

Immunoreactivity and Detection of Wheat Proteins by Commercial ELISA Kits

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Wheat proteins are responsible for sensitivities, including baker's asthma, immunoglobulin E (IgE)-mediated allergic reaction, wheat-dependent, exercise-induced anaphylaxis, and celiac disease. The detection of gluten/wheat traces in foods is important to safeguard the health of wheat-sensitive individuals and comply with food labeling. Many immunoanalytical-based commercial kits are available for the quantification of gliadin/gluten/ wheat proteins. We compared the immunoreactivity of wheat fractions with wheat-allergic human serum IgE and antibody conjugates used in six commercial immunoassay kits. Moreover, the performance of the kits was tested using corn flour spiked with gluten (5, 10, 25, and 50 ppm) and wheat flour (50, 100, 250, and 500 ppm). The albumin, globulin, gliadin, and glutenin fractions reacted with IgE from nine, eight, two, and eight patients' sera, respectively, out of nine wheat allergic patients tested. Among the antibodies from commercial kits, those from R-Biopharm, Morinaga, and Romer Labs reacted strongly with the gliadin fraction, whereas those from BioKits, ALLER-TEK, and ELISA Systems reacted strongly with the glutenin fraction. All kits showed minimal or no reactivity with albumin and globulin fractions. All kits detected the gluten and wheat flour in a corn flour matrix at the lowest spiked levels of 5 and 50 ppm, respectively. However, there was wide variation among the kits when comparing the recovery of gluten and wheat flour. The recovery was also dependent on the source material (gluten or wheat flour) used for spiking the corn flour matrix.

Wheat is one of the most widely consumed cereal grains in the world, with total production of about 685.6 million tons in 2009 (1). China is the highest wheat producer, followed by India. The United States produces about 60 million tons of wheat and ranks first in wheat export, with exports averaging about 50% of total production. The consumption of wheat varies depending on the geographic location and lifestyle. In the United States, wheat is commonly consumed in the form of pasta and bakery products. Although not a high dietary source of protein, the presence of a particular

type of protein called gluten makes wheat uniquely suitable in baking processes.

Wheat proteins can be classified based on their solubility into albumins (water soluble), globulins (salt soluble), gliadins (alcohol soluble prolamins), and glutenins (acid/alkali soluble glutelins). The heterogeneous proteins from gliadin and glutenin fractions are together called gluten. The gliadin and glutenin fractions show sequence similarity and can be classified based on disulfide bond formation (2). The gliadin fraction consists of monomeric proteins containing intramolecular disulfide bonds and can be divided into α -, β -, γ -, and ω -gliadins on the basis of their mobility at low pH in polyacrylamide gel electrophoresis (PAGE). On the other hand, the glutenin fraction consists of polymeric proteins linked together by intermolecular disulfide bonds and can be divided into high-molecular weight glutenin subunits (HMW-GS) and low-MW glutenin subunits (LMW-GS) on the basis of their mobility observed in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions.

Though enjoyed by most, wheat can cause cell-mediated food intolerance in celiac patients and immunoglobulin E (IgE)-mediated, life-threatening reaction to wheat-allergic individuals. Since there is no cure currently available for celiac disease or wheat allergy, a wheat- and/or gluten-free diet is the only effective treatment for sensitive patients. Wheat gluten, specifically the gliadin fraction, is the causative agent for celiac disease and has been reported to affect 0.5–1.0% of the U.S. population (3). The prevalence of wheat allergy in the United States has been reported to be 0.5% in adults (4) and 0.4–1.0% in children (5). Wheat is listed as one of the “Big 8” food groups responsible for more than 90% of food allergies. Unlike other common allergens, wheat is not only associated with classic IgE-mediated allergic reactions upon ingestion, but also with baker's asthma and wheat-dependent, exercise-induced anaphylaxis (WDEIA). Several allergens have been identified in different fractions of wheat proteins. Wheat ω -5 gliadin and HMW-GS are major allergens responsible for WDEIA (6), though a recent report suggests LMW-GS can also induce WDEIA (7). Most of the causative allergens for baker's asthma are derived from albumin and globulin fractions including enzymes (acyl-CoA oxidase, fructose bisphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenases, triosephosphate isomerase, and peroxidase); inhibitors (serpin, α -amylase inhibitor, and chymotrypsin inhibitor); thioredoxin; agglutinin; and lipid transfer protein (8, 9). There is considerable overlap among the proteins responsible for classical wheat allergy and other wheat sensitivities (celiac, WDEIA, and baker's asthma). Wheat fractions involving several proteins have been associated with wheat allergy, with major allergens being albumins/globulins for mainly children with atopic dermatitis and ω -5 gliadin for adults with anaphylaxis or urticaria (10).

