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Influence of Processing on the Allergenic Properties of Pistachio Nut Assessed in Vitro

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Pistachio (*Pistacia vera*) is a tree nut that has been reported to cause IgE-mediated allergic reactions. This study was undertaken to investigate the distinctions between different cultivars of pistachio nut and the influence of different processing on the IgE-binding capacity of whole pistachio protein extracts. The influence of different processes on allergenicity was investigated using competitive inhibition ELISA and Western blotting assays. The Western blotting results of extracts from pistachio cultivars showed no marked difference among them. The IgE-binding capacity was significantly lower for the protein extract prepared from steam-roasted than from raw and dry-roasted pistachio nuts. The results of sensory evaluation analysis and hedonic rating proved no significant differences in color, taste, flavor, and overall quality of raw, roasted, and steam-roasted pistachio nut treatments. The most significant finding of the present study was the successful reduction of IgE-binding by pistachio extracts using steam-roast processing without any significant changes in sensory quality of product.

KEYWORDS: Pistachio (*Pistacia vera*); Allergy; Cultivar; Processing; Sensory evaluation

INTRODUCTION

Allergic reactions to tree nuts can be serious and life-threatening (*1*). While fruits mainly cause oral symptoms, legume seeds, and nuts are likely to provoke acute generalized symptoms and even anaphylactic reaction (*2*).

Pistachio (*Pistacia vera*) belongs to the Anacardiacea family, which also includes mango and cashew. Because of its high nutritional value and split shell, pistachio is an increasingly important nut crop consumed as raw or roasted. Pistachio nuts are consumed as snack foods and used as ingredients in confectionery, chocolates, meat products, and ice-cream industries. So far, the major allergens have been identified in pistachio nut and characterized as Pis v 1, a 2S albumin, Pis v 2, an 11S globulin subunit, Pis v 3, a vicillin, Pis v 4, a manganese superoxide dismutase, and Pis v 5, an 11S globulin subunit (*3*−*5*).

Different food manufacturing condition may alter immunoreactive epitopes of allergenic proteins (*6*). For many years, pistachios have been roasted in Iranian homes. Different methods have been used resulting in different tastes. For example, lemon juice, saffron, or some other spices are often used as additives. Now, pistachio industry is moving forward to optimize the roasting process for better quality. Investigation among the ethnic people from the pistachio cultivation area in Iran revealed that some of the pistachio allergic patients traditionally use wet cooking method in combination with dry roasting to reduce pistachio nut allergenicity. In recent years, the challenge for food scientists, toxicologists, food manufacturers, and clinical allergists is to better understand the effects of food processing on allergenicity and then take actions to minimize the impact on allergic consumers. It is important to preserve food quality and food identity while altering food proteins to reduce food allergenicity. The aim of this study was to detect the differences between IgE-binding patterns of four pistachio cultivars and also identify processing methods that may decrease the allerginicity of pistachio nut while preserving the sensory quality.

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MATERIALS AND METHODS

Pistachio Cultivars. Many pistachio cultivars (cultivated varieties) are grown in Iran with different characteristics, like fruit shape and color of kernel. The Akbari, Ahmad Aghai, Kalle-Ghuchi, and Fandoghi are the major commercial varieties, which were selected for this part of the research. Pistachio nuts were harvested from Kerman province during summer 2007.

Pistachio Nut Processing. One of the pistachio cultivars, Akbari, was used for the processing. Whole raw pistachio nuts (control) were soaked in water containing lemon juice (pH3.2−3.5) and sodium chloride

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Table 1. Clinical Characteristics, Specific IgE-Reactivity and Skin Prick Test Responses of the Selected Patients with Allergy to Pistachio Nut

			pistachio nut extract	
patient no.	age (years)/sex	clinical characteristics[a]	specific IgE (OD)[b]	skin prick test (mm)
1	20/M	OAS, SI, C	1.2	5
2	21/F	OAS, SI, G	0.99	4
3	36/F	SI	0.80	5
4	36/F	OAS, SI	0.74	4
5	8/M	OAS, C, G, V	0.4	3
6	31/M	OAS, SI	0.68	5
7	32/F	SI, OAS	0.57	6
8	40/F	SI, C, G, V	0.66	5
9	44/M	R, SI	0.45	4
10	19/F	OAS, SI, C	0.81	6

[a] C, cough; R, rhinitis; G, gastrointestinal symptoms; SI, skin itching; OAS, oral allergy syndrome (OAS; defined as the onset of immediate oral itching with or without angioedema of the lips and oral mucosa); V, vomiting. [b] OD, optical density at 450 nm.

