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Study of the Thermoresistance of the Allergenic Ara h1 Protein from Peanut (*Arachis hypogaea*)

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Supporting Information

ABSTRACT: The effect of heat treatment on denaturation of Ara h1 protein, a major allergen from peanut, was studied using several techniques. Previously, Ara h1 protein was isolated from raw peanut using ammonium sulfate precipitation and chromatograpic techniques. Antibodies against Ara h1 protein were obtained in rabbits, conjugated with horseradish peroxidase, and used to develop a sandwich ELISA. Denaturation of Ara h1 protein was estimated by the loss of reactivity with its specific antibodies by ELISA. Kinetic and thermodynamic parameters of the denaturation process of Ara h1 protein were determined over a temperature range of 82−90 °C. Denaturation of Ara h1 was best described assuming a reaction order of 1.5. Thermal denaturation of Ara h1 protein was also studied by differential scanning calorimetry using several heating rates. The maximum peak temperature and the enthalpy of denaturation obtained by extrapolation to a scan rate of 0 °C/min were 90.22 °C and 1574 kJ/mol, respectively. The hydrophobicity of Ara h1 protein increased with the intensity of heat treatment, and aggregates were formed when the protein was treated at 90 °C for 10 min.

KEYWORDS: *Ara h1 protein, peanut, Arachis hypogaea, thermoresistance, immunoreactivity, differential scanning calorimetry, hydrophobicity*

INTRODUCTION

Ara h1 protein is a storage peanut (*Arachis hypogaea*) protein that accounts for 12−16% of the total protein contained in the seed. It belongs to the family of vicilins, which is included in the superfamily of cupins, with which it shares a common β barrel structure.[1] Ara h1 protein is a glycoprotein of molecular mass 63.5 kDa, with an isoelectric point of 4.55,[2] and contains a free sulfhydryl group in the molecule.[3] It is found in a trimer form that is stable in the presence of 1.8 M NaCl, indicating that the monomers are assembled primarily by hydrophobic interactions.[4]

Ara h1 protein is a major allergen of peanut. The frequency of sensitization of this protein is very high, as it has been reported that between 65% and 100% of peanut-allergic individuals have specific IgE for this protein.[2] In Ara h1 protein, up to 23 linear epitopes that interact with IgE from allergic individuals have been identified. Although these epitopes are distributed along the protein sequence, most of them are located in two areas which constitute the overlapping region between monomers. This location seems to explain why Ara h1 protein retains its allergenicity after being subjected to heat treatment or to proteolysis by digestive enzymes such as pepsin, trypsin, and chymotrypsin.[4]

In the Western world, the prevalence of allergy to peanut has been estimated to range between 1/10000 and 1/200,[5] and it has increased in the past few years. Peanut allergy deserves particular attention as it accounts for the majority of severe food-related allergy reactions, including life-threatening anaphylaxis. Furthermore, it tends to affect early in life, it does not usually resolve, and, in highly sensitized people, trace quantities can provoke an allergenic reaction.[6]

Peanuts are extensively used in the food industry, being part of many recipes such as chocolates, biscuits, candies, and confectionery products. For that reason contamination of raw ingredients or in production lines with peanuts may happen, so traces of peanut can be found in foods supposed to be free of it. Peanuts can be found in foods as raw ingredients as in butter and oil or added after being subjected to roasting, frying, or boiling processes.[4]

Although many studies have been published about the effect of several heating processes on the allergenicity of peanut protein extracts or specific peanut proteins using sera from allergic individuals, there is only scarce information about the effect of those treatments on the physicochemical properties of specific peanut proteins.[7]

In several works it has been reported that no reduction[7−9] or even an increase[4] of IgE binding to Ara h1 protein is observed after peanuts are subjected to some heat treatments. Thus, Burks et al.[10] observed that the heating at 100 °C for up to 1 h of a crude protein extract or fractions enriched in Ara h1 protein did not significantly change the IgE- or IgG-specific binding of the peanut-positive pooled human serum to Ara h1 protein when using an ELISA inhibition assay. Likewise, Koppelman et al.[7] used isolated Ara h1 protein from ground peanut that had been treated in an oven at temperatures between 50 and 140 °C for 15 min, and they did not find significant changes of the IgE-binding properties compared to those of protein isolated from unheated peanut when assayed in a binding ELISA assay.