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Undeclared gluten/wheat in foods poses a major risk to wheat-sensitive patients. Current regulations mandate declaration of wheat on the ingredient label if used in any food product. However, unintended addition of wheat or gluten during food processing as a result of cross-contamination or poor manufacturing practices may jeopardize the health of wheat allergic and celiac patients. Moreover, celiac patients are also at risk from other gluten sources, such as rye, barley, and possibly oats. Sensitive, robust, and reliable wheat/gluten detection and quantification methods are needed to ensure consumer safety and to comply with food labeling laws. Several polyclonal and monoclonal antibody-based ELISA kits are commercially available for the detection of gliadin/gluten/wheat. The current study evaluates the immunoreactivity of wheat protein fractions to wheat allergic human serum IgE and antibody conjugates from commercial test kits. We also estimated gluten and wheat flour recovery in food matrix spiked at various levels using the commercial test kits.

Experimental

Materials

Whole wheat flour and gluten-free corn flour were purchased from a local grocery store. Additionally, wheat flour standard reference material, SRM 1567a, was purchased from the National Institute of Standards and Technology (NIST; Gaithersburg, MD). Gluten, gelatin, alkaline phosphatase labeled goat anti-human IgE, and 4-methylumbelliferyl phosphate were purchased from Sigma Chemical Co. (St. Louis, MO). Black microtiter plates were purchased from BD Falcon (Bedford, MA) and clear Immulon 1 B microtiter plates from Thermo Electron Corp. (Milford, MA). The 660 nm protein assay reagent and enhanced chemiluminescence (ECL) substrate were purchased from Pierce (Rockford, IL). SureBlue 3,3',5,5'-tetramethylbenzidine (TMB) substrate was purchased from KPL Inc. (Gaithersburg, MD). Wheat-allergic human sera were purchased from PlasmaLab International (Everett, WA).

Commercial Test Kits

The test kits used in the study were RIDASCREEN® Gliadin (R-Biopharm AG, Darmstadt, Germany), wheat protein ELISA kit (Morinaga Institute of Biological Science, Inc., Yokohama, Japan), BioKits gluten assay kit (Neogen Corp., Lansing, MI), ALLER-TEK™ gluten ELISA assay (ELISA Technologies, Inc., Gainesville, FL), AgraQuant® gluten assay (Romer Labs UK Ltd, North Wales, UK), and Gliadin kit (ELISA Systems, Queensland, Australia).

Wheat Protein Fractionation

Unprocessed whole wheat flour purchased from a local grocery store was subjected to Osborne fractionation to separate four main fractions (albumin, globulin, gliadin, and glutenin) based on their solubility in different solvents (11, 12). Briefly, 20 g flour was suspended in 200 mL 0.5 M NaCl and magnetically stirred for 1 h at room temperature (RT), followed by centrifugation at 10000 g for 20 min at 4°C. The supernatant (containing albumins and globulins) was dialyzed against distilled water for 48 h with six water changes. The globulins

were separated by centrifuging at 15000 g for 20 min. The residual flour pellet was washed three times with 200 mL 0.5 M NaCl for 5 min each at RT before gliadin extraction. The washed residual flour was then suspended in 200 mL 75% (v/v) ethanol and magnetically stirred for 1 h at RT, followed by centrifugation at 10000 g for 20 min to obtain the gliadins. The pellet was washed three times with 200 mL 75% ethanol for 5 min each at RT. Glutenins were finally extracted by suspending the residual flour from ethanol extraction in 200 mL 50% (v/v) isopropanol containing 1% (v/v) acetic acid and 1% (v/v) β -mercaptoethanol (β -ME) for 1 h at RT, followed by centrifugation at 10000 g for 20 min. Both the gliadin and glutenin fractions were dialyzed against distilled water for 48 h with six water changes. All the protein fractions were lyophilized and stored at -20°C until further use.

Wheat proteins were extracted by vortexing whole wheat flour or protein fractions in an appropriate buffer for 1 h at RT followed by centrifugation at 10000 g for 15 min. The total protein in the supernatant was measured by 660 nm protein assay reagent using bovine serum albumin as the standard protein.

Fluorimetric ELISA (F-ELISA)

The F-ELISA was performed to evaluate the binding of wheat-allergic human serum IgE with wheat fractions (10). Total wheat proteins were extracted in cocktail solution containing 2 M guanidine hydrochloride (GuHCl) and 250 mM β -ME. Stock solutions of purified proteins were prepared by suspending 10 mg protein in 500 μ L buffer: albumin in carbonate buffer, pH 9.6; globulin in carbonate buffer containing 0.5 M NaCl; gliadin in 75% ethanol (v/v); and glutenin in carbonate buffer containing 0.1% SDS and 0.2% β -ME. Flat-bottom 96-well black microtiter plates were coated with total wheat proteins and wheat fractions at 10 μ g/mL in carbonate buffer (100 μ L/well) and incubated for 2 h at 37°C. The plates were washed three times with phosphate-buffered saline containing 0.05% (v/v) Tween 20 (PBS-T) and blocked with 200 μ L blocking buffer [0.5% (w/v) gelatin in PBS-T] for 1 h at 37°C. After washing the plates three times with PBS-T, plates were incubated for 2 h at 37°C with 100 μ L/well of wheat-allergic human serum diluted 1:5 (v/v) in the blocking buffer. The plates were washed three times with PBS-T and incubated for 2 h at 37°C with 100 μ L/well alkaline phosphatase-labeled goat antihuman IgE diluted 1:1000 (v/v) in the blocking buffer. The plates were washed again three times with PBS-T and incubated for 90 min in the dark at RT with 150 μ L/well of fluorescent substrate, 4-methylumbelliferyl phosphate, diluted 1:5 (v/v) in 1 M Tris-HCl, pH 9.8. The fluorescent product was measured in a microplate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA) using 360 and 440 nm filters for excitation and emission, respectively. Relative fluorescence for each serum was determined by the ratio of the fluorescence for coated antigen to that obtained with no antigen, and values greater than 2.5 were considered positive. Results were expressed as the mean of triplicate readings. Appropriate human serum controls, including tissue transglutaminase IgA positive (PlasmaLab International, Everett, WA), atopic (IBT Reference Laboratories, Lenexa, KS), and nonatopic serum (Centers for Disease Control, Atlanta, GA) were also used.

