

Development of a Sandwich Enzyme-Linked Immunosorbent Assay (ELISA) for Detection of Buckwheat Residues in Food

Rakhi Panda, Steve L. Taylor, and Richard E. Goodman

Abstract: Buckwheat is a pseudocereal (an eudicot with seed qualities and uses similar to those of monocot cereals, family Poaceae) that is consumed in some Asian countries as a staple, and in some western countries as a health food. Allergic reactions to buckwheat are common in some countries. The objective was to develop a specific and sensitive sandwich enzyme-linked immunosorbent assay (ELISA) to detect traces of buckwheat that might inadvertently contaminate other foods in order to assure accurate labeling and consumer protection. Buckwheat-specific antibodies produced in 3 species of animals were tested for specificity and titer by direct ELISA and immunoblot. A sandwich ELISA was developed utilizing pooled rabbit antibuckwheat sera to capture buckwheat proteins and pooled goat antibuckwheat sera, followed by enzyme-labeled rabbit antigoat immunoglobulin G (IgG), to detect bound buckwheat proteins. The lower limit of quantification (LOQ) of the sandwich ELISA was 2 parts per million (ppm) of buckwheat in the presence of complex food matrices. The ELISA is highly specific with no cross-reactivity to any of 80 food ingredients and matrices tested. Validation studies conducted with buckwheat processed into noodles and muffins showed greater than 90% and 60% recovery, respectively. The percent recovery of buckwheat from noodles was similar to that achieved with a commercial buckwheat ELISA kit (ELISA Systems Pty. Ltd., Windsor, Queensland, Australia) at high buckwheat concentrations. However, the sensitivity of this ELISA was greater than the commercial ELISA. This newly developed ELISA is sufficiently specific and sensitive to detect buckwheat residues in processed foods to protect buckwheat-allergic subjects from potential harm.

Keywords: allergen labeling, allergy, buckwheat, ELISA

Practical Application: Buckwheat is becoming a common food ingredient in a number of processed foods due to potentially beneficial nutritional properties, without the celiac disease inducing glutenin proteins of wheat and related cereals. However, buckwheat causes allergy in some individuals and must be labeled and tested accurately to protect those with allergy to buckwheat. We describe the development of a new test assay to help food producers ensure that buckwheat is not present in foods that are not intended to contain buckwheat.

Introduction

Buckwheat is an eudicotyledonous herb of the Polygonaceae family (<http://en.wikipedia.org/wiki/Polygonaceae>), which also includes the herbs rhubarb (*Rheum* sp.) and sorrel (*Rumex* sp.). In 2001, the world buckwheat production was approximately 3 million tons with US production of 65000 tons and consumption of 53900 tons (Umin 2002). Two species of buckwheat are cultivated for food consumption, *Fagopyrum esculentum* or common buckwheat and *Fagopyrum tartaricum* or tartary buckwheat (Ikeda 2002). Common buckwheat is primarily consumed in Asian countries. However, consumption in western countries including the United States is increasing as it is substituted for wheat flour for gluten-sensitive patients (thus found in many gluten-free products) and as a health food because of its nutrient content

(Wieslander 1996; Stember 2006). Common buckwheat flour is used to prepare noodles, crepes, pancakes, muffins, crackers, cereals, bread, biscuit, health food, veggie burgers, and griddle cakes. The groats (dehulled seeds) are also consumed (Li and Zhang 2001; Stember 2006). Buckwheat seed contains various essential vitamins and minerals as well as phenolic compounds such as flavonoids and flavones (Li and Zhang 2001). High dietary intakes of flavonoids are reported to be effective in reducing mortality from cancer and coronary heart disease (Steadman and others 2001). Rutin, the main flavonoid component in buckwheat seed has been reported to decrease vascular permeability and fragility, protecting the consumer from edema; inhibit hyaluronidase activity and prevent damage to various tissues due to antioxidative, hypotensive, and anti-inflammatory properties (Quettier-Deleu and others 2000). Buckwheat also contains plant sterols or phytosterols such as beta-sitosterol (most abundant), campesterol, and stigmaterol that are reported to lower serum cholesterol (Li and Zhang 2001).

Buckwheat is a common and occasionally severe cause of allergic reactions in Japan and Korea (Park and others 2000). The Japanese

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government now requires labeling of foods containing buckwheat (Ikeda 2002). Besides Japan and Korea, cases of buckwheat allergy have also been reported in Europe (Schumacher and others 1993; van Ginkel 2002; Plaza and Mahler 2005; Riffelmann 2005; Heffler and others 2007) and the United States (Smith 1909; Davidson and others 1992; Stember 2006). Various buckwheat proteins such as proteins of molecular weight 24 kDa (Urisu and others 1994), 14 kDa, 18 kDa (Yoshimasu and others 2000), 9 kDa (Park and others 2000), 10 kDa (Matsumoto and others 2004), 19 kDa (Choi and others 2007), and 16 kDa (Tanaka and others 2002) have been reported to bind IgE from buckwheat-allergic patients.

