

Research Note

An Autoclave Treatment Reduces the Solubility and Antigenicity of an Allergenic Protein Found in Buckwheat Flour

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ABSTRACT

The effects of an autoclave treatment of buckwheat flour on a 24-kDa allergenic protein were investigated by measuring reduction in solubility and antibody binding. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis showed that the intensity of the major bands, including that of the 24-kDa allergen, was reduced by the autoclave treatment. The protein solubility in buckwheat flour was variably decreased by the autoclave treatment. Enzyme-linked immunosorbent assay analysis using a monoclonal antibody specific for buckwheat 24-kDa protein showed that the reactivity of protein extracts (10 µg/ml) from buckwheat flour was lowered by the autoclave treatment. The autoclave treatment may reduce the major allergen content of buckwheat. Future studies will determine if autoclaving treatments affect the allergenicity of the 24-kDa buckwheat protein.

In recent years, buckwheat has become more and more popular in many countries as a kind of health food. However, buckwheat can also cause allergy and even anaphylaxis in a few subjects (7, 19). Buckwheat allergy is considered to be one of the most critical diseases, characterized by severe and dangerous symptoms following the ingestion of a small amount of flour or food products made from buckwheat (21). Buckwheat contains a number of allergens that react with immunoglobulin E (IgE) from buckwheat allergy patients (14, 26). The 24-kDa protein, the β chain of 11S globulin, has been considered to be the major allergen responsible for buckwheat allergy (16), and its nucleotide sequence has been reported (26).

Several research groups have exploited new techniques to eliminate food allergenicity and have developed low-allergenic food products (1, 18, 20, 23). However, there is little information on the reduction or elimination of buckwheat allergen related to food allergenicity.

A high-pressure treatment has been found to affect the structure of proteins (3, 5). Several previous articles have mentioned the effects of high pressure on the antigenicity (6, 8, 11, 24).

The objectives of this study were to investigate the effects of autoclave treatment of buckwheat flour on 24-kDa allergenic protein.

MATERIALS AND METHODS

Samples. Buckwheat seeds (*Fagopyrum esculentum* Moench) commonly cultivated in China were used. Whole seeds were

dehulled and then ground with a roll mill. The resulting flour was passed through a 100-mesh screen.

Autoclave treatment of buckwheat flour. Buckwheat flour flatly wrapped in aluminum foil was autoclaved at 121°C for 5, 15, and 30 min or 110 and 115°C for 30 min. To determine the total content of soluble proteins from autoclave-treated and untreated buckwheat flour, each type of flour was suspended in 20 mM phosphate buffer (pH 8.0) containing 3% NaCl. The suspensions were sonicated for 5 min, stirred at 60°C for 2 h, and centrifuged (3,000 × g for 10 min). Protein contents of the supernatant were determined by Quick Start Bradford dye reagent (Bio-Rad, Hercules, CA).

Preparation of monoclonal antibody. Buckwheat flour was suspended in 3% NaCl solutions. After being stirred for 2 h at room temperature, the suspension was centrifuged at 8,000 × g for 10 min. The supernatant was dialyzed against water for 12 h and then centrifuged at 8,000 × g for 10 min. The precipitate (globulin fraction) was lyophilized. Monoclonal antibody (MAb) to buckwheat globulin fraction was prepared as described in our previous study (17). The culture supernatants of the hybridoma population were screened for specific antibody production by ELISA or Western blotting with buckwheat globulin protein used as an antigen. Positive hybrids were cloned by the limiting dilution method. The culture supernatant of the cloned hybridoma was diluted with phosphate-buffered saline (PBS) and used for enzyme-linked immunosorbent assay (ELISA) (1:50) and Western blotting (1:10).

SDS-PAGE and Western blotting. Urea–sodium dodecyl sulfate (SDS) buffer (20 mM Tris-HCl, pH 8.0, 8 M urea, 2% SDS, 2% 2-mercaptoethanol) was used to extract total proteins from autoclave-treated and untreated buckwheat flour. The buckwheat flour was resolved in urea-SDS buffer, sonicated for 5 min, and stirred at 60°C for 2 h. The resultant sample solution was

