

Reactivity change of IgE to buckwheat protein treated with high pressure treatment and enzymatic hydrolysis

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ABSTRACT

BACKGROUND: Buckwheat is a popular food material for eastern Asian countries that can cause allergenic response. This study was conducted to evaluate the effects of hydrolysis with papain and high pressure (HP) treatment of buckwheat protein (BWP) on reactivity of IgE and its secondary structure.

RESULTS: Reactivity of IgE was examined using ELISA with serum samples from sixteen patients allergic to buckwheat. Reactivity of IgE to hydrolysate of BWP with papain showed a maximum decrease of 79.8%. After HP treatment at 600 MPa for 1 min, reactivity of IgE to BWP decreased up to 55.1%. When extracted, BWP was hydrolyzed with papain overnight following HP treatment at 600 MPa. Reactivity of IgE decreased significantly, up to 87.1%. Significant changes in secondary structure of BWP were observed in circular dichroism (CD) analysis after hydrolysis with papain following HP treatment.

CONCLUSION: Reduction of the reactivity of IgE showed a correlation with changes in the secondary structure of BWP, which may cause changes in conformational epitopes. This suggests the possibility of decreasing reactivity of IgE to BWP using combined physical and enzymatic treatments.

Keywords: buckwheat; reactivity of IgE; high pressure treatment; hydrolysis; papain

INTRODUCTION

Buckwheat (*Fagopyrum esculentum* Moench), a member of the *Polygonaceae* group of weeds and herbaceous species,¹ is a common food material in Eastern Asia. Consumption of buckwheat products has increased worldwide because of its balanced nutrients, which include carbohydrates, proteins, and fat.²

In Japan, buckwheat is one of the main causes of anaphylaxis as indicated by an incidence rate of 3.4% among 319 patients in a recent study.³ In South Korea, buckwheat is the most common cause of food anaphylaxis, as documented by a 29.3% rate of occurrence in a study of 41 patients under the age of 19.⁴ As part of an effort to protect the public from food allergies, the US Food and Drug Administration published a report titled ‘Approaches to establish thresholds for major food allergens and for gluten in food’ in 2006, suggesting proper ways to establish thresholds of eight major allergy foods including wheat.⁵ Major allergens of buckwheat are Fag e 1, Fag e 2, and Fag e 3. Buckwheat allergen Fag e 1, currently registered in Allergome,⁶ is classified as a 11S globulin-like legumin with a molecular weight of 22-24 kDa.⁷ Fag e 2 (16 kDa) and Fag e 3 (19 kDa) have been reported as major buckwheat allergens and are currently registered in the Allergen Nomenclature of the International Union of Immunological Societies (IUIS).⁸

Much research has been conducted on reducing the allergenicity of food. Efforts to reduce the reactivity of food allergens have been made using heat, enzymes, and chemicals. Soybean allergens showed heat resistant properties,⁹ but enzymatic hydrolysis combined with high pressure (HP) treatment could reduce the allergenicity of soybeans.¹⁰ The allergenicity of peanuts could be reduced with heat treatment such as through boiling and frying,¹¹ and hypoallergenic products from cow milk were introduced using enzymatic hydrolysis.¹²

There have also been efforts to reduce allergenicity using physical and chemical treatments of buckwheat. Chemical treatments such as treatments using acid, alkaline, and Maillard-type glycosylation, and physical methods such as boiling and autoclaving have also been studied.¹³⁻¹⁵ Enzymatic hydrolysis and treatment with high pressure have been investigated to reduce the reactivity of allergens in rice and soybeans.^{10, 16}

Buckwheat allergens are known to be resistant to gastric enzymes such as pepsin and chymotrypsin.¹⁷ Various commercial proteases including papain and alkaline protease were

used to evaluate buckwheat allergens. Among the various proteases tested, papain was a strong candidate to hydrolyze buckwheat allergens efficiently.¹⁸

Among the physical methods for reducing reactivity of IgE to allergenic food, HP treatment of food materials was introduced.¹⁹ The HP technique has been used for the reduction of microbial contaminants and it has demonstrated the advantages of retaining sensorial and nutritional values in comparison to thermal techniques. HP treatment causes denaturation through disruption of hydrogen bonds when treated above 150 MPa, changes in intramolecular forces above 200 MPa, and induction of refolding of low molecular weight proteins at pressures in 400 MPa.²⁰ The HP technique was applied for decreasing the allergenicities or antigenicities of soybean,^{10, 21} rice,¹⁶ apple juice²² and peanuts.²³

In this research, we explored multiplexed methods for reducing allergenicity. To find an effective control method of allergenicity, enzymatic hydrolysis with papain, HP treatment, and enzymatic hydrolysis with papain following HP treatment of buckwheat protein (BWP) were performed to evaluate the reactivity of IgE to BWP using the sera of sensitive patients. Furthermore, we examined the CD spectrum to understand the mechanism of the reduction of IgE reactivity by analyzing the secondary structural change of BWP.