(1.6% w/v) for 12 h. The sample was removed from the soaking solution, drained, and dried. Pistachio nuts were processed as follows:

To roast pistachio, the nuts were placed in a preheated oven (37 °C) and then heat increased to 150 °C over 8 h while stirring occasionally. To produce steam-roasted pistachio, the nuts were placed into the mesh basket and steam blanched for 10 min under atmospheric conditions by direct steam injection into the holding chamber and then soaked again in water containing lemon juice (pH 3.2−3.5) and sodium chloride (1.6% w/v) for 1 h. Pistachio nuts were dried and roasted in oven started from 37 °C and ended at 150 °C during over 8 h while stirring occasionally.

Protein Extract. Pistachio nuts (10 g) were ground using a domestic type mechanical high-speed grinder (Black and Decker, USA) for 20−30 s. Defatting was done using cold hexane (1/15 w/v) and shaking for 16 h. Hexane was removed by suction and then phosphate-buffered saline (PBS, pH: 7.4) containing Complete protease inhibitor cocktail (Roche, Mannheim, Germany) was added to the dried defatted powder (1/10 w/v) and shaken for 16 h. After centrifuging at 9000*g* for 30 min, the supernatant of the mixture was dialyzed in PBS for 24 h using dialysis membrane (Sigma, USA) with molecular weight cut off 12000 Da. The whole extraction procedure was performed at 4 °C to avoid any possible proteolysis during protein extraction. Protein concentration was determined using the Bradford protein assay with BSA as the standard protein (*7*). The results also were validated using, the BCA protein assay kit (Pierce Biotechnology, Rockford, IL).

Patients and Skin Prick Test (SPT). In total, 10 patients were included in this study on the basis of a case history of pistachio nut allergy (**Table 1**). Three subjects who showed negative SPT responses, and no specific IgE to pistachio nut extract were considered as negative controls. SPT with a wheal diameter >3 mm were considered positive compared with the results obtained with negative and positive controls. Two sets of pooled sera (A, patients nos. 1−5; B, patients nos. 6−10) were obtained by mixing equal-volume aliquots of the individual sera and then were stored in aliquots at −30 °C until use. The study protocol was approved by the Human Ethics Committee of the Avicenna Research Institute in Mashhad University of Medical Science.

Indirect ELISA for Specific IgE against Pistachio Allergens. Indirect ELISA was performed as described previously (*8*) with minor modifications. Polystyrene plates (Nunc Maxisorb, Roskilde, Denmark) were coated with 0.1 mL/well of a coating buffer (15 mM Na_2CO_3 and 35 mM $NaHCO_3$, pH 9.6) containing 10 μg/well of raw pistachio nut protein at 4 °C for 16 h. The amount of antigen to be used for plate coating was determined on the basis of preliminary titration experiments. The plates were washed with PBS, pH 7.2, containing 0.05% (v/v) Tween 20 (PBS). Remaining reactive sites on the solid phase were saturated with 200 μL of 2% bovine serum albumin (BSA) (overnight at 4 °C). After the plates were washed, 1:5 diluted human sera were incubated on the plates overnight at room temperature. The plates were then washed with PBS and incubated with biotinylated antihuman IgE (KPL, USA) (1:2000 v/v in PBS) for 2 h at room temperature. The unbound antibodies were removed from the wells by washing, which was followed by incubation with HRP-linked streptavidin (BD Biosciences, MD) (1:20000 v/v in PBS) for 45 min at room temperature. Visualization was performed using the enzymatic activity with H_2O_2/tetramethylbenzidine (TMB) as a chromogen substrate at 37 °C for 15 min, and the reaction was stopped by the addition of 100 μL of 3N HCl, then absorption was measured at 450 nm. Optical densities (OD450) greater than three times the median values of negative controls were considered as positive. The assays were performed in duplicate.