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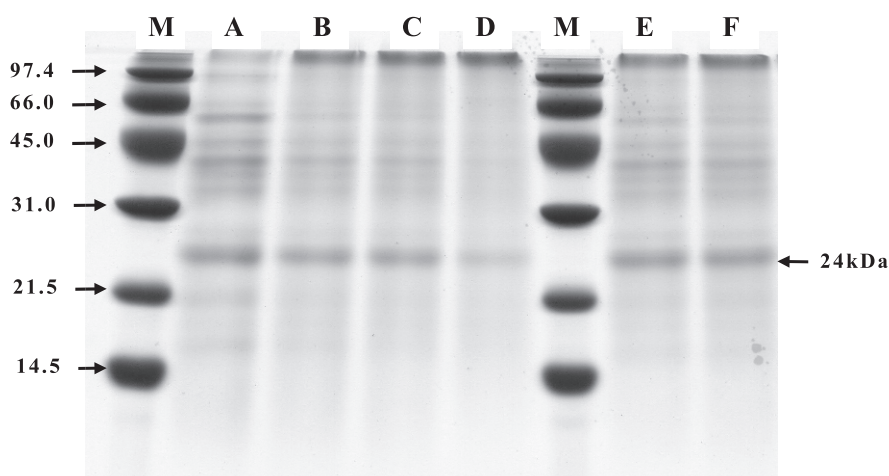


FIGURE 1. SDS-PAGE analysis of buckwheat proteins from autoclave-treated and untreated buckwheat flour. Buckwheat flour flatly wrapped in aluminum foil was autoclaved at 121°C for 5, 15, and 30 min and at 110 and 115°C for 30 min. The buckwheat flour was resolved in urea-SDS buffer and then sonicated for 5 min and stirred at 60°C for 2 h. The resultant sample solution was used for SDS-PAGE using 15% polyacrylamide gel. M, molecular weight marker. (A) Untreated buckwheat flour. (B through F) Autoclave-treated buckwheat flour at 121°C for 5 min (B), 121°C for 15 min (C), 121°C for 30 min (D), 110°C for 30 min (E), 115°C for 30 min (F).

used for SDS-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was carried out on 15% polyacrylamide gel according to the method of Laemmli (9). For Western blotting, buckwheat proteins were transferred onto the transfer membrane (Clear blot membrane P, Atto, Tokyo, Japan), and the blotted buckwheat 24-kDa protein was detected by subsequent reactions with anti-buckwheat 24-kDa protein mouse MAb, diluted (1:200) horseradish peroxidase-conjugated anti-mouse IgG antibody (titer 1:177, 550) (ICN Pharmaceuticals, Cleveland, OH), and the substrate mixture of 4-chloro-1-naphthol and H_2O_2 (25).

Purification of buckwheat 24-kDa protein. The buckwheat globulin fraction was dissolved in 3% NaCl (pH 8) solutions. The 24-kDa protein was purified on a preparative SDS-PAGE as described previously (12). The fractions containing protein bands of 24 kDa were pooled and dissolved in PBS.

ELISA. The purified 24-kDa protein fraction was diluted in PBS (2.5 to 15 μ g/ml), and 50 μ l was dispensed (0.125 to 0.75 μ g/well) directly into a 96-well ELISA plate (9018, Costar, NY) and left at 4°C overnight. Autoclave-treated and untreated buckwheat flour samples were extracted with 20 mM phosphate buffer (pH 8.0) containing 3% NaCl. The protein concentrations from both extracts were adjusted to 10 μ g/ml in PBS; 50 μ l was dispensed (0.5 μ g/well) directly into a 96-well ELISA plate. The wells were blocked with 300 μ l of 0.7% bovine serum albumin for 1 h at room temperature followed by the addition of 50 μ l of anti-buckwheat 24-kDa protein MAb and incubation for 1 h. After washing four times with PBS, 50 μ l of horseradish peroxidase-conjugated anti-mouse IgG (1:2,000) was added and left for 1 h. Each well was washed five times with PBS, and then the substrate mixture of H_2O_2 and ABTS (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was dispensed (50 μ l) into the well and left to undergo the enzyme reaction for 1 h at room temperature. The absorbance at 405 nm was measured on a model 680 microplate reader (Bio-Rad).

Statistical analyses. ELISA data are expressed as the means $\pm$ standard deviations. Differences in ELISA reactivity between treated and untreated samples were analyzed by the Student *t* test. Data analysis was performed using SPSS 17.0 for Windows. Significance of the difference was set at *P* values of <0.05 or <0.01.

RESULTS AND DISCUSSION

Effect of autoclave treatment of buckwheat flour on the protein solubility and the protein bands. The total

soluble protein contents of autoclave-treated flour and untreated buckwheat flour were compared. The treatment at 121°C resulted in protein concentrations of 12.0, 13.2, and 10.8 μ g/ml for 5, 15, and 30 min, respectively. The treatments at 110 and 115°C for 30 min resulted in 18.3 and 13.9 μ g/ml, respectively. The protein concentration of untreated buckwheat flour was 24.8 μ g/ml. As shown in Figure 1, the intensity of the major bands, including that of the 24-kDa allergen, was reduced by the treatments at 121°C for 5 and 15 min. A more pronounced reduction was observed by the treatment at 121°C for 30 min. Although the treatments at 110 and 115°C for 30 min also reduced the intensity of the major bands, the effects were lower than that at 121°C. New protein bands did not appear in the treated sample, but unclear bands appeared at the start lines of the gel.