Similar to other food allergies, there is no general cure, thus the only option for those allergic to buckwheat is complete elimination of buckwheat-containing foods and environmental exposure (Hong and Kim 2006). Accurate quantitative detection of buckwheat is crucial for proper labeling and consumer protection. A variety of enzyme-linked immunosorbent assays (ELISA) of high specificity, sensitivity, and accuracy have been successfully developed and utilized previously for detection of residues of allergenic foods such as peanut (Hefle and others 1994; Koppelman and others 1996), egg (Hefle and others 2001), soybean (Koppelman and others 2004), casein (Hefle and Lambrecht 2004), almond (Hlywka and others 2000), mustard (Lee and others 2008a), and lupine (Kaw and others 2008) in food. Three commercial buckwheat ELISA kits are currently available for detection of buckwheat in processed foods. FASTKIT ELISA is reported in the company brochure to have a detection range of 0.78 to 50 ng/mL buckwheat protein (Cosmo Bio Co. Ltd., Tokyo, Japan). The FASPEK kit instructions list the standard curve range as 1 to 64 ng/mL (Morinaga Inst. of Biological Sciences, Inc., Yokohama, Japan). The ELISA Systems kit instructions list a lower limit of quantification (LOQ) of 2.5 parts per million (ppm) soluble buckwheat proteins (ELISA Systems Pty. Ltd., Windsor, Queensland, Australia). However, there are no published reports on the methods utilized in development, validation, and application of these methods in detecting buckwheat residues in processed foods. The instructions for the ELISA Systems kit indicate it is useful for testing for the presence of buckwheat in a number of foods but do not provide validation data. The instructions for the FASTKIT ELISA provide a list of foods that may be tested and state validation was done in rice porridge and a few types of soups using 10- μ g/g buckwheat flour. Two of these kits (FASTKIT ELISA and ELISA System's Pty. Ltd. buckwheat ELISA kit) use buckwheat protein (soluble or total) as the standard for calibration. This approach does not allow correct quantification of the concentration of whole buckwheat present in food without the use of appropriate conversion factors. The instructions in the FASPEK buckwheat ELISA kit is in Japanese, thus the kit is not readily adapted for use in countries outside of Japan. Thus, our objective was to develop a sensitive, specific, robust, and well-validated sandwich-type ELISA, calibrated based on mass of buckwheat flour rather than soluble protein, for detection of buckwheat residues in various food matrices including processed foods.

Materials and Methods

Buckwheat-specific antibody production

Individual animals for immunization were selected based on the lowest preexisting level of antibodies against buckwheat, soybean, wheat, and amaranth proteins from 10 rabbits, 2 sheep, and 2 goats using direct ELISA assays. Stone ground flour produced from groats (hulled seeds) of common buckwheat by Hodgson

Mill (Effingham, Ill., U.S.A.) was used as a suspension to immunize 2 rabbits, 1 sheep, and 1 goat. A phosphate buffered saline (PBS) extract of the same buckwheat flour was used to immunize an additional 2 rabbits. Immunization was carried out by Covance Research Products (Denver, Pa., U.S.A.), an Association of Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited laboratory under an Institutional Animal Care and Use approved protocol. Initial immunizations used 250 μ g of flour or 250 μ g of soluble protein (rabbits) or 1 mg of suspended flour (sheep and goat) of immunogen as a suspension in Freund's Complete Adjuvant (FCA) by subcutaneous injection. The 1st booster injection for the sheep and goat was with 0.5 mg of immunogen on day 21 using Freund's Incomplete Adjuvant (FIA). Subsequent booster injections were given at a 21-d interval by using 0.25 mg for the sheep and goat. All booster immunizations in rabbits used 125 μ g of immunogen in FIA. Test bleeds were collected 10 d after each booster injection. The titer of buckwheat-specific serum was monitored throughout the immunization period by a noncompetitive ELISA as described by Lee and others (2008a). The titer was defined as the log reciprocal dilution of the mid-linear portion of the titration curve produced using 1 μ g/mL of soluble buckwheat protein coated onto microtiter plates with serially diluted serum samples in triplicate wells. Individual bleeds from 3 rabbits and 1 goat showing a titer value of ≥ 10000 were pooled separately. These serum pools were analyzed by immunoblots and then utilized in the development of reciprocal tests with each species as capture and detection antibody in the development of a sandwich ELISA.

Gel electrophoresis and IgG immunoblotting

The protein profile of buckwheat was examined after separating the proteins by SDS-PAGE using an XCell SureLockTM Mini-Cell (Invitrogen Corp., Carlsbad, Calif., U.S.A.). Buckwheat proteins extracted at 1:10 (w/v) with PBS were diluted to a protein content of 10 μ g and 1 μ g/10 μ L using Laemmli's SDS-sample buffer (Thermo Fisher Scientific, Rockford, Ill., U.S.A.), containing 2-mercaptoethanol (Bio-Rad Laboratory Inc., Hercules, Calif., U.S.A.) as a reducing agent. Samples were heated at 100 °C for 5 min and loaded in wells of 10 to 20% tris glycine gel (Invitrogen Corp.). Precision Plus protein standards (Bio-Rad Laboratory Inc.) were used as molecular weight markers. Electrophoresis was carried out at a constant 125 V for 1.5 h. Following electrophoresis, the gels were fixed in a solution of 7% acetic acid with 40% methanol and stained with Brilliant Blue G-colloidal concentrate (Sigma-Aldrich Corp., St. Louis, Mo., U.S.A.) for 2 h. The gels were destained for 1 min in a solution of 10% acetic acid and 25% methanol followed by multiple changes of 25% methanol.

