

Electrophoretic and Immunochemical Characterization of Allergenic Proteins in Buckwheat

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Key Words

Buckwheat · IgE-mediated allergy · IgE · IgG · Epitopes · ELISA · Immunoblot · Food allergy

Abstract

Background: Buckwheat allergies are not common, however, it is considered to be a very potent allergen. Ingestion of small amounts has been found to produce anaphylactic reactions, particularly in children. Identification and characterization of the major allergen(s) in buckwheat are currently underway, however, there are some discrepancies in the findings. **Methods:** Identification of the major allergen(s) was determined through Western blotting using buckwheat-allergic patients' sera. Once the allergenic proteins were identified, they were purified, their IgE-binding activity assessed through an indirect ELISA and the N-terminal amino acid sequence completed. To assess the stability of the IgE-binding epitopes, protein fractions were exposed to various treatments and assayed using an indirect ELISA. Lastly, the presence of anti-buckwheat IgG in the patients' sera was analyzed through Western blotting and ELISA. **Results:** IgE binding was detected to proteins with molecular masses of approximately 14 and 18 kDa. N-terminal

sequencing was completed and found to share some homology with rice proteins associated with rice allergies and cross-allergenicity with buckwheat proteins. When the water-soluble protein fraction was heated, exposed to acidic and alkaline conditions and fully denatured, IgE-binding activity was reduced. When the fraction was partially denatured through urea, IgE-binding activity increased. Furthermore, IgG-binding activity was detected with proteins only above the 20 kDa region. **Conclusions:** Proteins with molecular masses around 14 and 18 kDa were identified as the major allergenic proteins in the buckwheat-allergic patients' sera tested in this study. Results also indicate that these two proteins possess IgE-binding capability.

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Introduction

Buckwheat (*Fagopyrum esculentum*) is not taxonomically related to wheat. Buckwheat is grown in many Asian countries as well as Russia, Italy, the USA and Canada. Due to its unusually short growing season, it can be cultivated twice a year. It can be grown under poorer conditions than rice, at high altitudes, and both the grain and

Table 1. Clinical history of buckwheat-allergic patients' sera

Sera No.	Age	Sex	Immediate hypersensitivity reaction	IgE RAST PRU/ml
1	3	M	Anaphylaxis	10.7
2	2	F	Atopic dermatitis, wheezing	–
3	2	M	Atopic dermatitis, bronchial asthma	–
4	9	M	Urticaria, wheezing, vomiting	<17.5
5	10	M	Urticaria, eczema	2.2
6	6	M	Urticaria, wheezing, vomiting	4.7
7	14	M	Urticaria, wheezing, discomfort of larynx	<17.5
8	15	M	Gooseskin, vomiting	<17.5
9	33	F	Dyspepsia, wheezing, abdominal pain	<17.5
10	13	F	Vomiting	16.8
11	3	M	Erythema, eczema, vomiting, itching	1.0
12	14	F	Itching, urticaria, cough, vomiting, shock	14.7

leaves can be used as food [1]. Buckwheat products are increasing in popularity as a health food based on its high protein content, abundance of vitamins B₁ and P, dietary fiber and low fat content [2]. It is considered to be a major dietary source of rutin, a phenolic flavonoid found to possess antitumoral, antimutagenic, anticarcinogenic and anti-inflammatory activity [3]. It is also believed to reduce blood cholesterol counts [2].

Although buckwheat allergies are not common, it is considered to be a very potent allergen. Small amounts of this allergen can provoke serious symptoms in all types of exposure. Symptoms range from urticaria eruptions, gastrointestinal disorders, asthmatic attacks, nasal and eye symptoms to anaphylactic reactions [4]. A study done on 90,000 schoolchildren in Japan found a higher incidence of anaphylactic shock due to buckwheat compared to those with egg and milk allergies [5]. The mechanism of this allergy, a type I reaction or immediate hypersensitivity, is IgE-mediated. Characterization of buckwheat allergens has been limited. Buckwheat proteins of molecular masses 17, 50 and 100 kDa [6], 8–9 kDa [7] and 24 kDa [8] have been reported to exhibit strong IgE-binding activity. More recently, allergenic proteins of molecular masses 22, 36, 39–40 and 70–72 kDa were identified from water-soluble fractions of buckwheat [9, 10].