The aim of this work was to study the effect of heat treatment on Ara h1 protein to determine the kinetic and

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thermodynamic parameters of its denaturation. To perform this study, Ara h1 protein was purified from raw peanuts by using salting out and chromatographic techniques. Thermoresistance of Ara h1 protein was determined by an immunological technique, differential scanning calorimetry, electrophoresis, and fluorescence spectroscopy. To our knowledge, this is the first work in which kinetic and thermodynamic parameters of denaturation of Ara h1 protein have been calculated.

MATERIALS AND METHODS

Purification of Ara h1 Protein. Peanut protein extract was made by mixing 20 g of ground crude peanut with 100 mL of 50 mM Tris−HCl buffer, pH 8.2. After 2 h of gentle stirring at room temperature, the aqueous fraction was collected by centrifugation at 4000*g* for 30 min at 4 °C. The upper layer containing fat was removed, the pellet discarded, and the aqueous phase subjected to ammonium sulfate precipitation to 60% saturation, stirred at room temperature for 1 h, and centrifuged at 4000*g* for 30 min at 4 °C. The remaining supernatant was then taken to 80% ammonium sulfate and centrifuged. The pellet was solubilized in 3 mL of extraction buffer by sonication on ice and passed through a 0.45 μm filter.

The solubilized proteins were applied to a Sephacryl S-200 column (90 × 2 cm) (GE Healthcare, Piscataway Township, NJ) and eluted with the same extraction buffer at a flow rate of 30 mL/h. Fractions containing Ara h1 protein were collected and loaded onto a Q-Sepharose column (1.5 × 15 cm) (Sigma-Aldrich, St. Louis, MO). A linear gradient of NaCl (0−600 mM) was used to elute Ara h1 from the column at a flow rate of 30 mL/h.

Chromatographic fractions were analyzed by SDS−PAGE. Fractions containing pure Ara h1 were pooled, dialyzed against 25 mM ammonium bicarbonate buffer, freeze-dried, and stored at −20 °C until use.

Isolation and Conjugation of Anti-Ara h1 Protein Antibodies. Purified Ara h1 was used as an immunogen to obtain antisera in rabbits according to Wehbi et al.[11] All procedures were performed under Project License PI48/10 approved by the in-house Ethic Committee for Animal Experiments from the University of Zaragoza. The care and use of animals were performed following Spanish Policy for Animal Protection RD1201/05, which meets European Union Directive 86/609 on the protection of animals used for experimental and other scientific purposes.

Specific anti-Ara h1 antibodies were obtained by immunoadsorption using a column with Ara h1 immobilized in Sepharose 4B. A volume of 20 mL of anti-Ara h1 antiserum was applied to the column and washed with 150 mM NaCl, 15 mM potassium phosphate buffer, pH 7.4 (phosphate-buffered saline, PBS). Retained antibodies were eluted with 0.5 M NaCl, 0.1 M HCl−glycine buffer, pH 2.8, and immediately neutralized with 0.5 M Tris buffer, pH 8.0. Purified antibodies were conjugated with horseradish peroxidase (HRP) using the periodate method as previously described.[12]

Measurement of Ara h1 Protein Concentration. The concentration of immunoreactive Ara h1 was determined by a sandwich ELISA. Maxisorp microtitation plates were coated with 100 μL per well of anti-Ara h1 antibodies (5 μg/mL) in 50 mM sodium carbonate buffer, pH 9.6. After overnight incubation at 4 °C, the wells were washed three times with 300 μL of distilled water per well, and blocking of residual protein binding sites was performed with 300 μL of 2% (w/v) ovalbumin in PBS for 2 h at 37 °C. After being washing three times with distilled water, the plates were allowed to air-dry and stored at 4 °C. Then 100 μL volumes of Ara h1 standards or samples diluted in 0.1 M sodium borate buffer, pH 9.0, containing 0.5% bovine serum albumin were added to the wells and incubated for 30 min at 37 °C. Afterward, the plates were washed five times with PBS containing 0.05% Tween 20 (PBST) and incubated with 100 μL of HRP-labeled anti-Ara h1 antibodies in the same borate buffer for 30 min at 37 °C. After the plates were washed five times with PBST, they were incubated with 100 μL per well of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate for 20 min at room temperature. Finally, the enzymatic reaction was stopped by adding 50 μL of 2 M H_2SO_4 per well and the absorbance determined at 450 nm using a microplate reader (Labsystem Multiskan, Helsinki, Finland).