Proteins from identical sample gels were electro transferred to polyvinylidene difluoride (PVDF) membranes (0.45 μ m pore size, Invitrogen Corp.). The membranes were then blocked with 5% nonfat dry milk (NFDm) in PBS containing 0.05% Tween 20 (polyoxyethylene sorbitan, Bio-Rad Laboratory Inc.) (PBST) for 2 h at room temperature. The antibuckwheat rabbit and goat sera were diluted (1:10000 [v/v]) with 2.5% NFDm in phosphate buffered saline with 0.5% Tween 20 (PBST) and then incubated with identical blotted membranes for 2 h at room temperature. Unbound IgG was removed by 4 rinses for 5 min each with PBST. Then the membranes were incubated with corresponding detection of secondary antibodies (Immunopure[®], Pierce Biotechnology Inc., Rockford, Ill., U.S.A., horseradish peroxidase-conjugated rabbit anti-goat IgG and goat antirabbit IgG) diluted with 2.5% NFDm in PBST at 1:40000 (v/v) for 1 h. After

4 washes in PBST, the membranes were wet with Super-signal West Dura substrate (Thermo Scientific) and were covered with plastic transparent sheets before capturing the light emission using a Kodak Gel Logic 440 imaging system (Carestream Health, Molecular Imaging, Rochester, N.Y., U.S.A.). The prebleed sera of each species were used as negative controls using identical blots.

Standard and sample preparation

Buckwheat flour from Hodgson's Mill (Effingham, Ill., U.S.A.) was used as the standard throughout the study. Samples for cross-reactivity studies, the matrix interference study, and ingredients used in preparation of model foods were purchased from local grocery stores in Lincoln, Nebraska. Solid samples for the cross-reactivity study were ground to uniform consistency using an Osterizer® blender (Sunbeam Corp., Delray Beach, Fla., U.S.A.) or a commercial food processor (KPF600 Food Processor, Kitchen Aid®, St. Joseph, Mo., U.S.A.). Liquid samples were used as is. Standards, samples, and prepared noodles (model food) were extracted 1:10 using PBS containing 1% NFDM at 60 °C for 2 h using a shaking water bath (Model SW22, Julabo U.S.A., Inc., Allentown, Pa., U.S.A.). The extraction buffer used for the muffins (model food) was PBS containing 1% NFDM and 0.5% Tween 20. After extraction, the solutions were clarified by centrifugation at 3612 × g for 30 min at 10 °C using a tabletop centrifuge (Sorvall® Legend™ RT, Kendro Laboratory Products, Newton, Conn., U.S.A.). The supernatant was collected and stored at −20 °C until use.

Buckwheat sandwich ELISA

Pooled rabbit and goat antisera were utilized as capture and detection antibodies, respectively, in the development of the sandwich ELISA. A buckwheat standard of 1000 ppm ($\mu\text{g/mL}$) was prepared by spiking 0.01 g of buckwheat flour into 10 mL of PBS (representing the sample matrix), which is mixed by vortexing for 1 min to distribute the flour evenly. The entire 10 mL standard is subsequently "extracted" with 90 mL of extraction buffer and centrifuged to remove particulates to provide the 1000 ppm standard solution (this standard is similar to adding 0.01 g of buckwheat to 9.99 g of powdered muffin or noodle and extracting with 100 mL of extraction buffer and was used as an initial evaluation step of the assay). The clarified supernatant (1000 ppm) was serially diluted 1:3 using the extraction buffer to obtain standards of 333, 111, 37.0, 12.3, 4.12, 1.37, 0.46, 0.15, 0.051, 0.017 to 0 ppm. Microtiter plates (NUNC-Immuno™ MaxiSorp™ 96-MicroWell™ plates, Nalgel Nunc Intl., Rochester, N.Y., U.S.A.) were coated with 100 μL /well of pooled rabbit antisera, which was diluted (1:8000) with coating buffer (0.015M Na_2CO_3 , 0.035M NaHCO_3 , and 0.02% NaN_3 pH 9.6) prior to addition to the plates and then incubated overnight at 4 °C. The plates were then washed 4 times with wash buffer (PBS containing 0.05% Tween 20 and 0.02% NaN_3 pH 7.4) using a microplate washer (AM60, Dynex Technologies, Inc., Chantilly, Va., U.S.A.). Unoccupied sites on the wells were blocked with 350 μL /well of blocking buffer consisting of 1% bovine serum albumin (BSA) (RIA grade, Sigma Chemical Co., St. Louis, Mo., U.S.A.) in PBS, pH 7.4, followed by incubation at 37 °C for 1 h. The plate was washed 4 times with wash buffer before adding 100 μL /well of the serially diluted buckwheat standards (1000 to 0 ppm), followed by incubation at 37 °C for 1 h. Unbound protein was removed with 4 applications of wash buffer. Pooled goat antiserum was diluted 1:5000 with conjugate buffer (PBS containing 0.1% BSA, pH 7.4), 100 μL was added to each well,

and the plate was incubated at 37 °C for 1 h. The plates were washed 4 times with the wash buffer, and 100 μL /well of rabbit anti-goat IgG labeled with alkaline phosphatase (Immunopure®, Pierce Biotechnology Inc.), diluted 1:5000 with conjugate buffer, was added to each well. The plates were again incubated at 37 °C for 1 h. The plates were washed 4 times with wash buffer before adding 100 μL /well of *p*-nitrophenyl phosphate substrate (Sigma Fast™, Sigma-Aldrich Inc., St. Louis, Mo., U.S.A.). The plates were incubated in the dark for 30 min at room temperature before stopping the reaction by the addition of 100 μL /well of 1N NaOH. The absorbance of light was measured immediately at 405 nm using a microplate reader (ELx808 Ultraplate, Bio Tek Instruments Inc., Winooski, Vt., U.S.A.). Standard curves were generated using GraphPad Prism® Software (GraphPad Software Inc., San Diego, Calif., U.S.A.).