In this study, eight varieties of buckwheat seed were analyzed; Koban, Manisoba, Bota Soba, Mano, Mancan, BM94119.5, B640015 and BM94172.2. The allergenicity of crude and purified buckwheat proteins was assessed through Western blotting and an indirect ELISA using buckwheat-allergic patients' sera. Once identified, N-terminal sequencing was completed.

Materials and Methods

Buckwheat

Ten grams of finely ground buckwheat seed were defatted with cold acetone at 4 °C and thoroughly dried. Defatted flour was mixed with 100 ml deionized water and stored for 24 h at 4 °C. After centrifuging the extract at 8,000 g for 30 min, an equal volume of ethyl alcohol was slowly added to the supernatant at –20 °C. After centrifugation at 8,000 g for 30 min, the precipitate was resuspended in deionized water and stored at 4 °C in the presence of 0.05% sodium azide. Protein concentrations were determined through the Lowry method [11] using a commercially available kit (BioRad D_C Protein Assay, BioRad, Hercules, Calif., USA) and bovine serum albumin (BSA) as a standard.

Buckwheat-Allergic Patients' Sera

Serum was obtained from buckwheat-allergic subjects selected on the basis of their clinical records regarding ingestion of buckwheat products. Serum was collected from 12 subjects showing positive RAST to buckwheat antigens. Table 1 outlines a brief summary of their clinical history.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Western Blotting

SDS-PAGE was performed according to the method of Laemmli [12] in a Mini-Protein II Electrophoresis Cell (BioRad). Protein extracts were dissolved in sample buffer in the presence (reduced) or absence (non-reduced) of β-mercaptoethanol, heated for 5 min at 100 °C and loaded onto a 15% polyacrylamide gel. Gels were run at a constant current (20 mA/slab gel), and, after electrophoresis, were either developed or transferred onto nitrocellulose membranes (Micon Separations Inc., Westborough, Mass., USA) for Western blotting according to the method of Towbin et al. [13]. Electroblooming was carried out for 1 h at a constant voltage (100 V, 4 °C) on a Mini Trans-Blot Electrophoretic Transfer Cell (BioRad).

Specific IgE and IgG Immunoblotting

After electroblooming, membranes were blocked with 1% BSA in 20 mM Tris-buffered saline (TBS), pH 7.5, washed with TBS containing 0.05% Tween 20 and probed with buckwheat allergic pa-

tients' serum (diluted 1:10 in TBS) overnight at room temperature. Membranes were washed, incubated with either alkaline phosphatase-conjugated monoclonal anti-human IgE or anti-human IgG (Sigma Chemical Co., St. Louis, Mo., USA) diluted 1:1,000 in TBS containing 1% BSA for 1 h and developed with a nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate solution (Gibco BRL, Gaithersburg, Md., USA). Membranes were washed with distilled water to terminate development.

Enzyme-Linked Immunosorbent Assay

An indirect ELISA was performed according to a modified method of Engvall and Perlmann [14]. Medical grade high-binding polystyrene 96-well microplates (Corning, Cambridge, Mass., USA) were precoated with purified buckwheat proteins (0.5 µg/well in 100 mM carbonate buffer, pH 9.6) and incubated overnight at 4 °C. The plate was washed 3 times with phosphate-buffered saline containing 0.05% Tween 20 (PBST), pH 7.2, using a BioRad Immunowash Microplate Washer (BioRad). The plate was blocked with 2.0% BSA in phosphate-buffered saline (PBS) for 2 h at room temperature, washed and incubated with human IgE serum diluted 1:50 in PBS containing 1% BSA overnight at 4 °C. The plate was washed and incubated with either alkaline phosphatase-conjugated anti-human IgE or anti-human IgG diluted 1:1,000 in PBS containing 1% BSA overnight at 4 °C. The plate was washed and developed with *p*-nitrophenyl phosphate in 0.1 M diethanolamine buffer (pH 9.8) for 3 h at room temperature. The reaction was terminated through the addition of 3 N NaOH and read at 405 nm using a BioRad Microplate Reader (BioRad).

As a negative control, nonallergenic human serum was used throughout this study.