Competitive ELISA

The binding intensity of kit antibody conjugates to the wheat fractions was determined by competitive ELISA. The total wheat proteins and wheat fraction extracts prepared for F-ELISA were used. A clear 96-well microtiter plate was coated with total wheat proteins at 1 µg/well in carbonate buffer and incubated for 1 h at 37°C. The plate was washed three times with Tris-buffered saline containing 0.05% (v/v) Tween 20 (TBS-T) after each step. The unbound sites were blocked by incubation with 5% (w/v) nonfat dried milk (NFDM) in TBS-T for 1 h at 37°C. In a separate plate, 90 µL antiwheat/gluten/gliadin antibody suitably diluted in 1% (w/v) NFDM in TBS-T was added to each well. In the top well, 10 µL total wheat proteins or wheat fractions (1 mg/mL) were added and serially diluted 10-fold in the successive wells. The plate was incubated for 1 h at 37°C, and, thereafter, 80 µL contents were transferred to the respective wells of the coated plate. The coated plate was further incubated for 1 h at 37°C. Since all antibodies were conjugated to horseradish peroxidase (HRP), the color was developed by incubating with 100 µL/well TMB substrate for 30 min. The reaction was stopped by adding 100 µL/well 1 M HCl, and the optical density (OD) of the plate was read at 450 nm. The wells without the antibody were used as blanks. The % inhibition was calculated by the formula:

$$\% \text{ Inhibition} = 100 - \left\{ \frac{(\text{OD}_{\text{inhibitor}} - \text{OD}_{\text{blank}})}{(\text{OD}_{\text{no inhibitor}} - \text{OD}_{\text{blank}})} \times 100 \right\}$$

SDS-PAGE and Western Blotting

The protein extracts were boiled in SDS-PAGE sample buffer (50 mM Tris-HCl, pH 6.8, 1% SDS, 30% glycerol, 0.01% bromophenol blue, and 2% v/v β-ME) for 10 min, and suitable amount of protein was loaded on a NuPAGE® Novex 4–12% Bis-Tris mini gel (Invitrogen Corp., Carlsbad, CA). The gel was run at a constant current (30 mA) until the tracking dye migrated to the gel edge. The gel was stained overnight with 0.25% (w/v) Coomassie Brilliant Blue R (BBR) containing 50% (v/v) methanol and 10% (v/v) acetic acid. The gel was destained with 50% (v/v) methanol containing 10% (v/v) acetic acid until the background was clear.

For Western blotting, the gel was run as described above and polypeptides transferred onto 0.2 µm nitrocellulose membrane for 1.5–2 h at 10°C using NuPAGE transfer buffer. The transferred proteins were visualized with Ponceau S stain. The unbound sites on the nitrocellulose membrane were blocked by incubation for 1 h at RT in 5% NFDM prepared in TBS-T. The membrane was washed with TBS-T for 5 min and incubated overnight with appropriate dilutions of HRP conjugated antiwheat/gluten/gliadin antibodies at 4°C. The membrane was washed 3 times with TBS-T for 15 min each. The reactive bands were developed by incubating the membrane with ECL substrate for 1 min and exposing to the Kodak BioMax light film.

Preparation and Analysis of Spiked Corn Flour for Recovery Studies

Wheat- or gluten-spiked corn flour was prepared by adding 50 mg gluten or NIST wheat flour into 100 g corn flour

(equivalent to 500 ppm spiked level). The spiked samples were mixed overnight in a roller drum (Model No. TC-7, New Brunswick Scientific, Edison, NJ), followed by further dilutions with the same corn flour matrix and overnight mixing to obtain final spiked levels of 5, 10, 25, and 50 ppm gluten for gluten-spiked matrix, and 50, 100, 250, and 500 ppm wheat flour for wheat flour-spiked matrix. Four subsamples from each unspiked and spiked matrix were analyzed in duplicate by each commercial test kit, as per the manufacturer's recommendation.