Standard curves were also prepared in 2 different food matrices (muffins and noodles) to evaluate the effect of these matrices on the sensitivity of the ELISA. A standard was prepared by mixing 0.01 g of buckwheat flour with 9.99 g of powdered muffin or noodle. The mixture of solid material was extracted with 100 mL of extraction buffer and clarified by centrifugation as described earlier to obtain a 1000 ppm standard. The clarified supernatant was serially diluted 1:3 with extracts of buckwheat-free food matrix (10 g of powdered muffin or noodle extracted with 100 mL of extraction buffer and clarified by centrifugation) to obtain protein standards ranging from 1000 to 0 ppm, which were then tested in the sandwich ELISA. The absorbance curves from the spiked food matrices were compared with the standard curve developed previously in presence of PBS to evaluate possible interferences.

Evaluation of potential cross-reactivity

The specificity of the buckwheat-specific antibodies was tested for binding to other food ingredients that might be present in food matrices and might produce false-positive results due to cross-reactivity. The focus was on foods from botanically related species (amaranth, rhubarb, and quinoa) that would be expected to contain proteins similar to buckwheat. In addition, food sources that might be expected to be present in processed foods (Table 1) containing buckwheat were tested using the buckwheat ELISA as the sera of immunized animals might contain IgG against proteins from diverse sources, leading to false-positive detection. The protein content of each food extract was determined by the Lowry method using a DC protein assay kit using BSA as the standard (Bio-Rad Laboratory Inc.). Food samples were tested using 3 protein concentrations (10, 1, and 0.1 $\mu\text{g/mL}$) and 3 dilutions (1:10, 1:100, and 1:1000). Cross-reactivity was expressed as apparent buckwheat equivalents based on the absorbance readings obtained with the buckwheat standard curve.

Model food preparation with specific inclusion ratios of buckwheat

Muffins. The recipe for making the muffins was 140 g of all-purpose wheat flour, 50 g whole egg, 90 g whole milk, 50 g sugar, 50 g vegetable oil, and 15 g baking powder. A 100% all-purpose wheat flour sample was used as a negative control. Samples used to evaluate the detection of small quantities of buckwheat in wheat muffins were prepared by adding specific quantities of buckwheat flour to the all-purpose wheat flour, with a final mass ratio of 3, 10, 30, 100, 300, and 1000 μg buckwheat/g muffin mix (corresponding to 3 to 1000 ppm). To improve the accuracy of sample addition, a stock mixture of 1000 ppm ($\mu\text{g/g}$) buckwheat flour in all-purpose wheat flour was made by adding 1 g of buckwheat

Table 1—List of food and food ingredients showing no cross reactivity with buckwheat ELISA.

Foods botanically related to buckwheat	Egg	Color additive
Rhubarb (stem)*	Spray-dried egg white	Annatto
Quinoa flour	Oil	FD & C Blue 1
Amaranth seeds	Peanut oil	Caramel color
Cereals	Soybean oil	Paprika
Wheat flour	Canola oil	FD & C yellow 5
Rye flour	Olive oil	FD&C yellow 6
Barley flour	Vegetable oil	Other ingredients
Rice flour	Fruits	Baking soda
Corn flour	Cran berry	Lemon juice
Wheat flour	Raspberry	Peanut butter
White flour	Cherry	Soy lecithin
Legumes	Apple	Soy sauce
Black gram	Banana	Vanilla extract
Cowpea	Herbs and spices	Mint extract
Chick pea	All spice (ground)	Raisin
Kidney bean	Clove (ground)	Yeast-Brewer's
Dairy Products	Garlic (powder)	
Nonfat dry milk	Curry powder	
Butter milk	Coriander seed	
Casein	Cumin (ground)	
Whey powder	Ginger powder	
Yogurt	Mustard (ground)	
Shortening	Onion (powder)	
Nuts	Parsley flakes	
Almond	Pepper (white)	
Cashew	Pepper (black)	
Hazel nut	Salt	
Walnut	Thyme	
Peanut	Turmeric	
Pistachio	monosodium glutamate	
Poppy seeds	Preservatives	
Sesame seed	Citric acid	
Sweeteners	Potassium sorbate	
Sugar (white)	Ca ⁺ disodium ethylenediaminetetraacetic acid (EDTA)	
Sugar (brown)	Sodium benzoate	
Corn syrup	Vinegar	
Molasses		
Maltodextrin		

flour to 999 g of all-purpose wheat flour and extensively blended by sifting 7 times with a kitchen sifter after mixing thoroughly in a bowl. Samples (1.2 to 118.5 g) of the stock flour mixture (1000 ppm buckwheat) were mixed with all-purpose wheat flour in amounts ranging from 138.8 to 21.5 g, respectively, to produce samples with concentrations of 3 to 300 ppm buckwheat. To make the 1000 ppm level 0.4 g of buckwheat flour was added to 139.6 g of all-purpose wheat flour. The egg, milk, oil, and baking powder were then added to each flour mixture sample. The muffin mixtures were then thoroughly mixed in a Mixer (KSM150PS Artisan 5-Quart Stand Mixer, Kitchen Aid®, St. Joseph, Mich., U.S.A.) at medium speed for 3 min. Each mixture was transferred into medium size muffin cups in a baking pan, and the samples were baked in a preheated oven for 15 min at 350 ° F. The muffins were allowed to cool to room temperature. The weight of the muffins before and after baking was recorded in order to determine the moisture loss. The muffins were then stored at -20 °C until they were prepared for the ELISA tests. Potential contamination between buckwheat samples was avoided by preparing the negative control first, followed by the lowest dose mixture, and finishing with the 1000 ppm mix.