Carboxymethylation

Samples were carboxymethylated according to the method of Aitken and Learmonth [15]. Briefly, protein samples (5 µg) were dissolved in 50 µl of denaturation buffer (6 M guanidinium hydrochloride in 0.6 M Tris-HCl, pH 8.6) in a microcentrifuge tube and flushed with N₂ gas. An equal volume of 4 mM 1,4-dithiothreitol was added to give a final concentration of 2 mM. The tube was wrapped in aluminum foil and 40 µl of iodoacetic acid was added dropwise with stirring. The sample was incubated in the dark for 30 min at 37 °C followed by dialysis against 50 mM ammonium bicarbonate, pH 7.8, for 6 h.

Ultrafiltration

Ultrafiltration was carried out on a Stirred Ultrafiltration Cell, Model 8050 (Amicon Inc., Beverly, Mass., USA) using a 30,000 and 1,000 molecular weight cut-off (MWCO) membrane.

Reverse-Phase Chromatography

Crude extracts were purified through reverse-phase chromatography using a Source 15RPC ST 4.6/100 column (Amersham Pharmacia Biotech, Uppsala, Sweden). The column was equilibrated with 0.1% trifluoroacetic acid (TFA) and samples eluted with 0.1% TFA in 70% acetonitrile.

N-Terminal Sequencing

Protein samples (10 pmol) were blotted onto PVDF membrane (BioRad) and the N-terminal sequence determined using a PE Biosystems 41 Protein Sequencer (Foster City, Calif., USA).

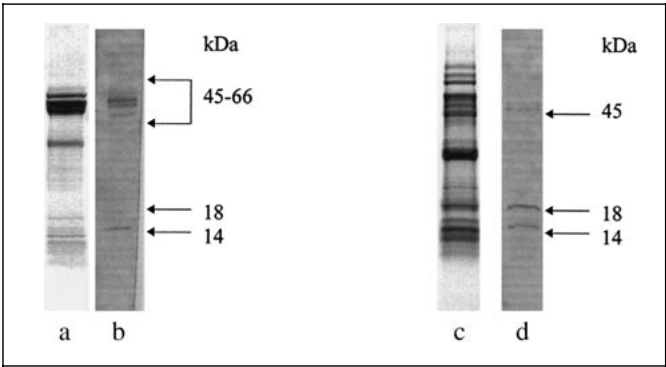


Fig. 1. Electrophoretic (a) and corresponding IgE immunoblot (b) of water-soluble buckwheat fraction under nonreduced conditions. Electrophoretic (c) and corresponding IgE immunoblot (d) under reduced conditions.

Table 2. Immunoreactivity of water-soluble buckwheat proteins excised from SDS-PAGE-reduced gels

Approximate MW, kDa	Absorbance (405 nm) ^a
24.0	0.068 (0.102–0.044)
18.0	0.450 (0.692–0.290)
17.5	0.100 (0.190–0.082)
16.0	0.188 (0.234–0.114)
15.0	0.196 (0.224–0.086)
14.5	0.232 (0.321–0.080)
14.0	0.418 (0.652–0.240)

^a Average from 7 different sera, range in parentheses.

Results

Immunoblot analysis of the water-soluble fraction under nonreduced conditions detected IgE activity around the 45- to 66-kDa region (fig. 1b). Recognition was also observed around the 14- and 18-kDa region, however, bands were faint. Under reduced conditions, recognition was lost around the 45- to 66-kDa region with the exception of a single band around the 45-kDa region while more intense coloration was observed around the 18- and 14-kDa region (fig. 1d).

Seven bands between the 10- and 20-kDa region were excised from SDS-PAGE-reduced gels and electroeluted. Fractions were lyophilized, resuspended and analyzed in an indirect ELISA. The 24-kDa protein was also examined. Strong IgE activity was observed with proteins extracted around the 14- and 18-kDa region while no activity was found with the 24-kDa protein (table 2).

Fig. 2. Electrophoretic (a) and corresponding IgE immunoblot (b) of extracted bands under reduced conditions. Lanes: (1) standard; (2) water-soluble fraction; (3) 66 kDa; (4) 64 kDa; (5) 63 kDa; (6) 61 kDa; (7) 60 kDa, and (8) 59 kDa.