Results and Discussion

Wheat Protein Fractionation

Wheat proteins were fractionated depending on their solubility. The SDS-PAGE of protein fractions is shown in Figure 1. Based on the CBBR stained polypeptide composition, there was no crossover of the bands observed between the fractions, indicating high purity of the fractions. The albumins were composed of polypeptides ranging from 12–70 kDa, with a major polypeptide at approximately 15 kDa. The globulins were composed of 15–75 kDa polypeptides, dominated by approximately 40, 60, and 72 kDa bands. Similar profiles of albumin and globulin have been observed previously (11, 13). The different types of gliadin and glutenin subunits in Figure 1 were identified based on their electrophoretic mobility under reducing conditions. The 15 kDa polypeptide observed in the gliadin fraction may be the LMW components of gliadin or a contamination from the albumin/globulin fraction. One of the important wheat allergens, α-amylase/trypsin inhibitor subunits (15–16 kDa), has been identified not only in the albumin/globulin fraction, but also in the gliadin and glutenin fractions (13).

Immunoreactivity of Wheat Fractions

Reactivity with wheat allergic patient sera.—The IgE reactivity of wheat-allergic patient serum to protein fractions was determined by F-ELISA. All wheat allergic patients recognized wheat extract while the IgE from the negative controls (transglutaminase IgA positive, atopic, and nonatopic) did not exhibit any IgE reactivity (Figure 2). Of the nine wheat-allergic patients tested, all reacted with albumin, eight each with globulin and glutenin, and two with gliadin. Among the gluten proteins, glutenin exhibited higher IgE reactivity than the gliadin fraction. The allergenic polypeptides within the protein fractions cannot be revealed by F-ELISA, and further studies involving immune-blot of purified proteins derived from the fractions and their proteomic analysis will be helpful in identifying specific IgE-reactive polypeptides. The allergen identified by patient serum IgE may be partially dictated by the type of wheat allergy. In a study by Mittag et al. (15) involving patients with baker's asthma, wheat allergy, and WDEIA, the albumin/globulin fraction showed the most intense IgE-reactivity in wheat allergic and baker's asthma patients, whereas WDEIA patients showed strong IgE-reactivity to gliadins and glutenins. Similar high reactivity to the albumin/globulin fraction (72%) has been reported by Battais et al. (10) in a study involving 60 wheat-allergic patients. Among the gliadin and glutenin fractions, they reported high IgE responses to ω5-gliadin and LMW-GS, respectively.

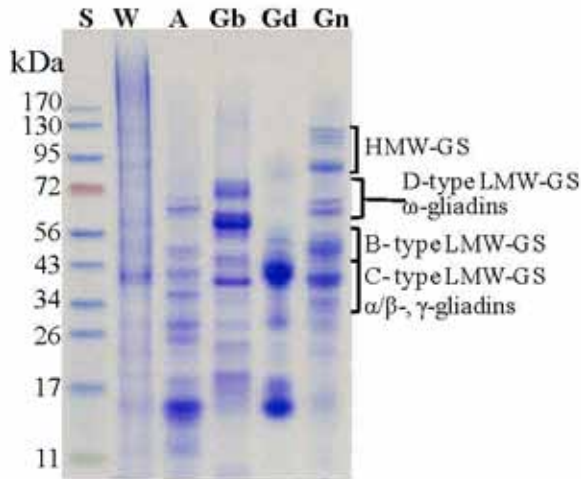


Figure 1. CBBR staining of wheat fractions. S-protein standards; W-whole wheat; A-albumin; Gb-globulin; Gd-gliadin; Gn-glutenin. HMW and LMW are high and low molecular weight glutenin subunits, respectively. Protein load in each lane was 10 μ g.

Reactivity with commercial kit antibodies.—Six different commercial ELISA kits detecting wheat proteins/gluten/gliadin were used to determine their reactivity with wheat fractions. Competitive inhibition ELISA curves for the wheat fractions to inhibit wheat proteins using the conjugate antibody from the commercial kits are shown in Figure 3. The inhibition in competitive ELISA is dependent on the concentration of the antibody and the inhibitor. Though the inhibitor concentration was constant, the antibody concentration may be different; hence, the inhibition cannot be compared quantitatively among the antibodies. Of the tested kits, R-Biopharm, Morinaga, and Romer Labs antibodies exhibited highest inhibition with the gliadin fraction, whereas BioKits, ALLER-TEK, and ELISA Systems antibodies exhibited highest inhibition with the

glutenin fraction. Interestingly, the antibodies from BioKits, ALLER-TEK, and ELISA Systems also showed similar high inhibition with total wheat protein extract. All of the antibodies showed very weak inhibition of wheat proteins when globulin fraction was used as an inhibitor.

Similar results were obtained when the antibody conjugates were incubated with wheat fractions in Western blotting (Figure 4). R-Biopharm, Morinaga, and Romer Labs antibodies reacted strongly to the gliadin, followed by glutenin and albumin. Although the above three kits recognized gliadin strongly, the Western blot profile of fractions identified by the R-Biopharm antibody was different compared to Morinaga and Romer Labs, indicating different specificity of the antibodies. Morinaga uses a proprietary polyclonal antibody for quantitative determination of wheat proteins (16, 17). Romer Labs uses gliadin-directed antibodies in its kit, which is evident from the high affinity of their antibody toward the gliadin fraction. Immunoblots are useful to determine the binding specificities of the antibodies used in ELISA, but they differ from ELISA in many aspects such as protein treatment (e.g., heat and chemical denaturation in Western blotting) and binding sites required (e.g., two sites in sandwich ELISA). The signal intensity of binding to gluten proteins cannot be compared among the antibodies due to varying concentrations of antibodies used in Western blotting. However, the qualitative binding pattern revealed that the antibodies from BioKits, ALLER-TEK, and ELISA Systems reacted similarly to wheat fractions, with high immunoreactivity to glutenin (>55 kDa), followed by gliadin. Biokits, ALLER-TEK, and ELISA Systems antibodies did not react to albumin. None of the antibody conjugates from the kits tested exhibited any reactivity with the globulin fraction.

