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Effects of Roasting, Blanching, Autoclaving, and Microwave Heating on Antigenicity of Almond (*Prunus dulcis* L.) Proteins

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Whole, unprocessed Nonpareil almonds were subjected to a variety of heat processing methods that included roasting (280, 300, and 320 °F for 20 and 30 min each; and 335 and 350 °F for 8, 10, and 12 min each), autoclaving (121 °C, 15 psi, for 5, 10, 15, 20, 25, and 30 min), blanching (100 °C for 1, 2, 3, 4, 5, and 10 min), and microwave heating (1, 2, and 3 min). Proteins were extracted from defatted almond flour in borate saline buffer, and immunoreactivity of the soluble proteins (normalized to 1 mg protein/mL for all samples) was determined using enzyme linked immunosorbent assay (ELISA). Antigenic stability of the almond major protein (amandin) in the heat-processed samples was determined by competitive inhibition ELISA using rabbit polyclonal antibodies raised against amandin. Processed samples were also assessed for heat stability of total antigenic proteins by sandwich ELISA using goat and rabbit polyclonal antibodies raised against unprocessed Nonpareil almond total protein extract. ELISA assays and Western blotting experiments that used both rabbit polyclonal antibodies and human IgE from pooled sera indicated antigenic stability of almond proteins when compared with that of the unprocessed counterpart.

KEYWORDS: Almond; amandin; processing; food allergy; ELISA; protein; antigenicity

INTRODUCTION

On a global basis almonds rank number one in tree nut production, and the U.S. has accounted for over 33% of the global almond production over the past 10 years. In 1999 the U.S. almond production was 360,000 metric tons, of which 210,000 metric tons were exported (*2*). Almonds are globally popular and are often used as a snack food as well as an ingredient in a variety of food products, notably bakery and confectionery products. Earlier, Sathe (*7*) has shown that a single water-soluble storage protein termed amandin or almond major protein (AMP) accounts for ∼65% of total aqueous extractable almond proteins.

Tree nuts are important food allergens and almonds are no exception. We now have an inhibition ELISA that can detect almond as low as 5−37 ppm in tested foods (*6*). Amandin is recognized by human IgE, rabbit polyclonal antibodies, and mouse monoclonal antibodies in as many as 60 different almond genotypes tested (*8*). There is an excellent correlation in polypeptide recognition by rabbit sera and human IgE (*8*). Therefore, use of rabbit reagent to detect and quantify almond proteins is relevant to almond-induced human allergies.

Almonds are often subjected to harsh heat processing conditions prior to or during their incorporation into foods. Thermal treatments promote protein denaturation, aggregation, and structure disruption and therefore have a potential to modify the allergenic properties of almond proteins. In our earlier investigations we have shown that almonds used as ingredients in commercial foods were easily detected (*6*). In the same study we also demonstrated that commercial processing (roasting and blanching) did not eliminate amandin antigenicity (*6*). In those studies, we had used anti-amandin sera and therefore may have been unable to evaluate potentially important non-amandin allergens that may be relevant to human allergies. The present study was, therefore, specifically designed to evaluate not only the antigenic stability of amandin but also that of total soluble proteins in almonds. We focused on subjecting almonds to carefully controlled heat processing treatments that almonds are likely to encounter during commercial food processing. Antigenic stability of amandin was determined by competitive inhibition ELISA using rabbit polyclonal antibodies raised against the purified amandin. Antigenic stability of total soluble proteins was evaluated by sandwich ELISA using goat and rabbit polyclonal antibodies raised against unprocessed Nonpareil almond total soluble protein extract.

MATERIALS AND METHODS

Materials. Whole natural raw Nonpareil almonds were obtained from Blue Diamond Growers (Sacramento, CA). Sources of electrophoresis

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chemicals have been reported earlier (*1*). DEAE DE-53 was from Whatman (Hillsboro, OR). Molecular weight standards and Sephacryl S-300 were from Pharmacia Inc. (Piscataway, NJ). Nitrocellulose paper and blotting papers were from Schleicher and Schuell, Inc. (Keene, NH). Acepromazine, bovine serum albumin, fentanyl, droperidol, complete Freund's adjuvant, incomplete Freund's adjuvant, alkaline phosphatase labeled goat anti-rabbit IgG and rabbit anti-goat IgG, Ponceau S, phosphatase substrate [*p*-nitrophenyl phosphate, disodium (PNPP)] were from Sigma Chemical Co. (St. Louis, MO). Microtiter ELISA plates were from Costar (Cambridge, MA). Tween 20 and other chemical reagents were from Fisher Scientific (Pittsburgh, PA).