Noodles. The recipe for making noodles was 453.6 g of semolina flour and 162 g of water. A negative control batch was

prepared without adding any buckwheat flour. In addition, a stock mixture of 2000 ppm buckwheat (1 g buckwheat flour added to 499 g semolina flour) was prepared and mixed thoroughly. This stock mixture was mixed in specific ratios with pure semolina flour to achieve 6 specific levels of buckwheat (3, 10, 30, 100, 300, and 1000 ppm) in noodles (for example, 0.9 g of stock plus 452.7 g of semolina for the 3 ppm sample). For each sample, the individual masses of stock buckwheat/semolina flour and semolina flour were mixed and the entire batch was sifted 3 times through a kitchen shifter. The individual batches were transferred into a home-style pasta maker (Lello Appliances Corp., Pasta Master, 3000 PRO, East Rutherford, N.J., U.S.A.) and mixed for 5 min while gradually adding 162 g of water. The dough was mixed for an additional 5 min before extruding the pasta using a spaghetti die. The extruded noodles were stored at -20 °C until tested for percent recovery by ELISA. Noodles were prepared in the order of buckwheat content (0, 3, 10, 30, 100, 300, and 1000 ppm) to reduce carryover errors.

Samples of the semolina flour noodles and wheat muffins were ground to a fine powder using a commercial food processor (KPF600 Food Processor, Kitchen Aid®, St. Joseph) before extraction and clarification as described earlier. The clarified supernatants were analyzed using the buckwheat sandwich ELISA. The standard curves developed in the prepared negative control (0 ppm) noodle and muffin matrix were used to estimate the relative amount of buckwheat recovered. The percent recovery of buckwheat from noodles was calculated as a ratio (in percent) of the estimated sample ppm obtained from the ELISA to the ppm of added buckwheat. For the muffins, moisture loss during baking was taken into account to calculate the actual ppm level of added buckwheat available in the muffins after baking. The percent recovery of buckwheat from the muffins was then calculated as a ratio (in percent) of the estimated sample ppm obtained from ELISA to the available buckwheat level (ppm) in the muffin after baking.

Samples of each manufactured noodle batch were also analyzed using a commercial buckwheat ELISA kit (ELISA Systems Pty. Ltd.) according to instructions on the kit insert. The values obtained using the newly developed ELISA was compared to those obtain with the ELISA Systems kit.

Statistical analysis

The lower limit of detection (LOD) of the buckwheat standard curves was determined as the mean absorbance value of the blank (wells without buckwheat) plus 3 times the standard deviation of the blank. The lower LOQ was determined as the lowest point on the relatively straight portion of the standard curve. The results were based on the mean of 4 replicates with 3 analyses per replicate. For the matrix interference studies, the differences in the slopes of the standard curves obtained in the food matrices samples and the standard curve obtained without added matrix were evaluated by Student's *t*-test using SAS software (SAS 9.1 software; SAS Inst. Inc., Cary, N.C., U.S.A.). The results of the percent recoveries of buckwheat from the manufactured model foods were expressed as the mean $\pm$ standard error of the mean based on determinations from triplicate extractions of each model food and analyzing each extract in triplicate on separate days. The percent recoveries of the buckwheat from the model semolina flour noodles using the developed buckwheat ELISA was compared with the percent recoveries obtained using the commercial buckwheat ELISA kit by Student's *t*-test using SAS software. A *P* value of less than 0.05 was considered as significant.

Results and Discussion

Polyclonal antibody production

Animal selection for immunization was done after a prescreening test by direct ELISA assay for the presence of preformed antibodies against buckwheat, soybean, wheat, and amaranth soluble proteins. Wheat and soybean are common ingredients used in buckwheat recipes, and amaranth is botanically related to buckwheat. Therefore, the presence of preformed antibodies against these ingredients would potentially confound development of the ELISA or compromise assay reliability. Based on the results of prescreening (data not shown), 4 rabbits, 1 sheep, and 1 goat (among the 10 rabbits, 2 sheep, and 2 goats prescreened) showing the lowest level of antibodies against all 4 ingredients were selected for immunization. The pooled antisera from 3 rabbits (2 immunized with PBS extract of buckwheat flour and 1 immunized with buckwheat flour in suspension) with a titer of 37300 and the pooled goat antisera with a titer of 45600 were used in the development of the buckwheat ELISA.

IgG immunoblotting

Development of a sensitive and specific sandwich ELISA requires that the capture and detection antibodies must recognize the same proteins, but different epitopes on the same proteins. It has been suggested that the antibodies should recognize all the major allergenic proteins of buckwheat (Jordan 2002). However, not all allergens in all food sources are known, so assays that are robust and able to detect a number of specific proteins from a complex food allergen would be useful for identifying possible unintended contamination (Hefle and others 2006). Stained SDS-PAGE gels of buckwheat extracts showed protein bands at molecular masses of approximately 50, 48, 31 to 40, 24, 18, 14, and 9 to 12 kDa (Figure 1), which correspond to the molecular weights of the major allergens of buckwheat reported by Urisu and others (1994) (24 kDa), Yoshimasu and others (2000) (18 kDa), Park and others (2000) (9 kDa), Matsumoto and others (2004) (10 kDa), Choi and others (2007) (19 kDa), Tanaka and others 2002 (16 kDa), and Nagata and others 2000 (56, 52, 45, and 43 kDa). The immunoblot-binding pattern of pooled rabbit and goat antisera to buckwheat proteins is shown in Figure 1. Although some minor differences in binding were observed between the rabbit and goat antisera, both showed binding to most of the Coomassie stainable proteins of buckwheat separated by SDS-PAGE in the range of approximately 10 to 90 kDa. Immunoblots with sera from these

animals taken prior to the 1st immunization did not show binding to buckwheat proteins (data not shown).

