheat patients ($n = 12$) revealed significant IgE reactive bands for wheat, barley, and rye protein fractions. Each cross-reacting serum presented a different IgE binding band from 25 kDa to 75 kDa for wheat, barley and rye extracts of varying sizes. Besides that, four minor IgE reactive bands in oat extracts were found in sera P6, P7, P9 and P12, while P3 and P9 sera showed weaker IgE reactive bands in buckwheat and quinoa, and lastly four of the twelve sera individuals (P1, P4, P10 and P12) showed clear immunoblot binding to maize protein fractions up to 50 kDa. Using sera of all patients, negligible IgE binding to rice extracts was obtained. There was no specific IgE reactivity in the two negative patient sera for wheat, barley, rye, oat, buckwheat, quinoa, rice and maize protein.

3.3. Indirect ELISA

IgE reactivity was further investigated by indirect ELISA for wheat, barley, rye, oat, buckwheat, quinoa, rice and maize extracts using sera from 5 allergic wheat patients (P1, P2, P6, P7 and P9) and a serum pool for two non-allergic wheat patients. As illustrated in Fig. 3, IgE reactivity of different cereal extracts with varying levels of IgE binding was observed in all five representative wheat allergic patients. Wheat, barley, and rye extracts had significantly higher IgE reactivity ($P < 0.05$) than oat, buckwheat, quinoa, rice, and maize extracts and the maximal IgE reactivity ($OD_{450\text{ nm}} \geq 0.54$) to rye was observed in all five wheat-allergic patients, followed by wheat ($OD_{450\text{ nm}} \geq 0.46$) and barley ($OD_{450\text{ nm}} \geq 0.20$) extracts. Patients 1 and 2 showed higher IgE reactivity ($P < 0.05$) to maize extracts than oat, buckwheat, quinoa, rice extracts, while patient 9 had higher IgE reactivity ($P < 0.05$) for oats, buckwheat, and quinoa extracts than for rice and maize extracts. Minimal IgE binding was obtained with the non-wheat allergic and non-atopic control sera. In addition, positive IgE binding values was considered as $OD \geq 2$ times non-wheat allergic serum IgE binding values plus 2S.D. According to Table 2, all five wheat-allergic patients present significant IgE binding to wheat, barley, and rye extracts ($P < 0.05$). However, patient 9 showed positive IgE binding to oat, buckwheat and quinoa while IgE was positive for maize extracts in patients 1 and 2.

3.4. Inhibition ELISA

In order to further evaluate the cross-reactivity, the ability of cereal extracts to inhibit IgE binding to wheat allergen extracts was assessed

individually using sera from five allergic wheat subjects (P1, P2, P6, P7 and P9). For all allergic individuals, different levels of inhibition were detected with specific cereal extracts (Fig. 4). Overall, wheat, barley and rye extracts caused an apparent dose-dependent inhibition of the wheat patients' serum (P1, P2, P6, P7 and P9), and >90% inhibition was obtained at 125.0 µg/mL of wheat and rye extract (final concentration) in all wheat allergic patients. Interestingly, higher levels of inhibition were observed with rye extracts compared to wheat extracts. Additionally, barley showed strong inhibition of IgE binding to wheat extract (30–76%), while oat, buckwheat, quinoa, and maize extracts showed a weaker inhibition (11–67%) at the maximum concentration of inhibitors whereas, rice extracts showed limited inhibition of IgE binding to all wheat allergic patients serum at a maximum concentration of inhibitors (22–42%).