Whole Almond Processing and Preparation of Nut Protein Extracts. Whole natural raw Nonpareil almonds were processed as follows.

Blanching Treatment in Boiling Water (100 °C) for 1, 2, 3, 4, 5, and 10 min. The ratio of almonds to water was 1:10 w/v. Samples were patted dry on paper towels and further air-dried at room temperature in a fume hood (until constant weight).

Pressure Cooking in Autoclave at 121 °C, 15 psi, for 5, 10, 15, 20, 25, and 30 min. Autoclaved samples were air-dried at room temperature in a fume hood (until constant weight).

Dry Roasting at 280, 300, and 320 °F for 20 and 30 min each, and at 335 and 350 °F for 8, 10, and 12 min each. Almonds were placed in crucibles and subjected to roasting in an oven (Thermolyne Corporation, Subsidiary of Sybron Corporation, Dubuque, IA).

Microwave Heating was done in a Panasonic microwave oven (Panasonic Company, Secaucus, NJ) at 50% power for 1, 2, and 3 min.

Defatted Almond Flour Preparation. Dry seeds were powdered manually using a mortar and pestle, and the resultant flours were defatted for 6 h using a Soxhlet apparatus (Fisher Scientific Co., Orlando, FL) and petroleum ether as a solvent (flour/solvent ratio of 1:10 w/v, boiling point range 38.2−54.3 °C). Defatted flours were dried in a fume hood, powdered again using a mortar and pestle (to obtain a homogeneous sample) and stored in screw-capped plastic vials at −20 °C until further use.

Protein Extraction. Buffered saline borate (BSB, 0.1 M H_3BO_3, 0.025 M $Na_2B_4O_7$, and 0.075 M NaCl, pH 8.45) was used to extract proteins from defatted almond flours. Typically, the flours were extracted with the buffer (flour/buffer ratio of 1:50 w/v) at room temperature for 1 h with vortexing at 10-min intervals. The extracts were centrifuged in a tabletop microcentrifuge for 10 min (13600*g*), and the supernatants were aliquoted and stored at −20 °C for further analyses.

Amandin Preparation. Amandin was purified from the defatted Nonpareil almond flour using anion exchange (DEAE DE-53) and gel filtration (Sephacryl S-300) column chromatography as described previously by Acosta et al. (*1*).

For competitive inhibition ELISAs, reference amandin was purified by isoelectric precipitation as described by Wolf and Sathe (*10*). Briefly, defatted almond flour was extracted with 10 vol 0.02 M Tris−HCl (pH 8.1) with stirring for 12 h, followed by centrifugation (12000*g*, 20 min) and filtration using glass wool. The supernatant was adjusted to pH 5.0 with magnetic stirring and allowed to stand for an hour. The precipitated amandin was centrifuged as described previously, dissolved in 0.02 M Tris−HCl (pH 8.1), and dialyzed against distilled water and lyophilized.

All protein preparations were conducted at 4 °C. The lyophilized protein preparations were stored at −20 °C.

Polyclonal Antibody (pAb) Production and Purification. Production and characterization of rabbit polyclonal antibodies to Nonpareil whole almond and purified amandin were done as described previously by Acosta et al. (*1*). Briefly, rabbits were immunized with immunogen (amandin or unprocessed whole almond extract) using Freund's complete adjuvant. After boosting the immunogen in Freund's incomplete adjuvant, the rabbits were bled, and the resultant serum was stored at −20 °C until used.

Goat polyclonal antibodies to the unprocessed Nonpareil almond protein extract were produced at Chemicon International Inc. (Temecula, CA).

For sandwich ELISA, rabbit anti-whole almond serum was purified by affinity (Protein G Sepharose, Pharmacia, Uppsala, Sweden) column chromatography. Briefly, the rabbit serum diluted in phosphate buffer saline (PBS, pH 7.2) was loaded onto a protein G Sepharose column (0.6 × 4 cm) equilibrated and washed in PBS until the $A_{280\ nm}$ was less than 0.01. The bound IgGs were eluted with 0.2 M glycine sulfate, neutralized with 1 M Tris, dialyzed against PBS (pH 7.2), and stored at −20 °C until further use. All purification steps were carried out at 4 °C.