MATERIALS AND METHODS

Patients' sera

Sera of sixteen patients with buckwheat allergies were selected among 1,150 patients with food allergies who visited the Allergy Clinic of Samsung Medical Center (Seoul, Korea). Twelve males and four females, ranging in age from 1 to 19 years were selected for this study (Table 1). Blood was collected from patients using Vacutainer SST Tubes (BD, NJ, USA), and stored at 4 °C for 10 min. After centrifugation at 1300 x *g* for 15 min, serum specimens were separated from the clot and freeze-dried at -80 °C. Measurements of total and specific IgE levels were performed using the Phadia UniCAP system according to the manufacturer's protocols (Phadia, Uppsala, Sweden). Anti-IgE (Phadia, Uppsala, Sweden) reacted with patient samples, and enzyme labeled antibody was added. After incubation with developing agent, fluorescence was measured. The specific IgE concentrations ranged from 1.11 to 57.6 kUA/L (Table 1). The specific IgE concentration of the normal serum was below 0.35 kUA/L (negative). After obtaining informed consent, blood samples were drawn and the sera were frozen at -80 °C until use. This study was approved by the Samsung Medical Center Institutional Review Board.

Extraction of buckwheat protein

Buckwheat protein was extracted according to the modified method of Tanaka *et al.*²⁴ Five grams of buckwheat powder (National Agricultural Cooperative Federation, Bong-pyung, Korea) were extracted overnight by shaking at 4 °C in 50 mL of 0.086 M NaCl (Showa, Gyoda, Japan) solution containing 0.033 M NaHCO₃ (Duksan, Ansan, Korea). After centrifugation at 12,000 x *g* for 30 min, the supernatant was filtered through a 3,000 Da ultrafilter (Millipore, Cork, Ireland), and freeze-dried at -80 °C. Protein concentration was

measured according to the BCA method using the Pierce BCA Protein Assay Kit (Thermo Scientific, IL, USA).

High pressure treatment

Extracted BWP was vacuum packed in polyethylene film bags. Samples were placed into the chamber and HP treatment was performed at 600 MPa (QUINTUS[®] QFP 35L-600, Avure Technologies AB, Sweden). Pressure was maintained for 1 min then depressurized within 5 min. After treatment, samples were transferred to a Falcon tube (BD Falcon™, BD, USA) and stored at -80 °C until use.

Hydrolysis with papain

Extracted BWP and HP treated samples were used for hydrolysis with papain according to the method of Adamczyk *et al.*²⁵ with slight modifications. Hydrolysis buffer contained 1 mM EDTA (Sigma, St. Louis, MO, USA) and 50 mM sodium phosphate (Duksan, Ansan, Korea), and was adjusted to pH 7.0. The protein concentrations of each sample were adjusted to 6 mg/mL using hydrolysis buffer, and the concentration of the papain (0.5 unit/g, Wako Chem., Japan) solution was 1 mg/mL. To initiate hydrolysis, samples were added to the pre-heated (37±2 °C) papain solution (enzyme solution:buckwheat sample = 6:1 (v:v)). Samples were incubated in a shaking incubator and each specimen was sampled at targeted intervals of 0 min, 60 min and overnight. Each specimen was quenched by heating at 95 °C for 10 min, stopping enzyme reactivity.