4. Discussion

Wheat allergy is a major public health issue and a global food safety problem (Cianferoni, 2016; Keet, Matsui, Dhillon, Lenehan, Paterakis, & Wood, 2009). Extraction of wheat allergens is crucial for proper diagnosis and quantitative detection of allergens. Because of significant differences in the solubility of individual wheat flour proteins, allergens considered important for wheat food allergic reactions are marginalized in total wheat fraction extract-based tests, which could be the main cause of undetected wheat-specific sensitivities, and the deficiency of decision levels for wheat IgE antibody predicting clinical reactivity (Nilsson et al., 2015; Sampson, 2001). Solvent-fractionated wheat proteins using sequential extraction methods require a lot of denaturants to fully dissolve fractions of glutenin and detailed analysis of individual protein fractions (Aalberse & Aalberse, 2015; Denery-Papini, 2003; DuPont, Chan, Lopez, & Vensel, 2005). Therefore, a reliable, fast and simple procedure for the extraction of allergens is considered necessary. In the present study, efficient extraction of whole wheat fractions with mild conditions were investigated. Considering the importance of the appropriate method for this study, it was observed that whole wheat flour allergens with mild conditions were effectively extracted, following which the possibility of cross-reactivity with other cereal protein fractions were analyzed. In order to evaluate and validate our methods of total wheat flour protein using sequential extractions can be clearly observed by SDS-PAGE in Fig. 1b and c. The results indicated that the protein profile from different fractions (W-S, S-S, E-S, and G-S) showed similarities to previous reports of (Sharma, 2012; Weiss, Vogelmeier, & Görg, 1993) (Fig. 2b), but there are many extraction steps.

Therefore, based on their differential solubility in a series of solvents, a different extraction buffer containing salt and alcohol with higher pH was analyzed by numerous pre-experiments to obtain total wheat fractions efficiently. One-step extraction of total wheat fractions using CBS (20 mM, pH 9.6, 0.5 M NaCl) containing 10%, 20%, 30%, 40%, 50%, 60% and 70% ethanol was carried out and optimized, respectively, and the results of extraction were analyzed by SDS PAGE (Fig. 1c). The extract pattern of proteins using CBS with 40% ethanol extraction solution showed the most bands (about 25). The widest range of molecular mass, and nearly all protein fractions using sequential extraction were included in the protein band in SDS gel. The SDS PAGE pattern of wheat extracts from single step extraction is almost the same as total protein fractions from six classes of wheat flour prepared using 8 M urea, 40 mM Tris, and 4% 3-[(3-cholamidopropyl) dimethylamino]-1-propanesulfonate (CHAPS) by Akagawa, Handoyo, Ishii, Kumazawa, Morita, & Suyama (2007). According to the previous reports, the water/salt-soluble albumin/globulin fraction mainly consists of non-specific lipid transfer protein 1 (9 kDa), amylase inhibitor (12 kDa), thio-redoxin (13 kDa), proflin (14 kDa), protein-like thaumatin (18 kDa) and triosephosphate isomerase (26 kDa), thiol reductase homologue (27 kDa), serpin (40 kDa) were identified as allergens in baker's asthma and urticaria. Most of the wheat proteins, known as prolamins (gliadins and

glutenins), are not soluble in saline solutions, primarily containing α/β-gliadin (28–35 kDa), γ-gliadin (28–35 kDa), ω_{1,2}-gliadin (40 kDa), ω₅-gliadin (65 kDa), low molecular weight glutenin (LMW-GS, 32–40 kDa) and high molecular weight glutenin (HMW-GS, 70–95 kDa) (Tri a 26) in wheat gluten have been reported as allergens (<http://www.allergen.org>) (Mamone, Picariello, Addeo, & Ferranti, 2011; Matsuo, Yokooji, & Taogoshi, 2015). In our research work, above mentioned wheat allergens albumin/globulin, gliadin and glutenin fractions, were extracted using CBS + 40% ethanol (Fig. 1c) with the protein recovery of 18.06 ± 0.79 mg protein/g wheat powder (S Table. 1). The lower recovery relative to sequential extraction might be attributed to the mild buffer without SDS and DTT or β-ME additions. While such chemical reagents might destroy or generate new IgE binding epitopes that cause inappropriately labeled allergens and inaccurate diagnoses. Wheat allergic patients may be susceptible to multiple proteins present in water-soluble, salt-soluble, ethanol-soluble and glutenin fractions. Whole protein fractions are crucial for their application in the diagnostic tests that are used to assess a patient's sensitization. Thus, the one-step mild extraction method to obtain the majority of wheat protein including the major wheat allergens, has a huge advantage in combining several fractions into a single test. Thus, protein recovery yields should not be paid too much attention in evaluating the advantage of one-step extraction and sequential extraction. Complete solubilization of wheat protein fractions in mild solution conditions could facilitate us to detect the predominant IgE-binding proteins in wheat flour efficiently.