Table 1. Effect of Blanching on Antigenicity of Almond Proteins[a]

blanching (100 °C, min)	IC_{50} processed/IC_{50} unprocessed	
	inhibition ELISA	sandwich ELISA
1	1.02 ± 0.49	1.14 ± 0.18
2	0.79 ± 0.33	1.36 ± 0.81
3	0.86 ± 0.43	1.37 ± 0.28
4	0.80 ± 0.28	0.96 ± 0.15
5	0.81 ± 0.36	0.77 ± 0.36
10	0.75 ± 0.39	1.00 ± 0.46
LSD[b] ($p = 0.05$)	0.62	0.70

[a] Data are mean ± standard deviation ($n = 6$). [b] LSD = least significant difference. Differences between two means within the same column exceeding corresponding LSD value are significant.

Monoclonal Antibody Preparation. Monoclonal antibody (mAb) preparation has been described earlier (*8*).

Electrophoresis and Immunoblotting. SDS−PAGE in the presence of β-mercaptoethanol (β-ME) was carried out according to the method of Fling and Gregerson (*4*). Proteins from electrophoresis gels were transferred onto 0.2-μm nitrocellulose (NC) paper according to the method of Towbin et al. (*9*). Unbound sites on the NC paper were blocked using 5% nonfat dry milk in Tris-buffered saline (TBS-T) [10 mM Tris, 0.9% (w/v) NaCl, 0.05% (v/v) Tween 20] for 2 h. The blots were washed twice, 5 min each, in TBS-T, and then incubated with rabbit antiserum (1:10,000 v/v) dilution in TBS-T for 1 h. NC sheets were rinsed twice with TBS-T, and washed once for 15 min, followed by three 5-min washes. The blots were then incubated for 1 h with horseradish peroxidase labeled goat anti-rabbit antibody diluted in TBS-T. The blots were washed as before and developed using a luminol/*p*-coumaric acid system. Western immunoblotting using pooled human sera was done as described earlier (*8*).

ELISA. Competitive ELISAs were done as described previously (*6*). Sandwich ELISAs were done using a 96-well microtiter plate coated with 50 μL of purified rabbit anti-whole almond antibody (0.2 μg/mL) in coating buffer (0.015 M Na_2CO_3, 0.035 M $NaHCO_3$, pH 9.6) and incubated for 1 h at 37 °C. Wells were washed thrice with BSB (pH 8.45) and blocked with 100 μL of 0.5% bovine serum albumin in 0.05% Tween-20 and 1 mM EDTA in PBS (10 mM, pH 7.2) for 1 h at 37 °C. Plates were washed, and 50 μL of sample serially diluted in 0.1% BSA in BSB was applied to coated plates and incubated at 37 °C for 1 h. After washing, the bound immunogen was captured using 50 μL of 1:5000 (v/v) diluted goat anti-whole almond antiserum (previously optimized) in 0.1% BSA/BSB and incubated for 1 h at 37 °C. Plates were washed and developed using 50 μL of alkaline phosphatase labeled rabbit anti-goat IgG (1:5000 dilution in 0.1% BSA/BSB) for 1 h at 37 °C and phosphatase substrate (50 μL of *p*-nitrophenyl phosphate, 1 mg/mL). Color development was stopped by addition of 50 μL of 3 M NaOH. Plates were read at 405 nm using a Powerwave 200 ELISA reader (Bio-Tek Instruments Inc., Riverton, NJ). All experiments were performed in duplicate and averages are reported.

Protein Determination. Soluble protein was determined according to the method of Lowry et al. (*5*). Bovine serum albumin (BSA) was used as a standard protein. Standard curves for BSA were prepared in appropriate buffer and suitable blanks were used in all assays.

Statistics. All experiments were done in triplicate, and data are reported as mean ± SEM. Appropriate data were analyzed for statistical significance by ANOVA procedures (SPSS software) and using Fisher's least significant difference (LSD, $p = 0.05$) test.