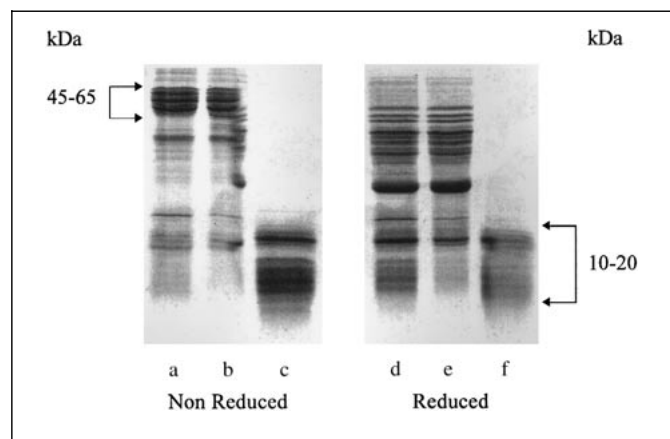
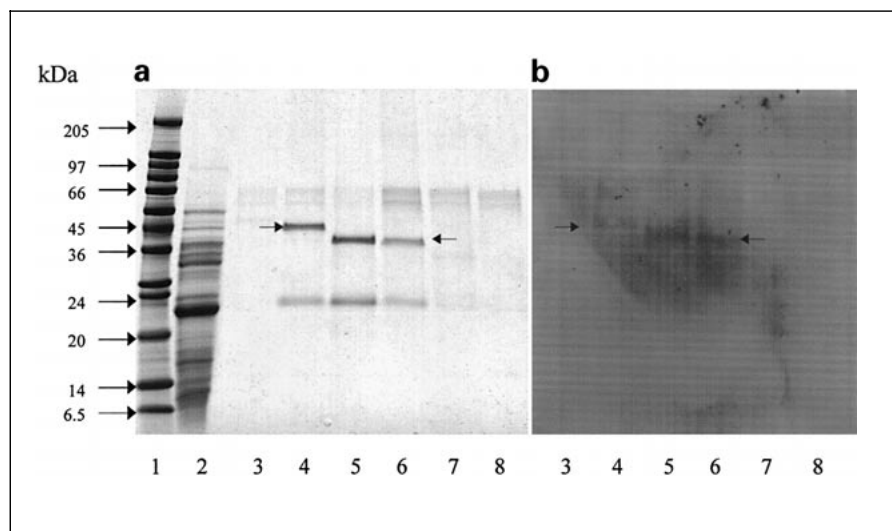


Fig. 3. Electrophoretic profiles of retentate and permeate. Lanes: (a) control; (b) retentate, and (c) permeate under nonreduced conditions. Lanes: (d) control; (e) retentate, and (f) permeate under reduced conditions.

Six bands around the 45- to 66-kDa region were excised from nonreduced SDS-PAGE gels, electroeluted and applied to SDS-PAGE under reduced conditions. Each protein contained a subunit with a molecular mass around the 24-kDa region, however, Western blot analysis revealed no IgE-binding activity (fig. 2). IgE-binding activity was only visible with a protein around the 45-kDa region. From the 12 human sera tested, 7 were found to exhibit strong IgE activity against the water-soluble fractions of buckwheat proteins. Thus, the remaining experiments were carried out with these sera.

Table 3. IgE ELISA activity of retentate and permeate exposed to various conditions

Treatment	Absorbance (405 nm) ^a	
	retentate	permeate
Control	0.569 (1.002–0.346)	0.386 (0.758–0.260)
Heated ^b	0.285 (0.354–0.170)	0.280 (0.302–0.140)
Acidic ^c	0.345 (0.444–0.174)	0.216 (0.338–0.086)
Alkaline ^c	0.371 (0.458–0.178)	0.216 (0.308–0.076)
Urea ^d	0.600 (1.034–0.232)	0.444 (0.672–0.256)
RCM ^e	0.134 (0.342–0.074)	0.151 (0.362–0.026)

^a Average from 7 different sera, range in parentheses.

^b Sample heated for 15 min at 100°C.

^c Sample exposed to acidic (pH 1.5) or alkaline (pH 12.0) conditions for 2 h.

^d Sample exposed to 6 M urea for 12 h at 40°C.

^e Reduced carboxymethylation.

Crude protein preparations were ultrafiltered using a 30,000 MWCO membrane in an attempt to separate out the low molecular weight proteins. The retentate was collected and permeate applied to a 1,000 MWCO membrane to concentrate. Through SDS-PAGE, the permeate was found to contain proteins below the 20-kDa molecular mass (fig. 3).