Buckwheat sandwich ELISA

Preliminary tests demonstrated that the best combination of sera for the sandwich ELISA was to use pooled rabbit sera for antigen capture and goat sera for detection of buckwheat proteins. Figure 2 shows buckwheat sandwich ELISA standard curve developed in PBS. The LOD and LOQ of the standard curve was 0.08 and 2 ppm (μg of whole buckwheat/mL), respectively. The sensitivity of detection is a critical parameter to consider in judging the suitability of the assay for detecting allergenic residues in food. Although an ideal assay would detect the allergenic food at a level below the threshold that elicits an allergic reaction in the most sensitive subject, the threshold of buckwheat is not known. However, an LOQ of 2 ppm ($\mu\text{g}/\text{g}$) was judged to be sufficient to ensure proper labeling of buckwheat-containing products based upon the threshold dose for peanut and the assumption that buckwheat and peanut are equivalently allergenic (Taylor and others 2009).

Food matrices have been found to mask allergenic proteins in some detection systems when testing food products (Poms and others 2004). Noodles and muffins were chosen as 2 foods that may include buckwheat, and thus could be sources of unlabeled

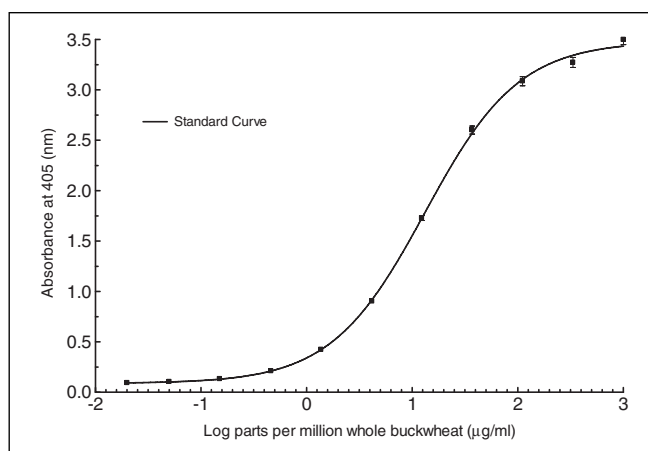


Figure 2—Buckwheat sandwich ELISA standard curve. Each data point represents mean of 4 replicates with 3 analyses per replicate with a standard deviation of <0.18 absorbance unit.

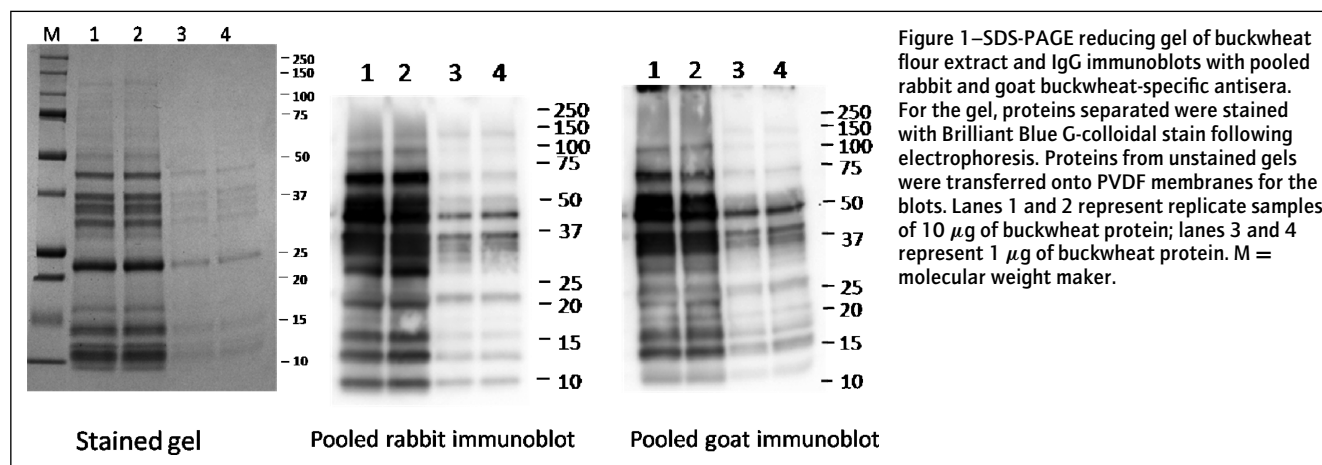


Figure 1—SDS-PAGE reducing gel of buckwheat flour extract and IgG immunoblots with pooled rabbit and goat buckwheat-specific antisera. For the gel, proteins separated were stained with Brilliant Blue G-colloidal stain following electrophoresis. Proteins from unstained gels were transferred onto PVDF membranes for the blots. Lanes 1 and 2 represent replicate samples of 10 μg of buckwheat protein; lanes 3 and 4 represent 1 μg of buckwheat protein. M = molecular weight maker.