RESULTS AND DISCUSSION

ELISA. ELISA results for almonds subjected to various processing treatments are summarized in **Tables 1−4**. Because

Table 2. Effect of Autoclaving on Antigenicity of Almond Proteins[a]

autoclaving (121 °C, min)	IC_{50} processed/IC_{50} unprocessed	
	inhibition ELISA	sandwich ELISA
5	1.03 ± 0.34	1.45 ± 0.31
10	1.37 ± 0.68	1.72 ± 0.45
15	1.70 ± 0.46	1.85 ± 1.00
20	1.19 ± 0.25	1.62 ± 0.46
25	1.61 ± 0.53	2.05 ± 1.10
30	1.29 ± 0.36	1.54 ± 0.33
LSD[b] ($p = 0.05$)	0.74	1.11

[a] Data are mean ± standard deviation ($n = 6$). [b] LSD = least significant difference. Differences between two means within the same column exceeding corresponding LSD value are significant.

Table 3. Effect of Microwave Heating on Antigenicity of Almond Proteins[a]

microwaving (min)	IC_{50} processed/IC_{50} unprocessed	
	inhibition ELISA	sandwich ELISA
1	0.81 ± 0.32	1.16 ± 0.67
2	1.45 ± 0.62	2.32 ± 1.69
3	3.49 ± 2.28	3.90 ± 2.26
LSD[b] ($p = 0.05$)	2.25	2.73

[a] Data are mean ± standard deviation ($n = 6$). [b] LSD = least significant difference. Differences between two means within the same column exceeding corresponding LSD value are significant.

Table 4. Effect of Roasting on Antigenicity of Almond Proteins[a]

roasting (°F/min)	IC_{50} processed/IC_{50} unprocessed	
	inhibition ELISA	sandwichELISA
280/20	0.81 ± 0.12	1.27 ± 0.56
300/20	0.88 ± 0.41	1.88 ± 0.96
320/20	1.04 ± 0.33	3.72 ± 2.48
280/30	0.83 ± 0.14	1.77 ± 1.51
300/30	1.21 ± 0.39	2.66 ± 1.79
320/30	1.39 ± 0.57	3.29 ± 1.29
335/8	0.79 ± 0.13	2.34 ± 1.18
350/8	0.77 ± 0.12	1.33 ± 0.55
335/10	1.19 ± 0.30	1.63 ± 0.61
350/10	1.42 ± 0.28	2.28 ± 2.08
335/12	1.28 ± 0.37	1.17 ± 0.69
350/12	1.52 ± 0.41	1.49 ± 1.00
LSD[b] ($p = 0.05$)	0.53	2.20

[a] Data are mean ± standard deviation ($n = 6$). [b] LSD = least significant difference. Differences between two means within the same column exceeding corresponding LSD value are significant.

heat denaturation typically decreases protein solubility, the ability to detect antibody reactive molecules in the soluble fraction would be correspondingly decreased and was therefore of concern. In our earlier investigations we had noted loss in protein solubility. However, SDS−PAGE analyses of almond proteins that remained soluble after heat treatments, at constant protein load, showed that the polypeptide profile remained essentially unchanged (qualitatively), indicating no loss of specific polypeptides (*1*). As expected, we did encounter loss in protein solubility in the present study (the extent depended on the severity and duration of heat treatment and ranged from 0 to 85%). However, the protein polypeptide profiles of soluble proteins extracted from processed samples remained the same when compared with that of the unprocessed control sample (data not shown). To compensate for reduced protein solubility we normalized all protein extracts to 1 mg/mL prior to ELISA assays. The antigenicity of processed samples typically decreased, as indicated by an increase in the IC_{50} ratio when sandwich ELISA was used. Data from direct inhibition ELISA assays indicated a decrease in the IC_{50} ratio in certain samples (e.g., 2−10 min blanched almonds and almonds roasted for various times and temperatures). Statistically, neither the decrease nor the increase in IC_{50} ratio was significant under most, but not all, of the processing conditions. A significant change in antigenicity (when compared with unprocessed sample) was observed only at certain extremes of prolonged roasting (320 °F for 20 and 30 min) and microwave heating (3 min) conditions. Data summarized in **Table 5** are for commercially processed samples. As the exact commercial processing conditions are not revealed by the processor, it is difficult to directly compare data from **Table 5** with data for samples processed in our laboratory (**Tables 1**−**4**). Because exact commercial almond processing conditions are difficult to know we carefully chose the heat processing conditions used in our laboratory to encompass those processing conditions normally used in industrial almond processing (*3*, *11*). Data from **Table 5** indicate that even under unknown processing conditions, almond proteins are relatively stable toward various heat processing methods commonly encountered by almonds used for food purposes, such that the presence of almond could be detected by this assay. The data also indicate that heat-induced changes in protein conformational epitopes are ineffective in reducing or eliminating almond protein antigenicity. The results from our investigation therefore suggest that linear (primary sequence) epitopes may be more important than conformational (some of which may be heat labile) epitopes in almond protein antigenicity.