In order to study the stability of the IgE-binding epitope(s), the retentate and permeate were exposed to various treatments and applied in an indirect ELISA (table 3). An absorbance of 0.569 was observed with

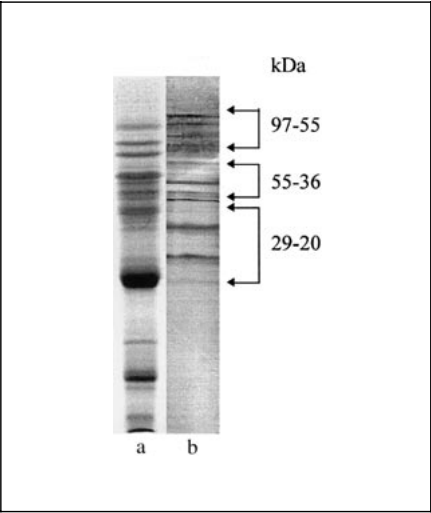


Fig. 4. Electrophoretic (a) and corresponding IgG immunoblot (b) profile under reduced conditions using anti-IgG as the secondary antibody.

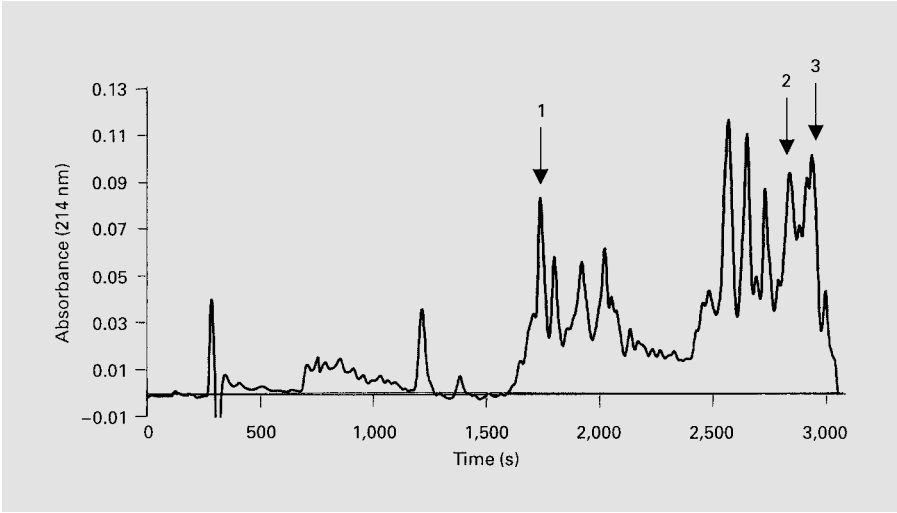


Fig. 5. Reverse-phase chromatographic profile of crude protein extract. Peak 1 represents the 14-kDa protein while peaks 2 and 3 represent the 18-kDa protein.

Table 4. N-terminal amino acid sequence of the 14- and 18-kDa buckwheat protein and its homology

Crop (protein)	Amino acid sequence
Buckwheat (14 kDa)	KYEGALKRIEGEGCK
Rice (phenylalanine ammonia lyase)	KIEGASLRIAQVA
Rice (endosperm protein)	KVEEALKEALEWLDE
Buckwheat (18 kDa)	RDEGFDLGETQMSSKCMR
Rice (<i>O. sativa</i> dehydrin)	RDLGEDLGETPRRATRAN
Rice (chilling-inducible protein)	SIEGFDLLDMLKKEEIV

the retentate (control), however, once heated, activity dropped by almost half. A similar trend was also seen when exposed to acidic (pH 1.5) and alkaline (pH 12.0) conditions. When completely denatured, the absorbance dropped to 0.134. Conversely, when the retentate was exposed to 6 M urea at 40 °C, IgE activity increased slightly to 0.600. Similar results were observed with proteins found in the permeate, however, the decrease in absorbance was not as significant (between 0.10 and 0.15 absorbance units) when exposed to heat, acidic and alkaline conditions (table 3). An increase in absorbance was also observed when treated with 6 M urea while a decrease from 0.386 to 0.151 was found when proteins were carboxymethylated.

Western blotting on the water-soluble fraction of buckwheat proteins was performed, however, anti-human IgG-conjugated alkaline phosphatase was used as the secondary antibody. Numerous bands were detected between the 24- and 70-kDa region, however, no detection was observed below the 20-kDa region (fig. 4). An indirect ELISA using anti-human IgG-conjugated alkaline phosphatase as the secondary antibody was also performed against the retentate and permeate fractions. An absorbance of 0.802 was observed with the native fraction of the retentate, however, when heated for 5 min at 95 °C, absorbance dropped to 0.405. IgG-binding activity was not observed with the permeate fraction.