SDS-PAGE and immunoblotting

SDS-PAGE of BWP was performed as described by Laemmli²⁶ using 12% Bis-Tris gel (NuPAGE, Invitrogen, USA). Samples were denatured using sample buffer containing reducing agent for 5 min at 95 °C, then electrophoresed for 40 min at 200 V. Dokdo-MarkTM broad-range protein marker (EBM-1032, ELPiS, Daejeon, Korea) was used to determine the protein molecular weights. Proteins were visualized by staining with Coomassie Brilliant Blue (Bio-Rad, Hercules, CA, USA). For immunoblotting, proteins from the gels were transferred to polyvinylidene difluoride (PVDF) membrane and processed at 20 V for 7 min using iBlot Dry blotting System (Invitrogen, Israel). The membrane was blocked with 2% nonfat dry milk for 1 hour at room temperature, and then incubated with pooled serum overnight at 4 °C. After washing three times with 0.03% PBST, the membrane was incubated with biotin labeled goat anti-human IgE (KPL, Gaithersburg, MD, USA) diluted 1:4000 in 2% nonfat dry milk for 1 hour followed by 30 min incubation with NeutrAvidin horseradish peroxidase (Pierce Chemical, Rockford, IL, USA) at room temperature. Then, the membrane was washed three times and treated with ECL reagent (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) for 2 min. The membrane was exposed to high performance chemiluminescence film (GE Healthcare, Buckinghamshire, UK), after which the film was developed.

Reactivity of IgE

Enzyme-linked immunosorbent assay (ELISA) was performed to evaluate the IgE reactivity of patients' sera with BWP. A 96-well microplate (Costar, Corning, NY, USA) was coated with 100 µL of buckwheat samples containing 50 µg/mL of protein diluted in 50 mM carbonate-bicarbonate buffer (pH 9.6, Sigma, St. Louis, MO, USA), and incubated at 4 °C for 16 h. The coated plate was washed with 0.05% PBS-Tween 20 for three times then blocked with 100 µL of 2% bovine serum albumin(BSA)-PBS for 2 h to prevent nonspecific linking.

Then, an aliquot of patient sera (320-fold-diluted) was added and incubated for 2 h at room temperature. After washing the plate, adsorbed IgE was detected with 100 μ L of goat anti-human IgE-HRP (1 μ g/mL) (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) diluted 1:2500 with 2% BSA-PBS. The plate was further washed and developed with 100 μ L of TMB peroxidase (3, 3', 5, 5'-tetraethylbenzidine, KPL, Gaithersburg, MD, USA) and then quenched with 1 M H_3PO_4 (Duksan, Kyunggi, Korea). Absorbance was measured in duplicate using a microplate reader (VersaMax™, Molecular Devices, CA, USA) at 450 nm.

Circular dichroism (CD) spectroscopy

Far-UV circular dichroism spectra of native, high pressure treated, hydrolyzed papain, and hydrolyzed papain following high pressure treated BWP were obtained to define the secondary structure of those samples using a Chirascan™-plus CD Spectrometer (Applied Photophysics, Surrey, UK). A 400 μ L sample was contained in a cuvette and scanned in the far-UV range from 190 to 250 nm at room temperature. CD analysis was performed in triplicate, and data was analyzed with Microsoft Excel and plotted with Prism 6.0.

RESULTS AND DISCUSSION

SDS-PAGE and immunoblotting analysis of BWP

Analysis of hydrolyzed BWP with papain following HP treatment was conducted using SDS-PAGE (Fig. 1). We could observe the well-known allergenic protein bands of BWP, which are referred to as P0, Fag e 1 (24 kDa), Fag e 2 (16 kDa), Fag e 3 (19 kDa), 10 kDa, and 9 kDa^{15, 24, 27, 28}. After hydrolysis with papain for 60 min, proteins were hydrolyzed and protein bands smeared below 7 kDa on SDS-PAGE. Protein bands above 15 kDa were no longer detected after hydrolysis for 60 min and overnight. Moreover, protein bands between 15 kDa and 10 kDa disappeared after being hydrolyzed overnight with papain (Fig. 1(A)).

Buckwheat protein treated with HP showed no significant change when compared to BWP using SDS-PAGE (Fig. 1(B)). Buckwheat protein with papain for 60 min following HP treatment (Fig. 1(B)) demonstrated more efficient hydrolysis relative to that without HP treatment (Fig. 1(A)). The impact of HP treatment on the proteins might be related to the rupture of the non-covalent interactions in the protein molecule, which has a substantial impact on protein conformation.¹⁹

Immunoblotting using patients' pooled serum was performed to evaluate the hydrolysis of BWP treated with HP (Fig. 2). Among major allergenic BWP bands currently registered on IUIS or Allergome, Fag e 1 and Fag e 2 were clearly observed in Fig. 2. Other protein bands, 30 kDa, 40-50 kDa, 60 kDa, and 70 kDa were also observed (Fig. 2). The intensity of the bands on immunoblotting showed no significant difference between BWP (P₁₀) and HP treated BWP (P₂₀). As shown in SDS-PAGE and immunoblotting (Fig. 1 and Fig. 2), all major allergenic protein bands of BWP disappeared after hydrolysis with papain overnight following HP treatment.