Western Blotting Assay. Total extract of pistachio nut from different samples were subjected to reducing 12% (w/v) SDS-PAGE (20 μg protein/lane) (*9*). Protein bands separated by electrophoresis were electrotransferred to polyvinylidene difluoride (PVDF) membranes (Immobilon P, Millipore Corp., Bedford, MA) (*10*). After blocking with 2% BSA for 16 h at 4 °C, the blots were incubated for 16 h at 4 °C with the pooled sera of five allergic patients, diluted 1:6 in PBS. The blots were then washed with PBS and incubated with biotinylated antihuman IgE (KPL, USA) (1:1000 diluted in BSA 1%) for 2 h at room temperature. The unbound antibodies were removed by washing, which was followed by incubation with horseradish peroxidase-linked streptavidin (BD Biosciences, MD) (1:40000 diluted). Blots were incubated with Supersignal West Pico Chemiluminescent Substrate Kit (Pierce, USA) for 5 min, and binding antibodies were then visualized by chemiluminescence using G-Box gel documentation system (Syngene, Cambridge, UK).

Competitive Inhibition ELISA. Ninety-six-well ELISA microplates (Nunc Maxisorb, Roskilde, Denmark) were coated with raw pistachio nut extract (10 μg/well in 0.1 M sodium bicarbonate, pH 9.6) and incubated overnight at 4 °C. Plates were then washed four times with PBS (pH 7.4, containing 0.05% Tween-20) and blocked with PBS containing 2% BSA for 1 h at 37 °C. After the plates were washed, 100 μL of pooled sera from five pistachio nut allergic patients were preincubated for 2 h at room temperature with 0.01, 0.1, 1, 10, and 100 μg of each inhibitor or with BSA as a negative control. Incubated sera (diluted 1:4 in PBS) were added to each well, and the plates were incubated overnight at room temperature. Plates were washed again and then incubated with biotinylated antihuman IgE (KPL, USA) (1:1000 v/v in 1% BSA) for 2 h at room temperature. The unbound antibodies were removed from the wells by washing, which was followed by incubation with HRP-linked streptavidin (BD Biosciences, MD) (1:20000 v/v in 1% BSA) for 45 min at room temperature. Visualization was performed using the enzymatic activity with H_2O_2/tetramethylbenzidine (TMB) as a chromogen substrate at 37 °C for 15 min, and the reaction was stopped by the addition of 100 μL of 3N HCl, then absorption was measured at 450 nm. The assays were performed in duplicate.

Digestion Reactions in Gastric Secretions (GS). Digestion reaction in (GS) was performed as described previously (*11*), with some modifications. GS were obtained from the stomach of a healthy fasting individual. Defatted pistachio powder samples of raw and steam-roasted pistachio were incubated in the presence of a 1:2 dilution of GS in PBS (pH 2) at 37 °C and shaking for 30 min. After centrifuging at 3000*g* for 3 min, aliquots of supernatant were taken for SDS-PAGE and Western blot analysis.

Sensory Evaluation. The sensory evaluation was done on the basis of five-point hedonic scale (5, excellent; 4, very good; 3, good, 2, bad; 1, very bad) using 17 trained and experienced participants. The panelists were trained on the use of hedonic scale and what they needed to consider during the evaluation. Samples were put at room temperature 2 h before evaluation to reach the ambient temperature. The panelists received three nuts per sample. Samples were labeled with three-digit random number and were presented in random order. The quality attributes evaluated were color, texture, flavor, taste, and overall palatability.

Statistical Data Analysis. Analysis of variance (ANOVA) was applied to the entire data set to determine the significance of the differences in the attribute ratings between the samples. The means were separated by use of the least significant difference (LSD) test. Differences were considered significant if $P < 0.05$.