The 14- and 18-kDa proteins were purified through reverse-phase chromatography. Peaks 2 and 3 were pooled and re-purified through reverse-phase chromatography (fig. 5). Once isolated, N-terminal sequence was completed. The N-terminal amino acid sequence of the 14- and 18-kDa proteins were KYEGALKRIEGEGCK and RDEGFDLGETQMSSKCMR, respectively.

Discussion

IgE activity was not evident with proteins around the 22- or 24-kDa region. This result differs from that of Nair and Adachi [9] and Urisu et al. [8] who found strong IgE activity with these proteins. On the other hand, Yanagiha-

ra [6] observed strong activity with a buckwheat protein around the 17-kDa region. Through N-terminal sequencing, both 14- and 18-kDa proteins were analyzed. Protein Prospector [<http://prospector.ucsf.edu>] and NCBI [<http://www.ncbi.nlm.nih.gov>] databases were accessed to search for some homology with other plant proteins. In particular, the search focused on rice (*Oryza sativa*) since studies have found cross-allergenicity with buckwheat proteins [16–18]. The first 10 amino acids were cross-referenced with a maximum of two amino acid substitutions. The 18-kDa sequence showed homology (80% identity) against a 16.7-kDa protein (*O. sativa* dehydrin, mRNA). Matsuda et al. [19] and Urisu et al. [20] reported a 16-kDa protein to be the major allergen present in rice. Some homology was also observed with a chilling-inducible protein (43.5 kDa) where 5 consecutive amino acids were detected when the first 7 amino acids were cross-referenced with two substitutions. Urisu et al. [20] also found allergenic activity with a small percentage of patients' sera against a rice protein around the 43-kDa region. The N-terminal sequence of the 14-kDa protein showed some homology with a 76.8-kDa rice protein (phenylalanine ammonia lyase) when the first 9 amino acids were analyzed with three substitutions (70% identity). A small percentage of IgE activity with a rice protein around the 78-kDa region has been observed [20]. Some homology was also found with an endosperm lumenal binding protein with a molecular weight of 73.5 kDa. Table 4 summarizes the N-terminal sequencing of the two buckwheat proteins and shared homology with other rice proteins.

After ultrafiltration on the crude extract, the retentate and permeate were exposed to a variety of conditions. A slight increase in absorbance compared to the control was observed when both fractions were treated with urea. These results were expected since it is well known that partially denatured allergens usually increase the IgE-binding activity. During sensitization, antigen-presenting cells in the human body are presented with denatured pieces of allergen to be processed. Samples were analyzed immediately after treatment with urea since partial refolding can occur over time [21]. Conversely, when the protein was fully denatured through chemical modifications (carboxymethylation), absorbance dropped (table 3). The addition of dithiothreitol causes the protein to unfold while treatment with iodoacetic acid prevents any disulfide bonds from reforming. When exposed to heat and acidic conditions, the absorbance decreased compared to the control (table 3).

A type II or cytotoxic hypersensitivity response involves antigen-antibody reactions at the cell surface

which result in cell damage and activation of the complement system. The antibodies usually associated with this type of response are IgG or IgM class [22]. The serum utilized in these experiments were a heterogeneous mixture of antibodies. Since buckwheat proteins were recognized when anti-human IgG was used as the secondary antibody, this would indicate the presence of anti-buckwheat IgG. Thus, it is possible that some of the allergic symptoms experienced by patients with buckwheat allergies are not only due to a type I response but also to a type II response. Takahashi [23] also reported the presence of IgE and IgG antibodies against buckwheat proteins. Results would also indicate that the 14- and 18-kDa proteins possess strict IgE-binding activity since no IgG-binding activity was detected below 20 kDa.

Indirect ELISA results assessing IgG activity also confirmed the findings of the IgG Western blot. IgG activity was not detected with the permeate (proteins below 20 kDa) while activity was detected with the retentate (proteins above 20 kDa). However, a drop in absorbance was observed when the retentate was heated. This drop in absorbance after heat treatment was still significant compared to the control which would be indicative of the heat stability of the IgG epitopes.

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