It is well known that a heat or high-pressure treatment causes the denaturation of proteins (3). High pressure can affect protein conformation and can lead to protein denaturation, aggregation, or gelation (5). Marcos et al. (10) reported that high pressure processing induced a reduction of protein solubility and modified the composition of the sarcoplasmic protein fraction. In the present study, an autoclave treatment of buckwheat flour markedly reduced the content of soluble proteins. Furthermore, the result of SDS-PAGE analysis indicated that the intensity of the major bands, including that of the 24-kDa allergen, was reduced by an autoclave treatment. This reduction of protein solubility seems to be due to protein denaturation or aggregation. It is of interest to establish in future studies whether the denaturation of 24-kDa proteins may affect their allergenicity. Cheah and Ledward (2) observed that the application of high pressure (300 to 400 MPa) induced a marked change in soluble protein patterns of minced pork. They suggest that decreased band intensities could be related to protein degradation or insolubilization of sarcoplasmic proteins due to protein denaturation. Ohshima et al. (13) suggested that certain fish sarcoplasmic proteins might become covalently linked together under application of high pressure and thus resistant to extraction with SDS. In the present study, autoclave treatment induced a reduction of protein solubility. Decreased band intensity could be related to insolubilization of protein. Although we used urea and SDS to extract total

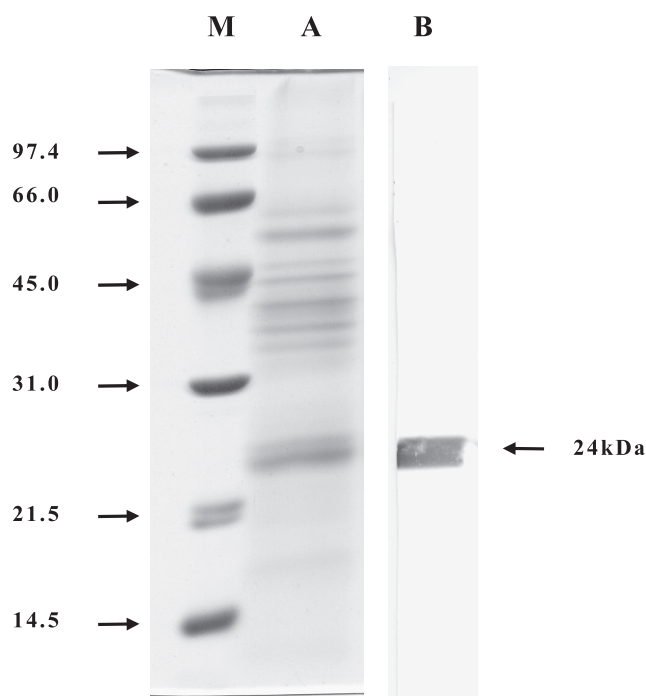


FIGURE 2. SDS-PAGE analysis (A) and Western blotting (B) using anti-buckwheat 24-kDa protein monoclonal antibody. SDS-PAGE was carried out on 15% polyacrylamide gel. Buckwheat proteins were transferred onto the transfer membrane, and blotted 24-kDa protein was detected by Western blotting with monoclonal antibody and horseradish peroxidase-conjugated anti-mouse IgG antibody. M, molecular weight marker; 24kDa, buckwheat 24-kDa protein.

proteins for sample preparation of SDS-PAGE, insolubilization of some buckwheat proteins might be induced by autoclave treatment. It was suspected from this result that the antigenicity of the 24-kDa allergen remains in the aggregated fraction.

Effect of autoclave treatment of buckwheat flour on the reactivity of protein extracts to MAb in ELISA. To determine the specificity of the prepared MAb, Western blotting of buckwheat protein was performed. As shown in Figure 2, the MAb reacted specifically with buckwheat 24-kDa protein. In the present study, we developed a simple ELISA system using MAb. The purified 24-kDa protein solution was adsorbed onto the ELISA plate and dose-dependently reacted with the MAb (Fig. 3A). However, the reactivity to the MAb of the protein extract (10 μ g of protein per ml) from autoclave-treated buckwheat flour at 121°C for 5, 15, and 30 min was significantly lower than that of the same protein concentration from untreated samples (Fig. 3B). Weak effects of autoclave on the ELISA reactivity were detected at 110 and 115°C for 30 min.