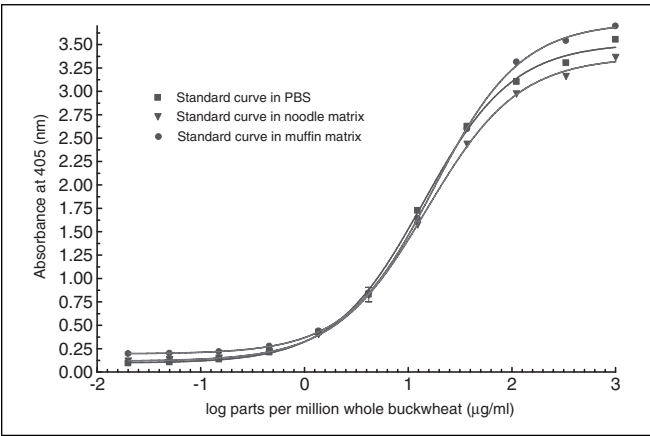


Figure 3—Standard curve of 1000 ppm buckwheat flour spiked into PBS, noodle food matrix, and muffin food matrix. Each standard curve was derived from 2 replicate trials with 3 analyses per replicate with a standard deviation of <0.2 absorbance units.

buckwheat. Standard curves of buckwheat standard in these matrices are shown in Figure 3 and are compared to the standard curve in PBS only. No significant difference in the slope of the curves developed in noodles or muffins were found compared to the PBS control standard curve ($P = 0.62$ for muffin matrix and $P = 0.53$ for noodle matrix). The LOQ of the standard curves in both food matrices was 2 ppm (μg of buckwheat/ g of food matrix). These results indicate that the buckwheat sandwich ELISA described here is efficient in detecting buckwheat in presence of these complex food matrices.

Cross-reactivity

The specificity of the buckwheat ELISA was evaluated by cross-reactivity studies using 80 different food ingredients (Table 1) including foods commonly used in buckwheat recipes and foods

botanically related to buckwheat (amaranth, quinoa, and rhubarb stem). No cross-reactivity was detected with any of the foods tested. Although phylogenetically related species have a greater chance of showing cross-reactivity because of the similarity in protein content and protein structure, no cross-reactivity occurred with rhubarb, which belongs to the same family as buckwheat (Polygonaceae). This may be because of the different protein content in the edible stem of rhubarb, which was analyzed for cross-reactivity, compared to the buckwheat seed storage proteins. Also no cross-reactivity was detected with amaranth and quinoa seed proteins, although they belong to the same taxonomic order as buckwheat (Caryophyllales). These results suggest that the buckwheat sandwich ELISA is highly specific for the detection of buckwheat proteins in the presence of a variety of other food ingredients.

Model foods

Two major commercial applications of buckwheat are noodles (for example, soba noodles) and baked products such as muffins. Although simple spiking and extraction of buckwheat from these 2 food matrices showed minimal effect on the sensitivity of the developed ELISA (Figure 3), verification of the ability of the sandwich ELISA to accurately measure buckwheat in presence of these food matrices after typical processing conditions is required to demonstrate its suitability for analysis of fully processed food.

The mean percent recovery of buckwheat from muffins (approximately 60%) containing different added levels of buckwheat is summarized in Table 2. The low recovery of buckwheat from muffins is likely explained as an effect of heat processing. Heat processing such as baking at high temperature can affect proteins by altering the 3-dimensional structure of proteins, destroying the structure, or by causing aggregation of proteins (Sathe and others 2005). Thermal processing can also result in various biochemical and enzymatic reactions in food such as Maillard browning

Table 2—Recovery and percent recovery of whole buckwheat flour from manufactured model wheat muffins determined by the developed buckwheat ELISA.

Added buckwheat level (ppm) ^a	Available buckwheat after baking (ppm) ^b	Levels detected by ELISA (ppm recovery) ^c			Average (ppm buckwheat) ^d	% recovery ^e
		Trial 1	Trial 2	Trial 3		
3	3.2	2.0 ± 0.0	2.5 ± 0.0	1.9 ± 0.0	2.1 ± 0.2	65.6 ± 6.2
10	10.7	6.3 ± 0.1	6.7 ± 0.4	5.8 ± 0.2	6.2 ± 0.2	58 ± 1.8
30	31.6	21.5 ± 0.7	24.4 ± 2.7	18.5 ± 1.0	21.4 ± 1.7	68 ± 5.4
100	105.6	80 ± 3.8	76 ± 8	63.6 ± 3.0	73.2 ± 5	69 ± 4.7
300	317.7	209 ± 27	193 ± 33	148 ± 12.8	183.4 ± 18	57.7 ± 5.6
1000	1073	760 ± 62	699 ± 111	639 ± 47	699.3 ± 35	65.1 ± 3.3

^appm = parts per million (mg of whole buckwheat flour/kg).
^bppm buckwheat available after baking was calculated from moisture loss from muffin samples after baking (mean moisture loss from muffin samples = 6.2% ± 0.7%).
^cData are mean ± SD ($n = 3$).
^dData are mean ± SE ($n = 3$).
^ePercent recovery calculated as ratio (in percent) of average ppm buckwheat flour recovered to the available (ppm) buckwheat after baking.

Table 3—Recovery and percent recovery of whole buckwheat flour from manufactured model semolina flour noodles determined by the developed buckwheat ELISA.