RESULTS

IgE-Binding by Pistachio Nut Cultivars. After the proteins in nut extracts of four cultivars were separated by SDS-PAGE, several IgE-binding protein bands were identified in a molecular weight range from 14 to 94 kDa by Western blot. SDS-PAGE of

protein extracts obtained from different varieties did not show any relevant difference in the protein bands, either in their pattern or intensity (**Figure 1A**). Western blotting results showed that the IgE-binding by pools of allergic patient sera to pistachio protein extracts from different varieties were very similar (**Figure 1B**).

IgE-Binding by Differently Processed Pistachio Nut. The same protein extraction method was applied to raw and processed pistachios. After solvent defatting and aqueous extraction, the protein extraction yield was 47% and 45% from raw and roasted pistachio samples, respectively, and 30% from the steam-roasted sample. Schmitt et al. showed that differences in the processing and preparation can drastically alter the overall protein solubility as well as the allergen and IgE-binding profiles of a particular extract (*12*).

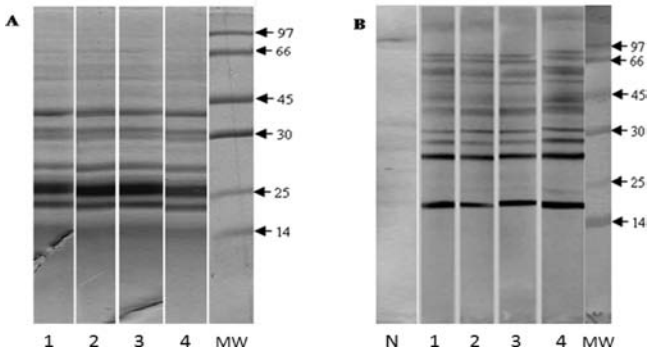

Figure 1. SDS PAGE (**A**) and Western blot (**B**) of raw whole pistachio nut cultivars probed with allergic patients' pooled sera. Akbari (lane1), Ahmad Aghai (lane2), Kaleh Ghochi (lane 3), Fandoghi (lane 4), mix of protein extracts from four pistachio cultivars probed with negative controls' pooled sera (**C**), low molecular weight marker (Amersham, UK) (MW).

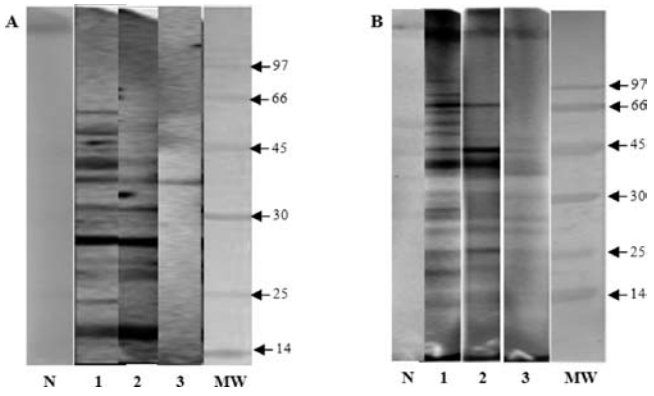

Figure 2. Western blot of the IgE-binding probed with two sets of allergic patients' pooled sera (**A** and **B**). Blotted extract: negative control (N), raw pistachio nut extract (lane 1), roasted pistachio extract (lane 2), steam-roasted pistachio extract (lane 3). Low molecular weight marker (Amersham, UK) (MW).

Figure 2 shows IgE-binding to raw and differently processed pistachio by two different pooled sera, each pool consisting of five patient sera (**Figure 2A**,**B**). In each lane, the level and pattern of IgE-binding is different, and with the exception of the negative control (N), lane 3 in both panels **A** and **B** of **Figure 2** shows the lowest IgE-binding properties. It demonstrates that IgE reactivity for steam-roasted pistachio is lower than raw and roasted pistachio nuts (**Figure 2**). **Figure 3** also reveals the inhibition of IgE-binding to immobilized raw pistachio protein when increasing concentrations of processed pistachio extracts were used as competitors. Compared to the raw extract, protein extracts from steam-roasted pistachio had distinctly reduced IgE-binding potency, but no marked decrease was seen in the IgE-binding of protein extracts from roasted pistachio. The results demonstrate that steam-roasted pistachio extract is a weaker competitor than roasted pistachio extract. The alteration of protein during steam-roasted processing could reduce the IgE-binding activity of the pistachio proteins, while proteins extracted from roasted pistachio showed strong IgE-binding activity.