Table 5. Effect of Commercial Processing on Antigenicity of Almond Proteins[a]

processing	IC_{50} processed/IC_{50} unprocessed
	inhibition ELISA
whole blanched	0.47 ± 0.12
whole dry roasted	3.01 ± 1.51
sliced blanched	1.50 ± 0.19
whole blanched and dry roasted	0.94 ± 0.53
LSD[b] ($p = 0.05$)	1.32

[a] Data are mean ± standard deviation ($n = 3$). [b] LSD = least significant difference. Differences between two means within the same column exceeding corresponding LSD value are significant.

Electrophoresis and Western Blotting. We conducted extensive Western blotting studies to further assess whether stability of almond protein antigenicity observed in ELISAs was relevant to human allergies (**Figures 1** and **2**). As can be seen from these figures, amandin polypeptides were recognized by all antibodies tested. Western blotting experiments revealed that non-amandin polypeptides are also generally heat stable, and reductions in antigenicity of certain polypeptides is observed only under extreme processing conditions [roasting (320 °F for 20 and 30 min) and microwave heating (3 min)]. These data indicate that the epitopes in almond proteins are generally stable toward heat processing. The ability of human IgE to recognize major almond polypeptides before and after the almonds were subjected to commercial processing (**Figure 2**, exact processing conditions unrevealed to us) also demonstrates that epitopes relevant to human IgE recognition of the major amandin polypeptides from ∼39 kDa to 66 kDa remained stable. However, the lower molecular weight polypeptide IgE binding is more easily abolished by processing than binding by rabbit IgG (compare **Figure 2** with the rabbit anti-total extract in **Figure 1**). Earlier we have shown that our ELISA is sensitive (can detect