Reactivity of IgE to hydrolysates of BWP with papain

To analyze the reactivity of IgE to BWP hydrolyzed with papain, we performed ELISA analysis using 16 serum samples from patients allergic to buckwheat. After hydrolysis overnight, IgE reactivity of 5 of the 16 samples (Serum # 4, 9, 10, 13, 16) decreased to the level observed with normal serum with rates ranging from 63.3% to 79.8% lower. The IgE reactivity of the remaining 11 serum samples (Serum # 1, 2, 3, 5, 6, 7, 8, 11, 12, 14, 15) decreased, but remained greater than that of the normal serum level. In these 11 samples, the decrease in IgE reactivity ranged from 52.0% to 78.4%. Papain digestion could not fully hydrolyze buckwheat allergens (Fig. 3). IgE reactivity of one serum sample displayed exceptionally high absorbance at 3.54, showing a decrease in absorbance of up to 68.8% after hydrolysis overnight with papain (Serum #11). This observation was similar to findings from Lee et al,¹⁷ in which buckwheat was hydrolyzed with the protease pepsin. Lee observed that out of a group of 6 serum samples, two still interacted significantly with the pepsin hydrolysate of buckwheat. These results may be due to variations in absorbance of the reactivity with individual sera, since each serum showed different reactivity with different epitopes in buckwheat allergens.

Reactivity of IgE to BWP treated with HP

Buckwheat protein was treated with high pressure at 600 Mpa for 1 min, 10 min, and 30 min. Because reactivities of IgE were similar after high pressure treatment for all time intervals, HP treatment of 600 MPa for 1 min was chosen as the standard protocol (data not shown). After HP treatment, reactivity of IgE in patient serum samples to BWP decreased by intervals of 21.2% to 55.1% when compared with that of T0 in Figure 3 and 4, although no difference was observed in the SDS-PAGE result (Fig. 1). The reduction of allergic response may be due to protein denaturation and change in protein conformation which are known to decrease allergenicity toward food allergens following HP treatment.¹⁹ Our results were similar to

those found in earlier studies using HP treatment at 300 MPa for 15 min on germinated soybean seed in which immunoreactivity was reduced by about 80%.²⁹

here have been several reports that HP treatment affects allergenicity. Mal d1, the major apple allergen, was treated with 500 MPa for 100 min, but there was no difference in allergenic response when compared to controls in western blotting analysis.²² Hu et al.²³ reported that antigenicity of Ara h 2, one of the major peanut allergens, decreased by 50% when treated with HP microfluidisation at 180 MPa. Moreover, after 300 MPa treatment, the reactivity of IgE to raw soybean seed increased by about 30%.²⁹ Allergen proteins have multiple antigen binding epitopes. In several studies, investigators have attributed the decrease in IgE reactivity to conformational changes of the allergen structure.^{30, 31} High pressure may act on the secondary, tertiary and quaternary structures of proteins which are regulated by hydrogen bonds.³²

Effects of hydrolysis with papain following HP treatment of BWP on reactivity of IgE

We also subjected HP treated (600 MPa for 1 min) BWP to enzymatic hydrolysis with papain. After hydrolysis with papain following HP treatment, only four patients' sera (serum #2, 3, 6, 11) showed higher reactivity than that of normal serum (Fig. 4). After hydrolysis overnight with papain following HP treatment, reactivity of IgE decreased by 58.6% - 87.1%.

High pressure treatment seemed to increase the efficiency of papain hydrolysis. Significant decreases in IgE reactivity were seen in BWP hydrolyzed with papain for 60 min (T60) and overnight (To/n) following HP treatment as compared to BWP that did not undergo HP treatment (T0 in Fig. 3). Reactivities of IgE decreased to normal levels in twelve out of sixteen serum samples (#1, 4, 5, 7, 8, 9, 10, 12, 13, 14, 15, 16) after hydrolysis with papain overnight following HP treatment. The other four sera (#2, 3, 6, 11) decreased by 77.7 % - 86.1% (Fig. 4). Notably, reactivity of IgE to the hydrolysate from sample #11 was

exceptionally high; the reactivity of IgE with this sample decreased up to 86.1% after hydrolysis overnight with papain following HP treatment. These results suggest that the allergic response, as indicated by reactivity of IgE, could significantly decrease in BWP samples that were first treated with HP and then digested with papain.