The R-Biopharm kit uses R5 monoclonal antibody raised against a secalin (prolamin in rye) extract that recognizes the potential celiac-toxic pentapeptide QQFPF, which occurs frequently in wheat α -, γ -, and ω -gliadins; hordeins (prolamin in barley); and secalins (18). The reactivity of the R5 antibody with the glutenin fraction in ELISA and Western blot may be due to the presence of R5 reactive epitope in the LMW-GS and/or traces of gliadin in the glutenin fraction.

The similar immunoreactivity of BioKits, ALLER-TEK,

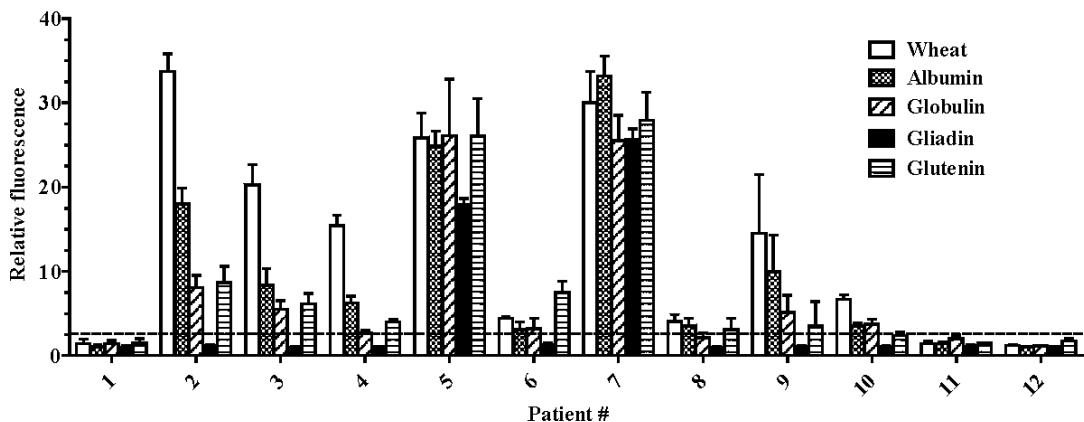


Figure 2. Immunoreactivity of wheat-allergic patient serum IgE to different wheat fractions (mean $\pm$ SD, $n = 3$). Patients No. 2–10 are wheat-allergic patients. Negative controls include patient No. 1 (tissue transglutaminase IgA-positive), No. 11 (atopic), and No. 12 (nonatopic). The horizontal dotted line (relative fluorescence = 2.5) denotes the cutoff for positive reading.