Digestion Reactions in Gastric Secretions (GS). The results revealed that soluble protein had been significantly decreased in steam-roasted pistachio extract compare to raw pistachio extract. It is possible that structural or chemical protein modifications that occur during steam-roasting process can reduce the solubility of pistachio proteins and therefore contribute to the alteration of IgE-binding activity (*12*). To see the IgE-binding reactivity of aggregated proteins as well as soluble proteins, defatted pistachio powder was incubated in the presence of GS for the indicated time and resolved by SDS-PAGE. **Figure 4A** compares the soluble proteins in raw and steam-roasted pistachio nuts. The Western blotting results of raw and steam-roasted protein extracts revealed decrease in IgE binding reactivity of soluble proteins using allergic patients' pooled sera (**Figure 4B**). The patterns of digested raw and steam-roasted pistachio proteins are presented in **Figure 4C**. Although the same amounts of protein extracts from raw and from steam-roasted pistachio nuts were loaded, the raw-pistachio-extract lane shows fewer protein bands. This could be due to the protein degradation to lower molecular weight proteins that were not visible in the SDS-PAGE. Results of Western blot demonstrated considerable reduction of IgE-binding reactions after digestion by GS, as well (**Figure 4D**). Gastric secretion of

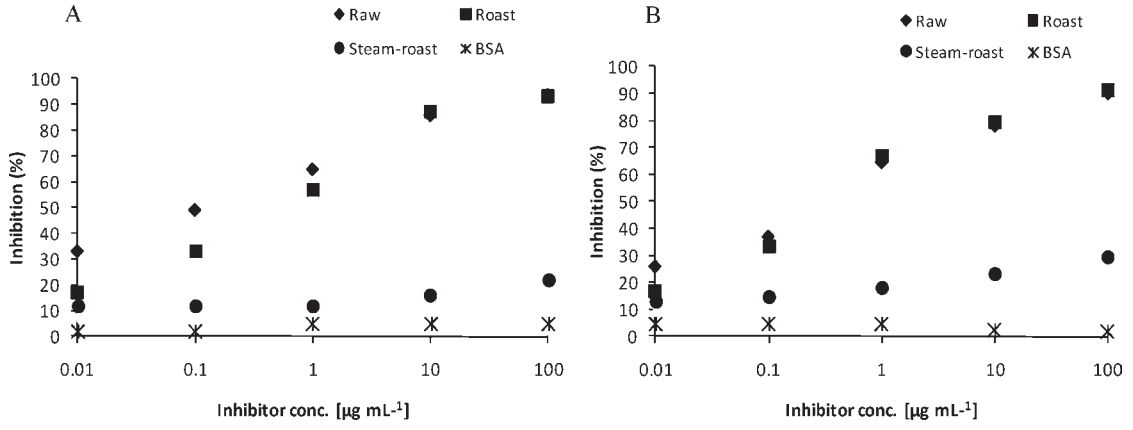

Figure 3. Competitive ELISA Inhibition of the IgE-binding to immobilized raw pistachio nut extract by increasing concentrations of raw, roasted, and steam-roasted pistachio extracts using two sets of allergic patients' pooled sera (**A**,**B**).