Previous studies have indicated that the combination of enzymatic hydrolysis and HP treatment could effectively decrease reactivity of IgE. The concentration of Gly m 1, a soybean allergen, decreased by about 0.5% with 300 MPa HP treatment, but with 300 MPa HP treatment followed by hydrolysis with Corolase PN-L, the concentration of Gly m 1 showed a 54% decrease.¹⁰ High pressure treatment of rice in the presence of Protease-N was also effective for releasing rice allergen proteins into the surrounding solution.¹⁶ These reports coincide with the findings of Huang et al.,¹⁹ stating that HP treatment could enhance enzymatic hydrolysis and extraction of allergens. In the present study, changes in protein conformation resulting from HP treatment may enhance papain hydrolysis of BWP.

Effect of HP treatment and hydrolysis with papain on secondary structure of BWP

The secondary structure of BWP with HP treatment, hydrolysis with papain, or hydrolysis with papain following HP treatment was analyzed using CD spectroscopy. The far-UV spectra are shown in Fig. 5. Buckwheat protein sample (T0) produced a large negative peak between 209 and 225 nm, and a positive peak at about 192 nm, a typical α -helix dominant structure. When BWP was treated with HP, the resulting spectrum seemed to contain less α -helical content (a→d in Fig. 5). Hydrolysis with papain caused a marked increase in random structure fractions in both untreated (a→b & c in Fig. 5) and HP treated BWP (d→e & f in Fig. 5) which showed a change in negative peak wavelength (209→204 nm). The amount of

random structural contents of BWP increased with HP treatment compared to that of untreated BWP. Hydrolysis with papain following HP treatment of BWP showed the most significant change in CD spectra (a→f in Fig. 5), which correlates to the results of reactivity with patient serum (Fig. 3 & Fig. 4). It was reported that the region containing EGVRDLKE may form an α -helix located on the surface of BWP, 16 kDa.³³ The 13S globulin allergen of buckwheat consists of three protomers exhibiting the cupin motif, which predominantly contain α -helices.³⁴ Hydrolysis with papain and HP treatment of BWP might have a synergetic effect on the loss of α -helical contents, thereby affecting the destruction of the conformational and sequential epitope of BWP, resulting in a significant decrease in IgE reactivity to patient sera.

These results suggest that HP treatment and hydrolysis with papain induce a significant decrease in IgE-binding properties of BWP due to changes in the secondary structure. Similarly, roasted peanuts in autoclave conditions were reported to lose significant α -helical contents, causing a notable reduction in IgE immunoreactivity.³⁵

CONCLUSION

In this study, the effects of HP treatment and hydrolysis with papain on BWP were evaluated. High pressure treatment on BWP induced modest decreases in IgE reactivity, while hydrolysis with papain showed more significant reductions. Combining both methods, i.e., hydrolysis of BWP with papain following HP treatment, induced an additive effect on decreased reactivity of IgE. These results might be explained by the denaturation of the secondary structural contents of BWP with HP treatment and hydrolysis with papain. The results of this study suggest the feasibility of decreasing reactivity of IgE to BWP, which may lead to the production of hypoallergenic buckwheat for safe consumption of allergic individuals.

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FIGURE LEGEND

Figure 1. SDS-PAGE of BWP hydrolyzed with papain (A) and BWP hydrolyzed with papain following HP treatment (B). (A) M: Molecular weight marker; P₁0, P₁60, and P₁o/n: BWP without hydrolysis at 0 min, 60 min, and overnight; T₁0, T₁60, and T₁o/n: BWP hydrolyzed with papain for 0 min, 60 min, and overnight. (B) M: Molecular weight marker; P₂0, P₂60, and P₂o/n: HP treated BWP without hydrolysis at 0 min, 60 min, and overnight; T₂0, T₂60, and T₂o/n: BWP hydrolyzed with papain following HP treatment for 0 min, 60 min, and overnight.