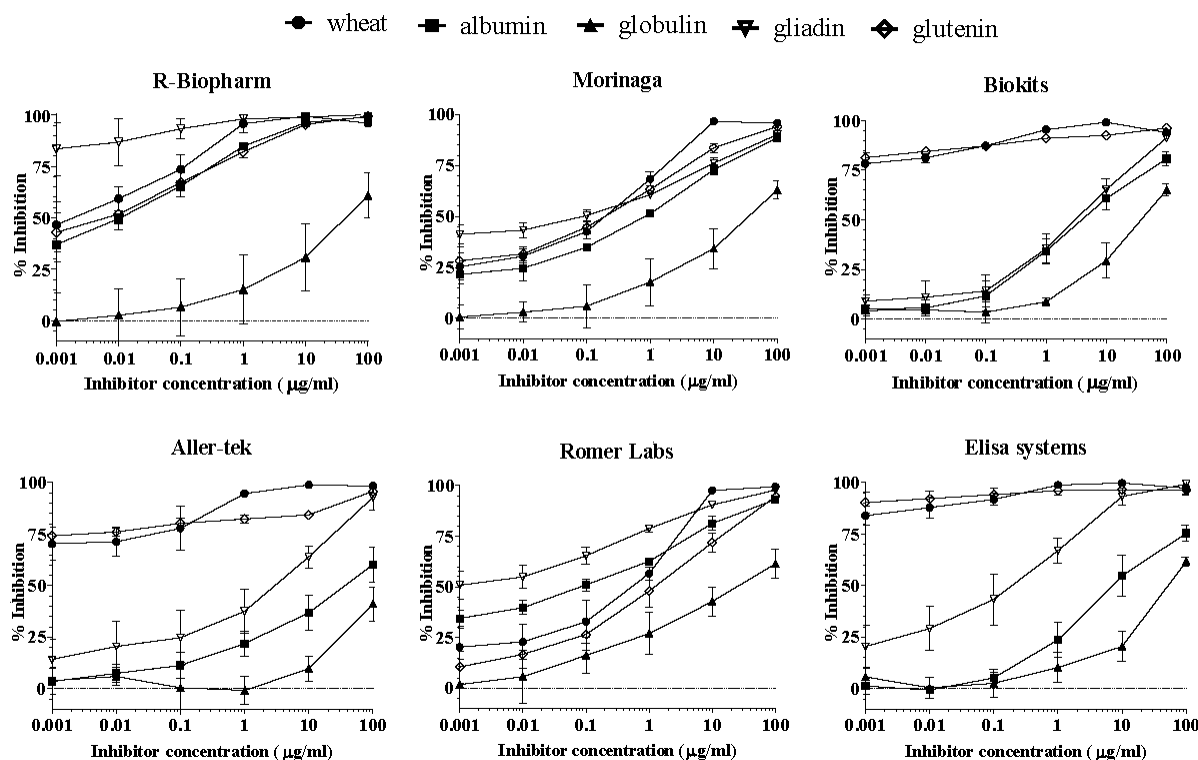


Figure 3. Competitive inhibition of total wheat proteins by wheat fractions as inhibitors using kit antibodies (mean $\pm$ SD, $n = 3$).

and ELISA Systems kits is not surprising since all three kits are based on the Skerritt monoclonal antibody [kit manual and (16)]. Though the Skerritt antibody is raised against the ω -gliadin fraction of gluten from the Australian wheat variety Timgalen (18), the antibody shows high reactivity with the glutenin fraction. A similar high affinity of the Skerritt antibody towards glutenin has been reported earlier (16, 19, 20). This could be due to the extraction of ω -gliadin along with the glutenin fraction or presence of gliadin-like subunits in the glutenin polymers. The D type LMW-GS is believed to be derived from modified ω -gliadin components that have acquired a cysteine residue (21).

Recovery of Gluten- and Wheat Flour-Spiked Corn Flour

The detection of gluten or wheat protein in spiked corn flour was assessed by the commercial ELISA kits. These kits vary in their LOQ and reporting unit of detection. The recovery of different gluten and wheat flour spiked samples by the commercial kits is presented in Table 1. Gluten content in the unspiked corn flour was below the LOQ for all kits except Morinaga, which detected a higher wheat protein amount (1.4 ppm) than its LOQ (0.3 ppm) in the unspiked corn flour. Though the amount detected by the kits at a given spiked level varied widely depending on the kit used for quantification, all of the tested kits were able to detect gluten and wheat flour at the lowest spiked level of 5 and 50 ppm, respectively. The average

recovery from different spiked levels of gluten- and wheat flour-spiked corn flour by various tested kits is shown in Figure 5.

Gluten-spiked corn flour was detected at similar levels to the actual spiked level by R-Biopharm, Morinaga, BioKits, and ELISA Systems kits, with an average recovery of 122, 166, 104, and 127%, respectively. The Romer Labs and ALLER-TEK kits detected three to six times higher gluten content than the spiked level. Geng et al. (22) reported differences in gluten recovery with commercial kits when gluten-spiked food matrix were extracted in ethanol versus cocktail solution. The differences in the gluten recovery from Skerritt antibody-based assays may be in part due to the use of different calibration standards by these kits. Overall, the variation in the recovery among the kits may be attributed to the differences in (i) extraction procedures, including buffer composition, sample to buffer ratio, extraction time, and temperature, (ii) specificity of the detection antibody, (iii) target analyte detected, (iv) calibration standards used, (v) conversion factor used in protein calculation, and (vi) reporting units. R-Biopharm and ELISA Systems kits measure gliadin content in the sample and use a conversion factor of 2 to calculate total gluten from gliadin content though both assays are based on different antibodies, R5 and Skerritt, respectively. Such a conversion factor is not used by the user for other Skerritt antibody-based kits (BioKits and ALLER-TEK). It may be possible that the factor is already included in the calibrators or gluten is used as a calibration standard. This conversion factor has been questioned recently, as it may vary among cereal grains. Wieser and Koehler (23) reported a prolamin to glutenin ratio of 1.50–3.14 for common wheat