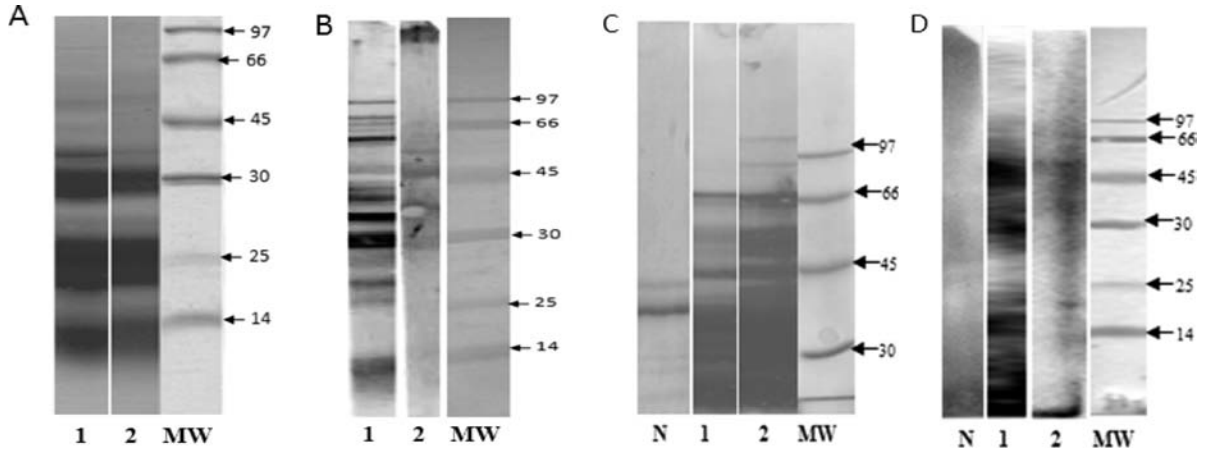

Figure 4. SDS-PAGE (**A**) and Western blot (**B**) analysis of raw and steam-roasted pistachio probed with allergic patients' pooled sera. SDS-PAGE (**C**) and Western blot (**D**) analysis of digested raw and steam-roasted pistachio using gastric secretions probed with allergic patients' pooled sera. GS as a negative control (N), raw pistachio (lane 1), steam-roasted pistachio (lane 2), and low molecular weight marker (Amersham, UK) (MW).

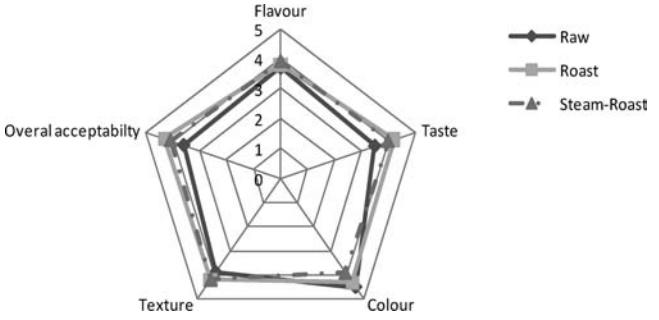

Figure 5. Diagram of the sensory scores from the descriptive analysis of raw, roasted, and steam-roasted pistachio.

a healthy fasting individual was used as a negative control to see any possible nonspecific IgE reactivity due to the gastric secretion's protein.

Sensory Evaluation. Descriptive sensory analysis was conducted to explore the effects of commercial processing, roasting (dry roast), and the process used in this study to reduce allergenicity (steam-roast) on the four sensory attributes of pistachio nut (flavor, taste, texture, and color) as well as total acceptability. The comparison of the attributes for raw, dry roasted, and steam-roasted pistachio nuts is illustrated in **Figure 5**. The sensory panels exhibited preferences for the flavors of steam-roasted pistachio and a higher score for flavor, taste, texture, and overall acceptability noted for roasted and steam-roasted pistachio nuts compared with raw pistachio and lower for color. All the differences were not statistically significant ($p < 0.05$). The results showed the high acceptability of steam-roasted pistachio. IgE-binding has nothing to do with flavor or acceptability.

DISCUSSION

The study shows that pistachio nuts' IgE-binding activity can be reduced by processing without any significant changes in the sensory quality of the product.