Added buckwheat level (ppm) ^a	Levels detected by ELISA (ppm recovery) ^b			Average (ppm buckwheat) ^c	% recovery
	Trial 1	Trial 2	Trial 3		
3	3.0 ± 0.0	2.95 ± 0.0	2.86 ± 0.0	2.94 ± 0.0	98 ± 0.0
10	9.8 ± 0.4	12 ± 0.3	10.7 ± 0.3	10.8 ± 0.6	108 ± 6
30	28.1 ± 0.6	27.4 ± 1.3	26.8 ± 0.6	27.4 ± 0.3	91.3 ± 1
100	87.3 ± 12	109 ± 12	89.5 ± 5.1	95.2 ± 7	95.2 ± 12
300	261 ± 7.5	254 ± 10	231 ± 18	248 ± 9.2	82.6 ± 3.0
1000	967 ± 92	926 ± 32	1003 ± 88	965 ± 22	96.5 ± 2.2

^appm = parts per million (mg of whole buckwheat flour/kg).
^bData are mean ± SD ($n = 3$).
^cData are mean ± SE ($n = 3$).

Table 4—Recovery and percent recovery of buckwheat soluble protein and whole buckwheat flour from manufactured model semolina flour noodles determined by the buckwheat ELISA test kit from ELISA Systems Pty. Ltd.

Added buckwheat level (ppm) ^a	Levels detected by ELISA (ppm recovery) ^b			Average (ppm buckwheat protein) ^c	Average (ppm whole buckwheat) ^d	% recovery
	Trial 1	Trial 2	Trial 3			
3	ND ^e	ND	ND	ND	ND	ND
10	ND	ND	ND	ND	ND	ND
30	ND	ND	ND	ND	ND	ND
100	6.9 ± 0.0	6.7 ± 0.1	7.5 ± 0.5	7.1 ± 0.2	85.2 ± 2.4	85.2 ± 2.4
300	26.6 ± 3.2	22.3 ± 0.9	22.1 ± 0.2	23.6 ± 1.4	283 ± 17	94.3 ± 5.6
1000	78.2 ± 11	76.9 ± 5	82.9 ± 5.1	79.3 ± 1.8	952 ± 22	95.2 ± 2.2

^appm = parts per million (mg of total buckwheat/kg).^bData are mean ± SD (*n* = 3).^cData are mean ± SE (*n* = 3).^dThe ppm whole buckwheat was obtained by multiplying the average levels of buckwheat proteins detected in ELISA by the conversion factor of 12, which was derived from the soluble protein content of buckwheat flour of 8.34% as measured by the Kjeldahl method.^eND = not detected.

reactions resulting in amino acid modification and protein cross-linking (Maleki and Hurlburt 2004). Thermal processing can also decrease the solubility of proteins, thereby making them less extractable and thus less detectable (Poms and Anklam 2004).

The percent recovery of buckwheat from the semolina flour noodles was higher compared to muffins (Table 3) indicating that the extrusion processing involved in making the noodles using the home-style pasta maker has minimal effect on the structure and solubility of buckwheat proteins, while high temperature baking does impact the solubility or structure of the same proteins. This example of buckwheat recovery from 2 foods prepared by diverse processes clearly illustrate that processing can markedly impact the accuracy of measurement of proteins from allergenic foods. The assay described here can reliably identify buckwheat in both muffin and noodle samples, however the absolute amount of buckwheat present in heat-processed foods cannot be assumed without further evaluation of processing effects.

The noodle samples were also evaluated using a commercially available buckwheat ELISA kit (ELISA Systems Pty. Ltd.), in order to compare the detection efficiency of both methods. The average ppm recovery of buckwheat from the noodles using the commercial buckwheat ELISA Systems kit was lower compared to that obtained using the newly developed ELISA (Table 4). This can be explained by the measurement systems adopted by each of the ELISAs. The newly developed buckwheat sandwich ELISA is based on detection of whole buckwheat flour as ppm ($\mu\text{g/g}$). Alternatively, the ELISA Systems' buckwheat ELISA kit is based on detection of buckwheat as ppm of buckwheat flour soluble protein (μg of buckwheat flour soluble protein/g of noodle). Thus, a more appropriate comparison requires conversion from ppm of buckwheat flour soluble proteins into ppm whole buckwheat flour to avoid underestimation (Lee and others 2008b). The soluble protein content of buckwheat flour utilized by ELISA Systems buckwheat ELISA kit was 8.34% as measured by the Kjeldahl method. Based on the protein content, a conversion factor of 12 ($100/8.34$) was calculated. Table 4 summarizes the ppm and percent recoveries of whole buckwheat obtained from noodles using the kit, after multiplying with the conversion factor of 12 to convert the results from soluble buckwheat protein to whole buckwheat. After converting the results from the ELISA Systems method, results from the 2 ELISAs were not significantly different ($P = 0.98$). However, the sensitivity of ELISA Systems kit was not sufficient to detect buckwheat in noodle samples containing 3, 10, and 30 ppm buckwheat flour, as the absorbance values were out of range of the standards provided by the kit. Because allergen detection kits are intended to protect the highly sensitive allergic

consumer, it is essential that the kit be able to detect allergenic food at levels equal to or below the expected threshold of elicitation. Based on previously determined thresholds for peanut, the newly developed sandwich ELISA is sufficiently sensitive to protect highly sensitive buckwheat-allergic consumers.

Conclusion

The sandwich ELISA developed in this study is sufficiently sensitive and specific to be utilized by the food industry for the accurate detection and quantification of buckwheat levels in presence of various food matrices. This may help in proper labeling of buckwheat-containing products and in reducing the risk of accidental exposure. Although the developed ELISA is highly sensitive and accurate in detecting buckwheat from processed foods such as noodles undergoing minimal processing, additional research is needed to improve the detection of buckwheat from foods undergoing heat treatment such as baked products, where there is a greater chance of protein denaturation and modification.

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