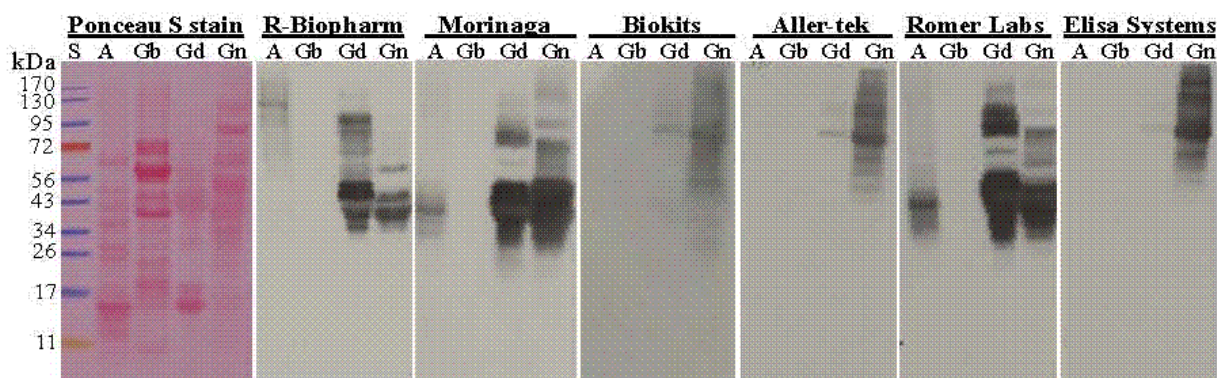


Figure 4. Ponceau S stain of transferred proteins and Western blot of wheat fractions with kit antibodies. S-protein standards; A-albumin; Gb-globulin; Gd-gliadin; and Gn-glutenin. Protein load in each lane was 20 μ g.

analyzed by a combination of Osborne protein fractionation and RP-HPLC. Morinaga measures wheat protein content in ng/mL, and uses a conversion factor to calculate wheat protein in ppm from the standard curve. BioKits, ALLER-TEK, and Romer Labs kits measure the gluten content in the sample that can be read directly from the standard curve.

It has been well known that extraction buffer plays a vital role in ELISA since the target analyte has to be extracted into the solution in order to be detected by the detection antibody. All commercial ELISA kits tested, except Romer Labs, come with its own sample extraction mixture/solution, and all except Morinaga need aqueous ethanol during the extraction process. The kits also use different sample-to-buffer ratio, extraction time, and temperature, further affecting protein recovery. The R-Biopharm kit uses the two-step extraction with cocktail solution and 60% ethanol (final concentration). The presence of a disaggregating agent (GuHCl) and a reducing agent (β -ME) in cocktail extraction solutions helps improve gliadin recovery in heat-treated foods as compared to the 60% aqueous ethanol extraction (24). Morinaga uses a proprietary extraction solution

containing SDS and β -ME for high protein recovery; moreover, the samples are extracted overnight or for at least 12 h. The BioKits, ALLER-TEK, and ELISA Systems buffer consists of an unknown extraction additive/solution along with 40% aqueous ethanol, whereas Romer Labs uses only 40% aqueous ethanol for protein extraction.

In the case of wheat flour-spiked samples, the lower detection values observed as compared to the spiked level is due to the fact that the kits are estimating the protein content (gluten or total wheat protein). The gluten content is approximately 10% in wheat flour; thus, the expected values for gluten in Table 1 for wheat flour-spiked samples would be 10% of the spiked level. The nitrogen (micro-Kjeldahl method) in wheat flour used for spiking was estimated to be 2.2015% ($n = 2$; Micro Analysis Inc., Wilmington, DE). Considering the conversion factor of 5.7, the protein content in wheat flour was 12.55%. The average recovery of wheat flour shown in Figure 5 was calculated by assuming that 80% of total wheat protein is gluten. Hence, the amount of wheat flour detected by all kits estimating gluten was calculated by dividing the obtained gluten content by 0.1004

Table 1. Gluten content measured by ELISA kits in gluten- and wheat flour-spiked corn flour^a

Spiked material	Spike level, ppm	R-Biopharm ^c (5 ppm) ^b	Morinaga (0.3 ppm)	BioKits (3 ppm)	ALLER-TEK (5 ppm)	Romer Labs (4 ppm)	ELISA Systems ^c (5 ppm)
Unspiked	0	<LOQ	1.4 $\pm$ 0.4	<LOQ	<LOQ	<LOQ	ND ^d
Gluten	5	5.9 $\pm$ 1.5	8.2 $\pm$ 2.3	6.2 $\pm$ 1.5	32.9 $\pm$ 15.2	24.3 $\pm$ 9.8	7.1 $\pm$ 1.9
	10	16.0 $\pm$ 9.3	16.4 $\pm$ 7.0	12.2 $\pm$ 3.1	44.1 $\pm$ 15.3	30.9 $\pm$ 11.8	12.4 $\pm$ 4.3
	25	33.6 $\pm$ 7.7	45.1 $\pm$ 19.0	26.0 $\pm$ 2.4	112.0 $\pm$ 34.5	72.7 $\pm$ 19.0	35.0 $\pm$ 4.1
	50	37.7 $\pm$ 17.3	77.9 $\pm$ 14.0	32.5 $\pm$ 2.6	264.4 $\pm$ 70.2	137.3 $\pm$ 8.0	50.6 $\pm$ 12.6
Wheat flour	50	5.2 $\pm$ 0.9	8.1 $\pm$ 0.6	8.8 $\pm$ 1.9	73.7 $\pm$ 7.0	16.8 $\pm$ 3.5	15.5 $\pm$ 3.0
	100	8.0 $\pm$ 2.1	16.0 $\pm$ 2.3	18.5 $\pm$ 1.6	165.8 $\pm$ 10.9	32.5 $\pm$ 4.6	41.6 $\pm$ 3.1
	250	15.8 $\pm$ 1.6	42.0 $\pm$ 7.8	27.4 $\pm$ 1.3	392.0 $\pm$ 60.6	68.7 $\pm$ 6.3	107.3 $\pm$ 7.3
	500	26.0 $\pm$ 3.5	72.0 $\pm$ 4.1	57.4 $\pm$ 4.6	844.9 $\pm$ 117.5	122.6 $\pm$ 9.4	215.7 $\pm$ 22.8

^a Values reported as measured gluten content by the kits, except Morinaga measured as wheat protein.