Wheat is being consumed in diverse cooked formulations and a variety of food products, such as noodles, baked goods, pizza, bulgur, and couscous, as one of the staple dietary source for people all over the world (Nakase, Usui, Alvarez-Nakase, Adachi, & Matsuda, 1998; Quirce, Boyano-Martínez, & Díaz-Perales, 2016). However, wheat belongs to the family of Poaceae, so it can cross react with many members of this family and can lead to many adverse reactions such as allergic reactions (Pourpak, Mesdaghi, Mansouri, Kazemnejad, Toosi, & Farhoudi, 2005; Quirce, Boyano-Martínez, & Díaz-Perales, 2016; Tatham & Shewry, 2008). Cross-reactivity among the Poaceae family was observed in the present study including wheat, barley, oat, rye, and maize, respectively. Our results confirmed by western blotting, indirect, and inhibition ELISA have shown that cross-reactions occur between cereals (barley, rye, oat, and maize), involving several proteins in this phenomenon. Western blotting analysis showed that wheat allergenic protein bands have broad IgE-reactive protein bands ranging from 25 to 75 kDa (α/β-gliadin, γ-gliadin, ω₅-gliadin and LMW-S) in all reactive sera with 12 kDa (amylase inhibitor) and 18 kDa (thaumatin like protein) in one of the twelve reactive sera (P9) (Fig. 2), which suggested that gliadins and glutenins could be important allergenic components. Specific IgE-reactive scattering bands were also found in total wheat flour protein fractions using 8 M urea combined ultrasound with 7 mixed sera wheat allergic patients (Akagawa, Handoyo, Ishii, Kumazawa, Morita, & Suyama, 2007). Wide range of IgE reactive bands in rye extracts showed in all wheat patients, which could be due to structurally similar epitopes of various proteins that are taxonomically closely related (Bietz, 1982). In some previous research studies, patients with wheat allergy, water/salt-insoluble wheat proteins (gliadins and glutenins) were seen as causative allergens (Battais et al., 2005; Hofmann, Fischer, Eriksson, Bengtsson Gref, Biedermann, & Jakob, 2012; Kliever, Venter, Cassin, Abonia, Aceves, Bonis, et al., 2016). Specific IgE binding in all wheat allergic patients found that barley extracts with molecular weight 25 to 65 kDa, an IgE band with P7 and 18 kDa serum (identified as protein-like thaumatin) indicated significant cross-reactivity among wheat, barley and rye. Extracts of oat and maize appeared to have a poor cross-reactivity to wheat, rye and barley, as shown in Fig. 2, in which the IgE-binding is very weak in wheat, rye and barley in the majority of patient's sera, suggesting a weaker cross-reactivity.