Heat Treatment. A 40 μL volume of Ara h1 protein solution (200 μg/mL) in PBS was introduced into glass capillary tubes (1.5−1.6 mm outer diameter, 1.1−1.2 mm inner diameter) which were sealed with a microflame, and their hermeticity was checked by immersion in tepid water. The capillaries were immersed in a temperature-controlled water bath (±0.1 °C) at five temperatures: 82, 84, 86, 88, and 90 °C. Heated samples, in duplicate, were removed from the bath at different intervals for each temperature and immediately cooled by immersion in an ice−water bath. The concentration of immunoreactive Ara h1 protein was measured by sandwich ELISA as described above.

In another series of experiments, 100 μL of Ara h1 protein solution (2 mg/mL) was added to tubes (12.0 mm outer diameter, 11.6 mm inner diameter) containing 900 μL of preheated PBS, and the final solution was heated at 72, 85, and 90 °C for 15 s and 10 min.

Kinetic and Thermodynamic Parameters for Heat Denaturation of Ara h1 Protein. The denaturation process of a protein can be described by the general equation

$$-dc/dt = kc^n \quad (1)$$

where $-dc/dt$ represents the rate of protein denaturation, k is the rate constant, c is the residual protein concentration at each temperature, and n is the reaction order.[11]

To determine the n and k values, the least-squares criterion by the Solver function of the Excel 5.0 package (Microsoft, Seattle, WA) was used. Correlation coefficients (r^2) and the root mean square error (RMSE) between predicted and observed data were used to test the fitness of the model.[13]

The apparent energy of activation was calculated from the Arrhenius equation, and the thermodynamic parameters, such as the change of enthalpy of activation ($\Delta H^{\ddagger}$), the change of entropy of activation ($\Delta S^{\ddagger}$), and the change of free energy of activation ($\Delta G^{\ddagger}$), were calculated as described previously.[11]

Differential Scanning Calorimetry (DSC). Solutions of Ara h1 protein were prepared in PBS buffer at a protein concentration of 100 mg/mL. Samples and references (10 μL) were introduced into aluminum pans (TA Instruments, New Castle, DE) and sealed for analysis. The references consisted of pans containing the same volume of PBS.

DSC of protein was performed in a DuPont thermal analyzer, model DSC 10 (Weinheim, Germany), using the Thermal Analyst 2000 system. DSC scans were programmed in the temperature range of 35−110 °C at different heating rates: 3, 5, 10, 15, and 20 °C/min. After treatment of proteins, denatured samples were left to cool at room temperature and scanned again at 10 °C/min in the same conditions to evaluate if there was renaturation of the protein.

Calculation of Thermodynamic Parameters. Several thermodynamic parameters were obtained from the transition peaks of Ara h1 protein obtained by DSC, such as the temperature of maximum heat absorption (T_p), the onset temperature (T_i), and the enthalpy change (ΔH_{ap}) for its denaturation process. The value of the van't Hoff enthalpy and the value of the ratio $\Delta H_{VH}/\Delta H_{ap}$ were also determined.[14]

The value of the activation energy (E_a) for the denaturation process of Ara h1 was calculated by Kissinger's method.[15] This method is based on the relationship between the maximum heat temperature and the heating rate. Data obtained were statically evaluated by the Student test.

Determination of Free Sulfhydryl Groups. The presence of free sulfhydryl groups in Ara h1 protein treated at 72, 85, and 90 °C for 15 s and 10 min was determined by Ellman's procedure.[16] Briefly, a 50 μL volume of a 20 mM solution of 5,5′-dithiobis(2-nitrobenzoic acid) (DNTB) in PBS was added to 1 mL of Ara h1 solution (0.2 mg/mL) in the same buffer. The evolution of the absorbance at 412 nm was monitored in a double-beam UV spectrophotometer (Unicam, Cambridge, U.K.) until the absorbance value was stable. The amount of free sulfhydryl groups was calculated from the molar absorption coefficient of TNB (14 150 cm^{-1} M^{-1}).

Determination of Surface Hydrophobicity. The initial intrinsic fluorescence intensity (F_0) at 337 nm by excitation at 295 nm of untreated and Ara h1 treated at 72, 85, and 90 °C for 15 s and 10 min was measured in a Cary Eclipse spectrofluorometer (Varian, Palo Alto, CA). Aliquots of a 5 M acrylamide solution were added to the protein sample to reach a final concentration of 0.2 M, and the fluorescence intensity was recorded (F). When the F_0/F ratio was plotted versus the quencher concentration (Stern−Volmer plot), a linear relationship was obtained, whose slope corresponds to the apparent hydrophobicity constant (K_{ap}).