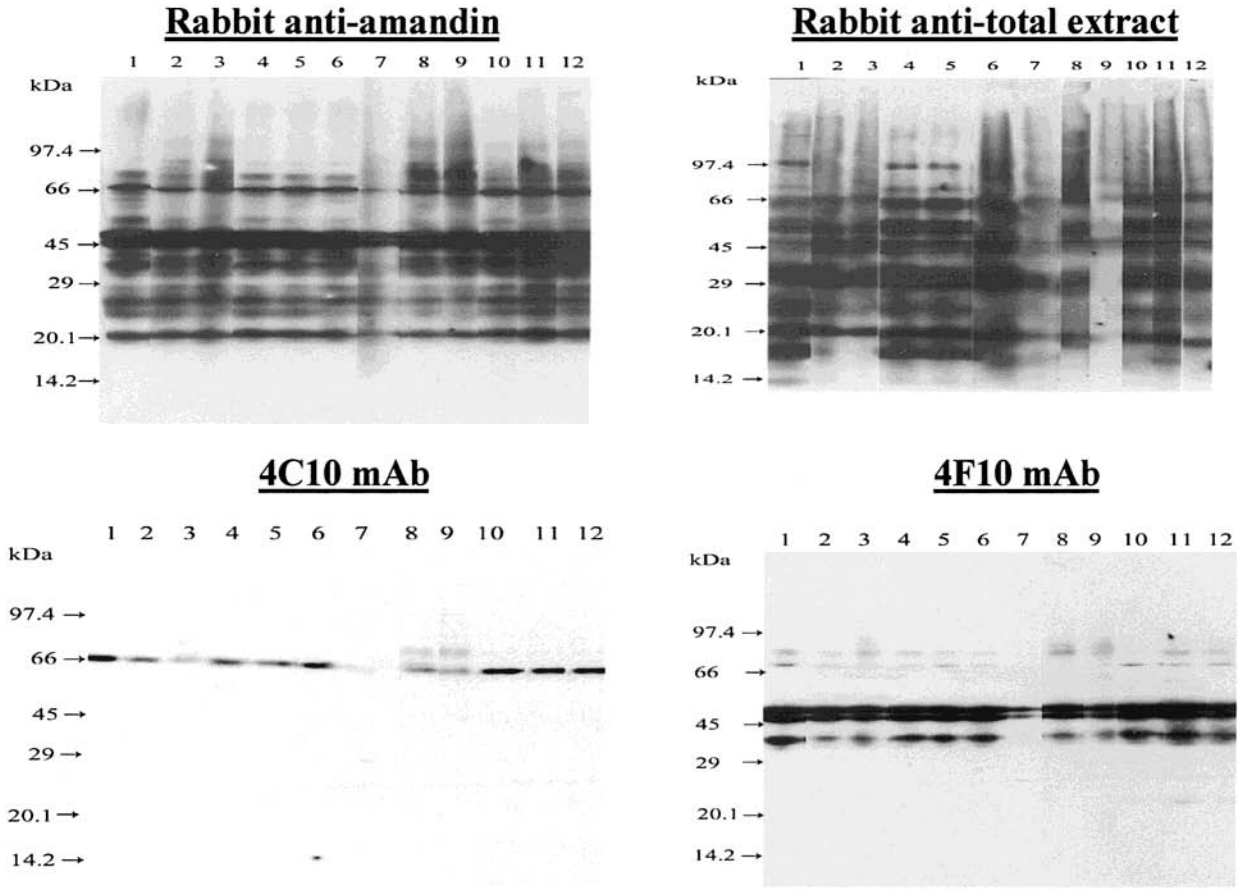

Figure 1. Western blots of processed samples probed with rabbit anti-amandin pAbs, rabbit anti-Nonpareil almond whole protein pAbs, 4C10 mouse anti-amandin mAb, and 4F10 mouse anti-amandin mAb. 1, Unprocessed; 2 and 3, autoclaved for 15 and 30 min, respectively; 4 and 5, blanched for 5 and 10 min, respectively; 6 and 7, microwave heated for 1 and 3 min, respectively; 8, 9, 10, 11, and 12, roasted for 20 min/320 °F, 30 min/320 °F, 8 min/350 °F, 10 min/350 °F, and 12 min/350 °F, respectively.

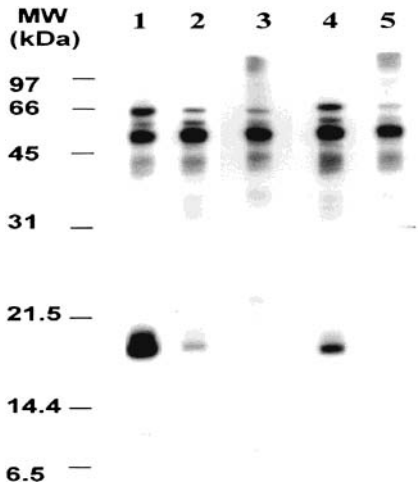

Figure 2. Western blots of commercially processed samples probed with human IgE. 1, Whole unprocessed; 2, whole blanched; 3, whole dry-roasted; 4, sliced blanched; and 5, whole blanched dry-roasted.

5−37 ppm almond in tested foods), robust, and specific for almonds (*6*). Results of the present and some of the earlier investigations (*1*, *6*) from our laboratory indicate the antigenic stability of almond proteins toward commonly encountered food processing conditions. Additional work is necessary, however, to improve the utility of our ELISA assay. Specifically, it would be useful to (a) evaluate influence of multiple processing procedures on antigenic stability of almond proteins, (b) test the assay procedure using several commercial foods that contain almonds or have the potential to contain trace amounts of almonds as contaminant, and (c) thoroughly evaluate the effects of several food components on possible interference in the assay.

CONCLUSIONS

The results of these investigations demonstrate that almond protein antigenicity is stable and readily detectable regardless of which antibodies (rabbit pAbs, mouse monoclonal Abs, or human IgE) are used. The data also show that amandin is an excellent marker protein for the purposes of assessing presence of almonds in processed foods.

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