It has been shown that different food-processing methods have different effects on food protein structure (*13*); thus, some methods may increase, decrease, or have no effect on allergenicity of specific food proteins. Such effects may be governed by the molecular properties of an allergen and its interactions with other food components (*13*). Heat-induced denaturation of proteins and/or reaction of food proteins with the food matrix could reduce the allergenic potency of the food product (*14*).

In this vitro investigation of the processed pistachio extracts, ELISA inhibition experiments verified by Western blotting results show that the IgE-binding of pistachio allergens are diminished by steam-roast processing but are little changed by dry roasting. It has been demonstrated that certain methods of cooking peanuts, such as boiling or frying, appear to be more effective at reducing allergenicity compared with dry roasting (*15*). Decreased allergenicity of canned tuna and salmon was demonstrated by ELISA-inhibition assay and oral challenges with canned salmon in two patients allergic to salmon (*16*). Steam cooking at 100 °C for 5 min and homogenization has been reported to eliminate kiwi fruit sensitivity in certain Kiwi-allergic children (*17*). In contrast, thermal processing has been shown to increase the IgE-binding activity of peanut extracts (*11*), suggesting that thermal processing may enhance peanut allergenicity, however, it has been shown that the allergenicity of hazelnut is reduced by roasting (*18*). Vinegar addition during cooking may decrease lentil and chicken allergenicity (*19*).

Food-processing treatments often cause some protein denaturation, degradation, and aggregation, all of which can change protein solubility. The extent or loss of protein solubility depends on the severity and duration of processing (*20*). Significant alterations in protein structure may occur during heat treatments, the nature and extent of which depend on the temperature and the duration of the thermal processing. Typically, loss of tertiary structure is followed by reversible unfolding, loss of secondary structure (70−80 °C), formation of new intra/intermolecular interactions, rearrangements of disulfide bonds (80−90 °C), and formation of aggregates (90−100 °C) (*21*). Alteration of protein structure could also depend on the conditions of heat processing, e.g., dry vs wet treatment heating (*22*).

The considerable reduction of protein solubility in steam-roast processed pistachio suggests possible irreversible changes in the secondary structures of proteins. It is reported that a high level of aggregation could be responsible for low access to proteolytic enzymes and might, therefore, decrease the protein digestibility (*23*). Protein aggregation during steam-roast processing of pistachio nuts was proved by analysis of FTIR results (data not shown). The Western blot result of steam-roasted pistachio protein suggests that aggregation of pistachio protein may afford some protection from digestion by gastric secretions. It has been suggested that molecular structure of allergens may protect them from protease digestion (*24*).

Composition of protein isolated from pistachio nut indicated that 66% of the total protein was globulin, while albumins,

glutelins, and prolamins, respectively, contributed 25%, 7%, and 2% of the total protein (*25*). The globulin storage proteins all share a propensity to form large thermally induced aggregates (*26*). On the other hand, three out of five identified allergens in pistachio nut have been characterized as globulin storage proteins (*3*, *5*). It has been reported that 7S globulins have their major thermal transition at around 70 ± 75 °C, while 11S globulins unfold at temperatures above 94 °C, as determined by differential scanning calorimetry, with the precise values varying between plant species and with protein concentration and ionic strength (*26*).

In conclusion, the method of steam-roasting process, as practiced in this study, appears to reduce the IgE-binding of pistachio nut to a greater extent than does the method of dry roasting, a practice widely used in the pistachio industry worldwide. Our results showed protein alterations might be occurring as a result of ionic strengths of soaking solution and heat-processing conditions applied for steam-roasted pistachio nut. These protein alterations might be responsible for the reduction in IgE-binding. All sensory attributes (color, flavor, taste, texture, and overall acceptability) were rated equivalent for raw, dry roasted, and steam-roasted pistachio nuts ($P < 0.05$). Taken together, producing pistachio nut with reduced allergenic properties and high sensory acceptability could be commercially viable. In vivo tests need to be performed to determine the safety of the product from steam roasting when consumed by allergic patients.

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Note Added after ASAP Publication.

This paper was published on the Web on August 25, 2010, with an error to an author's name (SOHEILA J. MALEKI). The corrected version was reposted on September 15, 2010.

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