Subsequently, we further analyzed the potential cross-reactivity of wheat protein extracts with barley, rye, oat, buckwheat, quinoa, rice, and maize extracts using indirect and inhibition ELISA using 5 standard

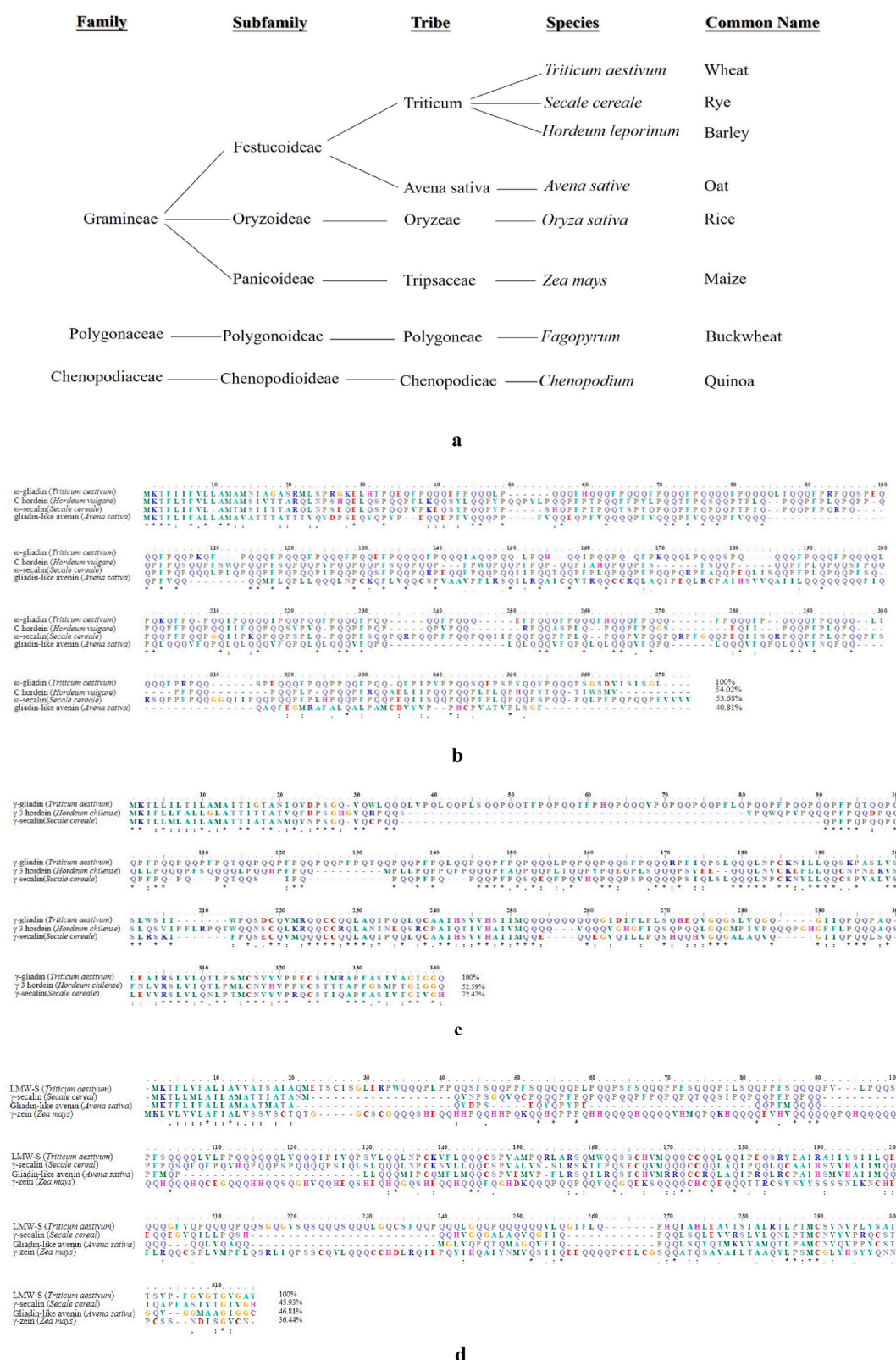


Fig. 5. Taxonomic relationship analyses and sequence alignment. (a) Taxonomic relationship between cereal grains and grasses of family Poaceae. Alignment of allergen sequences with *Triticum aestivum* β -gliadin (GenBank accession number: AAA34289.1) (b), α -gliadin (GenBank accession number: AJG03079.1) (c) and LMW-S (GenBank accession number: AAP44989.1) (d) as reference using NCBI Protein BLAST. Species include *Secale cereale*, *Hordeum leporinum*, *Avena sativa*, *Oryza sativa*, *Zea mays*, *Fagopyrum*, *Chenopodium*. No significant similarity had been analyzed by sequence alignment. Sequence identity is also shown.