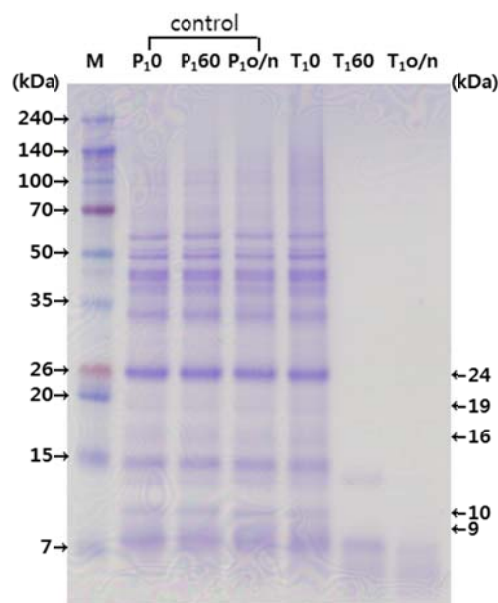
Figure 2. Immunoblotting of BWP with papain hydrolysis following HP treatment with pooled patients' serum. M: molecular weight marker; P₁0: BWP; P₂0 and P₂o/n: HP treated BWP without hydrolysis at 0 min and overnight; T₂0 and T₂o/n: papain hydrolysates following HP treatment at 0 min and overnight.

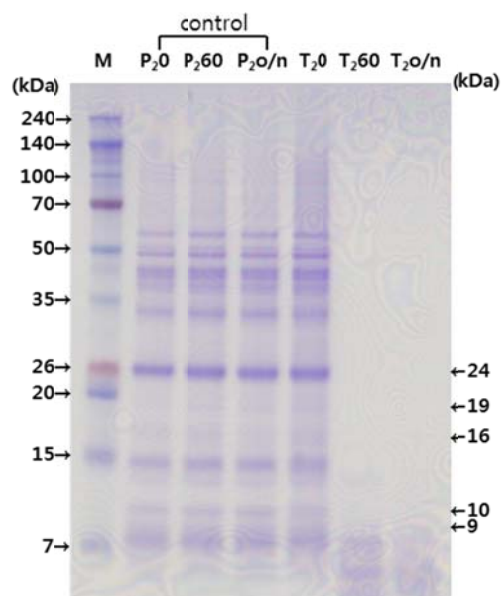
Figure 3. IgE reactivity in sera of 16 patients with buckwheat allergy: effect of hydrolysis of BWP with papain. BWP was hydrolyzed with papain for the indicated interval as described in the Experimental section. One sample of normal serum was also included in the analysis. Blank: serum-free 2% BSA-PBS; T0: papain treatment for 0 min; T60: papain treatment for 60 min; To/n: papain treatment overnight. Error bars indicate standard deviations.

Figure 4. IgE reactivity in sera of 16 patients with buckwheat allergy: effect of hydrolysis of BWP following HP treatment. BWP was treated with HP at 600 MPa for 1 min then hydrolyzed with papain for the indicated times. One normal serum sample was included in the analysis. Blank: serum-free 2% BSA-PBS; T0: papain treatment for 0 min; T60: papain treatment for 60 min; To/n: papain treatment overnight. Error bars indicate standard deviations.

Figure 5. CD spectra of BWP treated with HP and hydrolysis with papain. T0, T60, and To/n: BWP hydrolyzed with papain for 0 min, 60 min, and overnight; HP T0, HP T60, and HP To/n: hydrolysis with papain following HP treatment for 0 min, 60 min, and overnight.

(A)



(B)**Figure 1.**

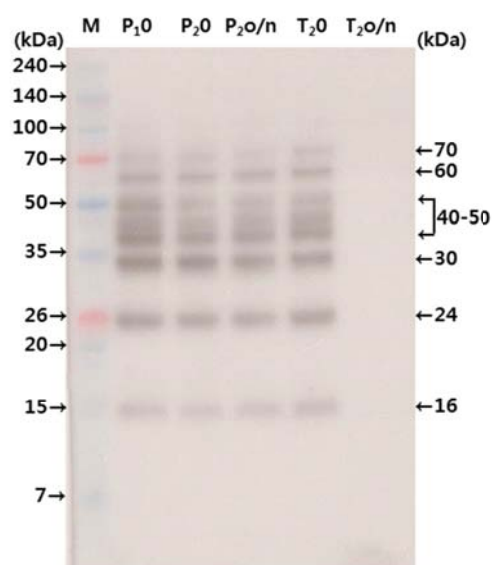


Figure 2.