SDS−PAGE. SDS−PAGE was performed using 8−25% or high-density polyacrilamide gels on a Phast System (Pharmacia, Upsala, Sweden). The samples were diluted in 10 mM Tris−HCl buffer, pH 8.0, containing 1 mM EDTA, 2.5% SDS, and 0.01% bromophenol blue in the presence or absence of 2.5% mercaptoethanol and boiled for 5 min. The proteins were stained with Coomassie blue and destained until a colorless background was obtained.

RESULTS AND DISCUSSION

Determination of Immunoreactive Protein. In this work, the effect of heat treatment on denaturation of Ara h1 was studied by measuring the loss of reactivity with specific antibodies using a sandwich ELISA. The relationship between the values of absorbance and the logarithm of the concentration of Ara h1 standards was represented to obtain calibration curves; the relationship found was linear within the range of concentration between 20 and 400 ng/mL with a correlation coefficient $r^2 > 0.98$.

Some preliminary experiments were carried out to determine an appropriate range of temperatures to study the thermoresistance of Ara h1 protein. We observed that Ara h1 was denatured very slowly at 80 °C, more than 2 h of heat treatment being necessary to obtain 25% denaturation of the protein. On the contrary, the denaturation of Ara h1 was very rapid at 92 °C, resulting in overly shortened sampling intervals. Therefore, a temperature range between 82 and 90 °C was chosen to perform a kinetic study.

For each temperature of treatment, regular time intervals were selected to take the samples. A standard curve was included in every assay, and the concentration of immunoreactive protein in untreated and heat-treated samples was calculated by interpolating their values of absorbance.

The loss of concentration of immunoreactive Ara h1 increases with the time of treatment (Figure 1). The experimental data obtained in this work were analyzed using eq 1 to determine the rate constant (k) and the reaction order (n) of denaturation. When using the least-squares criterion, n values obtained ranged between 1.3 and 1.7 (results not shown). As the comparison of k values is only possible for the same reaction order, the mean value of n was calculated, which was 1.5. Then the experimental data were evaluated again considering the fixed reaction order (Table 1). The values of the coefficients of correlation were higher than 0.909, and the values of the root mean square errors were lower than 0.0578, which indicates that the measured data were well described by the kinetic parameters determined for $n = 1.5$. The correlation between the concentrations experimentally determined and those calculated for the experiments at all temperatures was 0.974 (Figure 2). Kinetic parameters obtained in this study would allow predicting the heat-induced denaturation of Ara h1 protein on the basis of the temperature and time applied.

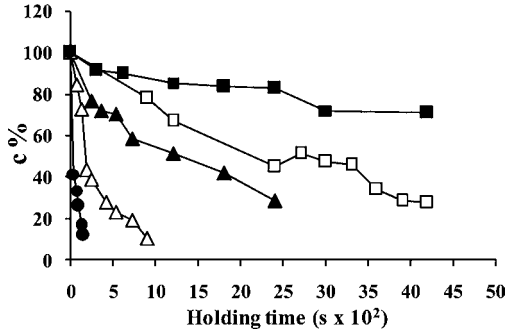

Figure 1. Effect of heat treatment on the denaturation of Ara h1 protein at different temperatures (■, 82 °C; □, 84 °C; ▲, 86 °C; Δ, 88 °C; ●, 90 °C). c is the concentration of immunoreactive protein at each holding time expressed as the percentage of the protein concentration of the untreated sample.

Table 1. Kinetic and Thermodynamic Parameters for Heat Denaturation of Ara h1 Protein at Different Temperatures, Estimated by Loss of Immunoreactivity and Assuming a Reaction Order of $n = 1.5$[a]

T (°C)	k (10^4 s^{-1})	r^2	RMSE	$\Delta H^{\ddagger}$ (kJ/mol)	$\Delta S^{\ddagger}$ [kJ/(mol·K)]	$\Delta G^{\ddagger}$ (kJ/mol)
82	1.14	0.921	0.0345	732.23	1.74708	112.02
84	4.15	0.910	0.0513	732.22	1.74704	108.52
86	7.90	0.974	0.0394	732.19	1.74699	105.03
88	47.83	0.970	0.0578	732.18	1.74694	101.53
90	322.50	0.909	0.0406	732.16	1.74690	98.05
			E_a = 735.28 kJ/mol			

[a] k = rate constant, r^2 = correlation coefficient, RMSE = root mean square error, $\Delta H^{\ddagger}$ = change in the enthalpy of activation, $\Delta S^{\ddagger}$ = change in the entropy of activation, $\Delta G^{\ddagger}$ = change in the free energy of activation, and E_a = activation energy. Samples were assayed in triplicate in at least two independent experiments.