^b Values in parenthesis represent LOQ as ppm of gluten for all kits, except for Morinaga reported as ppm of wheat protein. LOQ reported as the minimum concentration of standard provided with the kit for those kits that did not report LOQ.

^c Data reported as gluten content by multiplying measured gliadin by 2.

^d Not detected in three subsamples and 2.3 ppm gluten detected in one subsample.

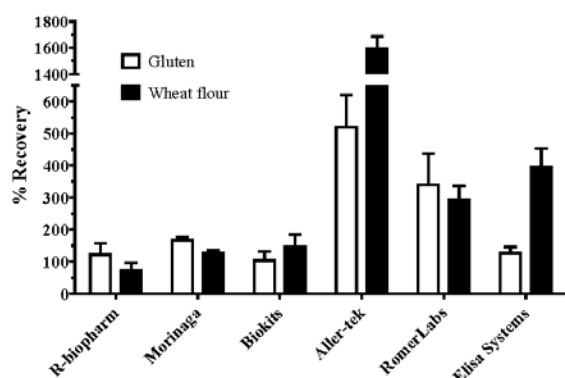


Figure 5. Average recovery of gluten and wheat flour in spiked corn flour using ELISA kits.

($0.1255 \times 0.8 = 0.1004$). Since the Morinaga kit estimates total wheat protein, the amount of wheat flour detected was calculated by dividing the obtained total wheat protein content by 0.1255. Based on these calculations, all kits except R-Biopharm overestimated the gluten or wheat protein content in wheat flour-spiked samples. R-Biopharm exhibited average wheat flour recovery of 74%. Among the kits exhibiting high wheat flour recovery, the Morinaga kit estimated wheat proteins in wheat flour close to the spiked level with an average wheat flour recovery of 126% followed by BioKits (146%) and Romer Labs (294%).

Since most of the kits estimate gliadin or gluten content in the sample, their recovery studies involve gliadin- or gluten-spiked food matrixes (processed or unprocessed). However, under a practical food industry scenario, the cross-contamination of gluten in foods can occur not only with gluten, but also with cereal grains containing gluten storage proteins. The wheat flour contamination in food will further increase the gluten detection complexity, as there is a strong correlation ($r > 0.80$) between the protein content of flour and the amount of total gliadin (25), and different wheat varieties are known to have different protein contents. It was not surprising to observe an overestimation of gluten in wheat flour-spiked matrixes for kits that overestimated gluten in gluten-spiked matrixes (e.g., Romer Labs, ALLER-TEK). However, although kits such as ELISA Systems exhibited close to 100% recovery for gluten-spiked matrixes (127%), the recovery was much higher for wheat flour (395%). The variation in gluten quantitation observed for the same kit when using different spiking material can be attributed to the presence of different amounts of target analyte in the spiking material. Seilmeier and Wieser (26) reported different proportions of ω -gliadin (target analyte for Skeritt antibody-based assays) in wheat varieties, which may affect the binding of the antibody to gliadins from different wheat varieties. In a study involving 16 wheat cultivars, the amount of ω -gliadin varied from 6.2 to 20% of total gliadin (25). We evaluated the ratio of wheat flour to gluten recovery in corn flour spiked with wheat flour and gluten, respectively. The ALLER-TEK and ELISA Systems kits showed a higher ratio (approximately 3.1), compared to that obtained from other kits (0.6–1.4). Both the ALLER-TEK and ELISA Systems kits are based on Skeritt antibody. The other Skeritt antibody-based assay, BioKits, showed 1.4 times higher wheat flour recovery than gluten. Allred and Ritter (16) analyzed 40 unknown samples by four commercial gluten assays and found

Skeritt antibody-based assays estimate gluten 4–10 times higher than the R5-based assays. Apart from the differences in the antibody affinity to gluten proteins, the lack of standard reference material for gluten estimation and use of different calibration standards may also be responsible for the observed variation. It should be noted that the recoveries reported are for unprocessed spiked matrix and may not necessarily apply to complex processed foods. Protein recovery has been shown to be drastically reduced upon processing as a result of thermal aggregation of proteins (27, 28).

In summary, albumin and globulin fractions from wheat contain major IgE-binding allergens, and the commercially available ELISA kits are primarily directed against gluten proteins. All of the kits tested were able to detect gluten and wheat flour at the lowest spiked level of 5 and 50 ppm in corn flour, respectively. However, there was considerable variability in the recovery of the gluten and wheat flour using different kits. Also, the recovery of the proteins may change significantly under incurred conditions involving different food matrices and thermal treatments. Further studies are warranted to evaluate the effect of food processing on gluten- and wheat-flour incurred food matrices.

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