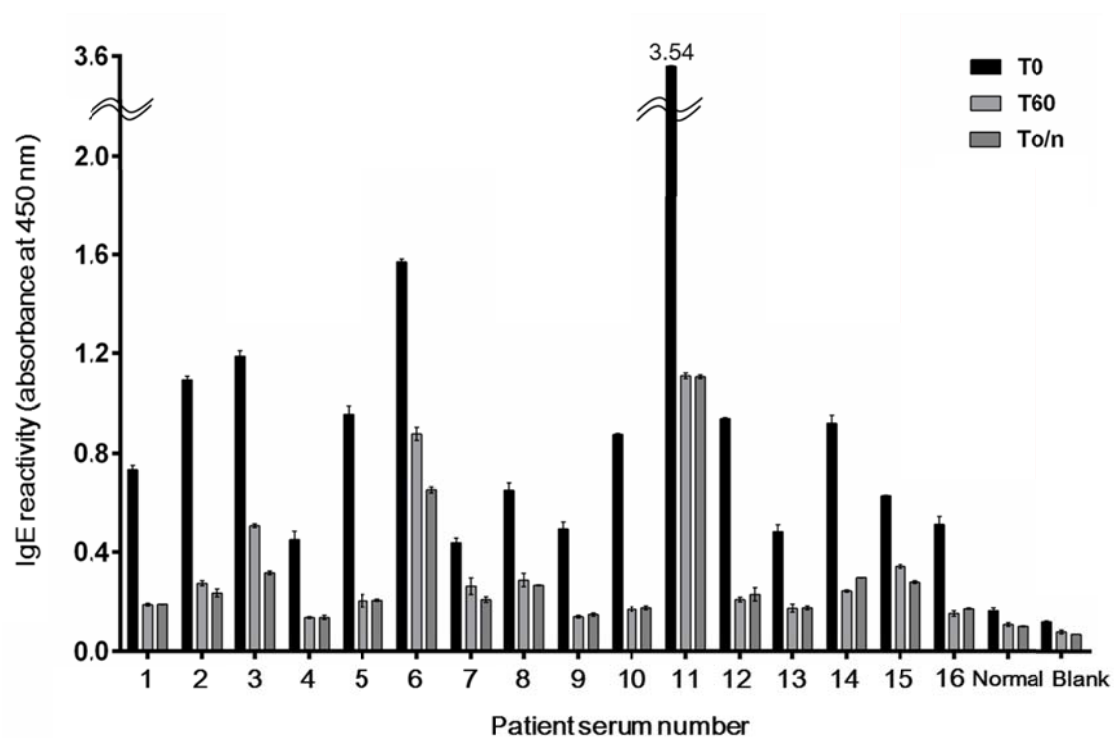


Figure 3.