Buckwheat frequently elicits severe allergic symptoms (4, 22), and about 0.2% of Japanese children have allergy to buckwheat (15). In a previous report 24-, 19-, and 16-kDa proteins were identified as strong candidates for major buckwheat allergens (14). Tanaka et al. (16) reported that 24-kDa buckwheat proteins were bound to IgE antibodies present in the sera from almost all subjects carrying positive

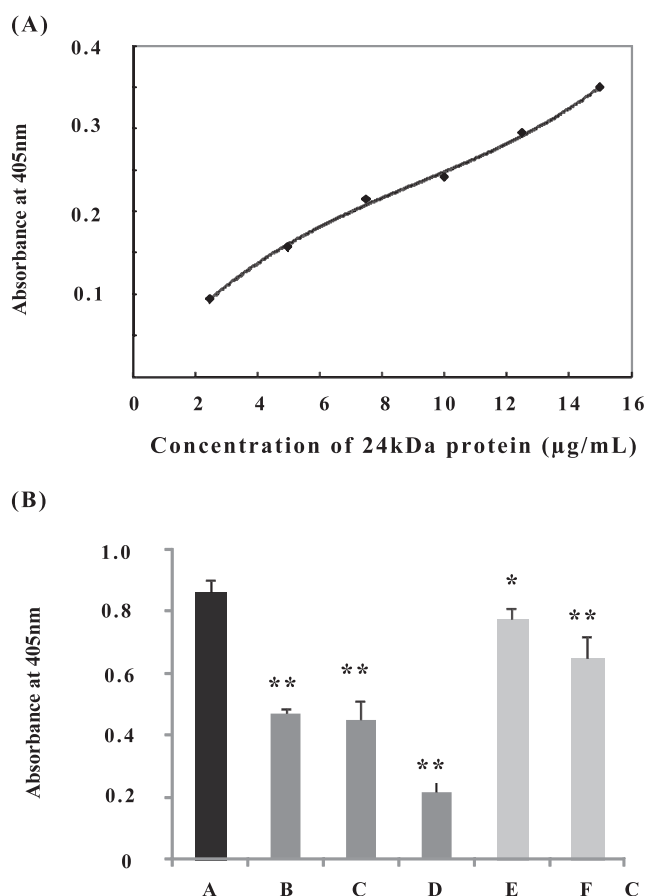


FIGURE 3. ELISA analysis of purified buckwheat 24-kDa protein (A) and protein extracts from autoclave-treated and untreated buckwheat flour (B). (A) Buckwheat 24-kDa protein was diluted in PBS (2.5 to 15 μ g/ml), dispensed (50 μ l; 0.125 to 0.75 μ g per well) into a 96-well ELISA plate, and left at 4°C overnight. (B) Autoclave-treated buckwheat flour and untreated buckwheat flour were extracted with 20 mM phosphate buffer (pH 8.0) containing 3% NaCl. The protein concentrations from both extracts were adjusted to 10 μ g/ml in PBS and dispensed (50 μ l; 0.5 μ g per well) directly into a 96-well ELISA plate. ELISA was performed as described in "Materials and Methods." In B, each value is the mean $\pm$ standard deviation ($n = 3$ or 4). * $P < 0.05$; ** $P < 0.01$.

buckwheat-specific IgE. To date, there is no method for the detection of major allergens in buckwheat flour by use of a specific antibody. For the development of hypoallergenic buckwheat products, it is essential to establish a method for the detection and measurement of major allergens in the products. In the present study, we have prepared successfully the MAb specific for the 24-kDa allergen. This MAb could be useful for the measurement of 24-kDa allergens in various buckwheat products, using ELISA or Western blotting.

Yamamoto et al. (24) reported the effects of a high-pressure treatment on the IgE-specific binding activity and structural changes of bovine gamma globulin. They found that the IgE-specific binding activity and allergenicity of bovine gamma globulin were decreased by the high-pressure treatment. In the present study, ELISA analysis using MAb showed that the reactivity of protein extracts from buckwheat flour was lowered by the autoclave treatment. As the

treatment time became longer, this phenomenon became more evident. The results suggest that the 24-kDa allergen in buckwheat flour was insolubilized by the autoclave treatment or that the epitopes on the allergen against the MAb might be modified during the degradation of protein and that the MAb could not bind to the modified epitopes.

Kato et al. (8) reported that release of allergenic proteins from rice grains was induced by high hydrostatic pressure. When polished rice grains were immersed in distilled water and pressurized at 100 to 400 MPa, considerable amounts of proteins were released. In the present study, we autoclaved buckwheat flour directly. The technique that may release allergenic proteins from immersed buckwheat seed could be utilized as a processing tool for reducing the allergenicity. Further study on the high-pressure treatment combined with other food processing techniques is necessary to eliminate allergenicity of buckwheat.

In conclusion, we demonstrated that the reactivity to specific MAb of the 24-kDa buckwheat protein, which is the major allergen based on its IgE-binding capability, was decreased by the autoclave treatment. Autoclave treatment is simple and economically advantageous and can be easily accomplished on an industrial scale. We also suggest that a specific MAb is useful for the measurement of major allergens in buckwheat products or the development of hypoallergenic buckwheat products.

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