positive wheat allergic sera as per western blotting assay. The cutoff value for indirect ELISA significant IgE binding has been identified as OD ≥ 2 of normal human serum plus 2S.D. In all five wheat allergenic patients, we recorded increased IgE binding for wheat, barley, and rye extracts, oat and maize provided lower IgE binding for two out of five serum samples, while buckwheat, quinoa, and rice showed unacceptable IgE binding for all serums (Fig. 3). These findings are in accordance with western blotting assay analyzes that suggest a high cross-reactivity between wheat, barley and rye and a lower cross-reactivity in oat and maize (Kliewer, et al., 2016; Pourpak, Mesdaghi, Mansouri, Kazemnejad, Toosi, & Farhoudi, 2005; Tatham & Shewry, 2008). The results of the inhibition ELISA showed that all patients were highly inhibited by IgE binding in barley and rye extracts, and little affected by oat and maize extracts, further verifying the cross-reactive effect between wheat, barley, rye, oat and maize (Fig. 4). Many studies have shown that a large number of allergenic wheat patients have adverse reactions from barley, rye or oat, but none of them have allergic reactions to rice, which may be the result of their taxonomic relationship (Allen et al., 2014; Pourpak, Mesdaghi, Mansouri, Kazemnejad, Toosi, & Farhoudi, 2005).

Our work has shown significant cross-reactivity between cereal grains (barley, rye, or oat) examined in wheat allergic patients through western blotting, indirect, and ELISA inhibition assays, while our findings differ in case of maize. Cereal grains are taxonomically classified into the Gramineae family by phylogenetic relationships (Fig. 5a), including wheat, barley, rye, oat, rice and maize, while wheat, barley, rye and oat belong to the Festucoideae subfamily; rice and maize belong to the Oryzeae and Tripsaceae subfamilies, while buckwheat and quinoa belong to subfamilies (Polygonaceae and Tripsaceae) (Jones, Magnolfi, Cooke, & Sampson, 1995). The close taxonomy and evolutionary relationship may be responsible for the strong cross-reactivity between cereal grains, including wheat, barley and rye. Typical repetitive units are QQPQQPFP in γ -gliadins, QQQFP in ω -gliadins, QQQPFPs in LMW-GS with high sequence similarity among hordein and secalin using sequence alignment analysis (Fig. 5 b-d). The whole sequence identity of allergens from wheat (γ -gliadin, ω -gliadin and LMW-S), barley (γ 3 hordein, C hordein) and rye (γ -secalin, ω -secalin) is higher than 50%, whereas no significant similarity was found between buckwheat, quinoa and rice allergens (<30%). Furthermore, our findings suggested that rice, buckwheat and quinoa proteins represented negligible IgE reactivity including inhibition of IgE binding to wheat allergic serum due to taxonomic difference and reduced. Thus, it can be pointed out that other grains such as rice, non-grain buckwheat and quinoa may be adequate and effective substitutes for patients with wheat allergy.

5. Conclusion

In this current research work, a reliable, efficient and measurable one-step extraction method was developed using Na₂CO₃-NaHCO₃ (20 mM, pH 9.6, 0.5 M NaCl, 40% ethanol, 1 mM PMSF) extraction solution to extract whole wheat protein fractions, including maximum wheat flour allergens that could be used for accurate diagnosis and quantitative detection of wheat allergens. In addition, the total protein fractions obtained from common dietary cereal were further used in the analysis of cross-reactivity using 12 wheat allergenic serum. Results showed that substantial cross-reactivity among cereals, including wheat, barley, and rye, was established due to the close phylogenetic relationships and high sequence identity (>50%), slightly lesser in oats and maize, whereas rice, buckwheat and quinoa share a relatively insignificant cross-reactivity. In particular, people with wheat allergy should specifically avoid eating barley and rye while being careful in the consumption of oat and maize. Therefore, it is recommended that rice, non-grain quinoa and buckwheat can be appropriate alternatives for wheat allergy patients.