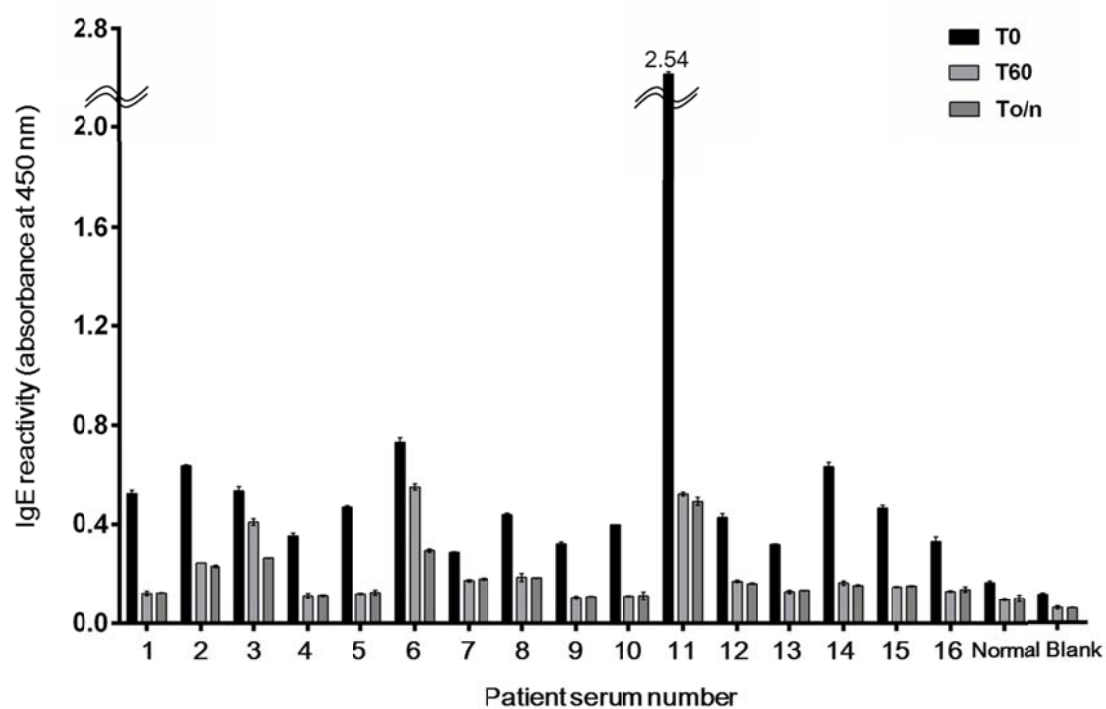


Figure 4.

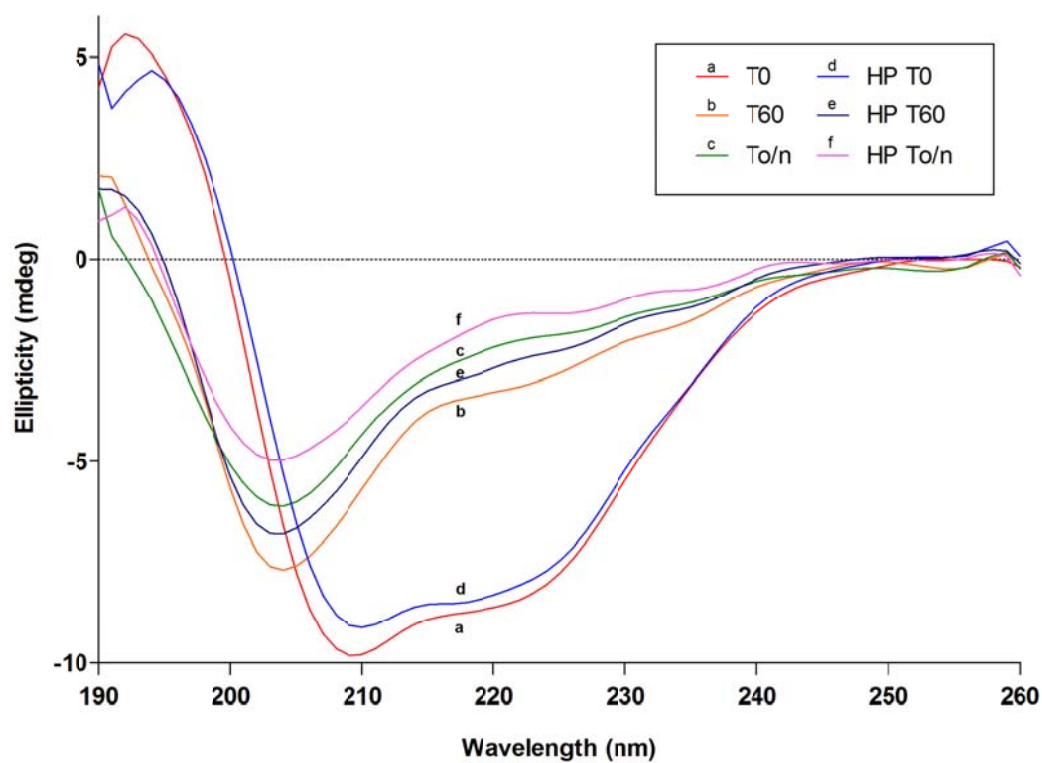


Figure 5.

Table 1. Coated allergen particle (CAP) IgE fluorescent enzyme immunoassay (FEIA) results from buckwheat (kUA/L) in serum from 16 patients allergic to buckwheat.

Patient No.	Age (years)	Sex (M/F)	CAP IgE FEIA buckwheat (kUA/L)
1	3	F	10.5
2	4	M	57.6
3	16	F	41
4	3	M	13
5	2	M	51.7
6	4	F	47.2
7	5	M	1.11
8	19	M	9.8
9	6	M	1.58
10	3	M	51.4
11	2	M	55
12	2	F	24.6
13	3	M	4.18
14	10	M	7.59
15	2	M	1.95
16	1	M	5.54