CRedit authorship contribution statement

Jinlong Zhao: Investigation, Methodology, Writing-original draft. **Zhenxing Li:** Conceptualization, Methodology, Writing-review&editing, Supervision, Funding acquisition, Data curation, Supervision. **Mati Ullah Khan:** Writing-review&editing, Formal analysis, Validation, Resources. **Xiang Gao:** Conceptualization, Validation, Resources, Formal analysis. **Min Yu:** Validation, Resources. **Hongyan Gao:** Validation, Resources. **Yonghong Li:** Methodology. **Hewen Zhang:** Methodology. **Binaka Prabashini Dasanayaka:** Writing-review&editing. **Hong Lin:** Data curation, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2021.129064>.

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- 1 S Table 1 Protein recovery in defat wheat flour samples using different extraction methods as determined by the
- 2 Bradford assay.

Extraction methods		Protein recovery (mg protein/g wheat powder)
Sequential extraction	W-S	22.16±0.69
	S-S	11.65±0.12
	E-S	47.68±0.40
	G-S	26.41±0.41
	ddH ₂ O	40.73±0.24
	CBS	25.92±1.30
One-step extraction	CBS+10%	22.57±0.04
	CBS+20%	18.85±0.33
	CBS+30%	18.98±0.29
	CBS+40%	18.06±0.79
	CBS+50%	16.47±0.21
	CBS+60%	18.89±0.14
	CBS+70%	22.37±0.67

4 **S Table 2** The OD (450 nm) values of IgE-binding activity to cereal protein extracts incubated with sera from
5 wheat allergic patients.

Sera	OD (450 nm)							
	Wheat	Barely	Rye	Oat	Buckwheat	Quinoa	Rice	Maize
P1	0.46±0.04 ^c	0.28±0.03 ^b	0.54±0.05 ^c	0.12±0.01 ^b	0.09±0.01 ^c	0.08±0.00 ^c	0.08±0.00 ^b	0.20±0.00 ^a
P2	0.72±0.01 ^a	0.33±0.01 ^a	0.98±0.01 ^a	0.06±0.01 ^c	0.06±0.00 ^c	0.07±0.00 ^c	0.09±0.01 ^b	0.16±0.00 ^a
P6	0.49±0.02 ^c	0.20±0.03 ^c	0.55±0.02 ^c	0.07±0.01 ^c	0.06±0.00 ^c	0.07±0.00 ^c	0.08±0.00 ^b	0.07±0.01 ^c
P7	0.67±0.04 ^{ab}	0.25±0.01 ^b	1.00±0.02 ^a	0.13±0.01 ^b	0.06±0.00 ^c	0.07±0.01 ^c	0.07±0.00 ^b	0.07±0.01 ^c
P9	0.60±0.05 ^b	0.36±0.01 ^a	0.74±0.03 ^b	0.22±0.00 ^a	0.24±0.04 ^a	0.25±0.01 ^a	0.10±0.02 ^a	0.12±0.02 ^b
N	0.13±0.01 ^d	0.13±0.01 ^d	0.13±0.01 ^d	0.12±0.01 ^b	0.13±0.01 ^b	0.14±0.01 ^b	0.16±0.01 ^a	0.14±0.01 ^b

6 Results are expressed as mean ± standard deviation (n=3). Values with different superscript lowercase letters in
7 the same column mean differ significantly ($P < 0.05$). The values of N serum expressed as 2 OD mean ±standard.