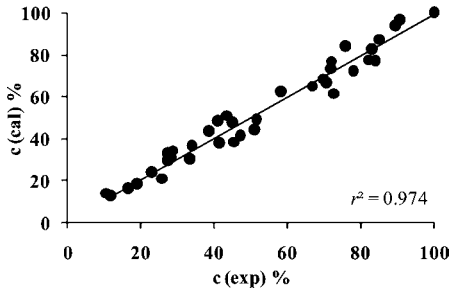

Figure 2. Correlation between values experimentally determined (exp) after heat treatment and those calculated (cal) by means of kinetic parameters for Ara h1 protein assuming a reaction order of $n = 1.5$. c is the concentration of immunoreactive protein at each holding time expressed as the percentage of the protein concentration of the untreated sample.

The graphical representation of the natural logarithm of the rate constant versus the reciprocal of the absolute temperature (Figure 3) gave a linear relationship in the temperature range studied ($r^2 = 0.96$), and the calculation of the activation energy value was obtained from the slope, which was 735.28 kJ/mol. The value of the activation energy of Ara h1 protein is high, which indicates that a large number of low-energy bonds are broken during denaturation.

Values obtained for the thermodynamic parameters were similar at the different temperatures studied (Table 1). The

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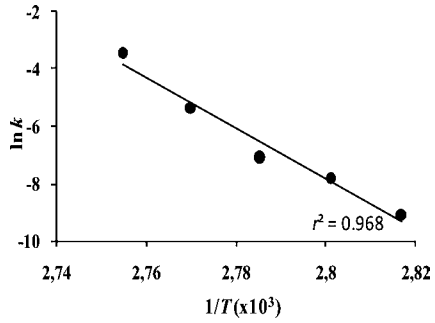

Figure 3. Effect of temperature on the rate constant (k) of denaturation of Ara h1 protein for n = 1.5. $1/T$ represents the reciprocal of the absolute temperature.

value of the change in the enthalpy of activation estimated for Ara h1 protein was about 732.2 kJ/mol. This value is higher than that reported using immunochemical techniques for other globular proteins such as bovine lactoferrin (199 kJ/mol)[17] and bovine IgG (368 kJ/mol),[18] which suggests a large change in Ara h1 protein conformation. The variation in entropy of activation had positive values, which indicates that there is not a significant process of aggregation. Therefore, the values of the thermodynamic parameters obtained for Ara h1 protein are typical of a process in which denaturation predominates over aggregation during heat treatment. The value of the free energy of activation decreased slightly from 112.02 to 98.05, with increasing temperature, similar to that reported for the denaturation process of other globular proteins.[11,17,19] It is assumed that the free energy of activation should be maintained at the same value, in a short range of temperature.

Our results are in contrast with those found when performing studies on the allergenicity of Ara h1. The work of Koppelman et al.[7] was the first report on the study on the allergenicity of Ara h1 protein isolated from heat-treated peanut. These authors found that the IgE-binding properties of Ara h1 were slightly affected when the ground peanut was heated in an oven at temperatures between 50 and 140 °C for 15 min. Similar results have been also obtained for Ara h1 when studying the reactivity of IgE from sera of patients allergic to peanuts, with protein extracts obtained from peanut subjected to roasting, frying, or boiling treatments, by an immunoblot technique.[20−22]

It has been reported that Ara h1 contains 23 linear IgE-binding epitopes, 4 of them being immunodominant. By using point mutations in the epitope regions, it was also demonstrated that mainly hydrophobic residues are of importance for IgE binding. Although the IgE-binding epitopes had more even distribution in the primary structure, they were located in two main regions, which are the overlapping regions between the monomers of the trimer protein structure, and thus, they may be protected from denaturation by the monomer−monomer contacts.[22]

The observation that the reactivity of Ara h1 with rabbit polyclonal IgG decreases with heating whereas the IgE-binding sites remain unaltered indicates that epitopes reacting with both immunoglobulin classes are different. Therefore, we suggest that several of the IgG-binding sites are conformational epitopes and they are located in parts of the protein that are sensitive to heat denaturation. However, conformational IgE-binding epitopes are not present or restricted to parts of the protein that are not affected by heat denaturation. This assumption would explain the heat-stable allergenic nature but the heat-sensitive antigenic nature of Ara h1.

Differential Scanning Calorimetry. Thermal denaturation of Ara h1 protein was studied by DSC. Figure 4 shows a

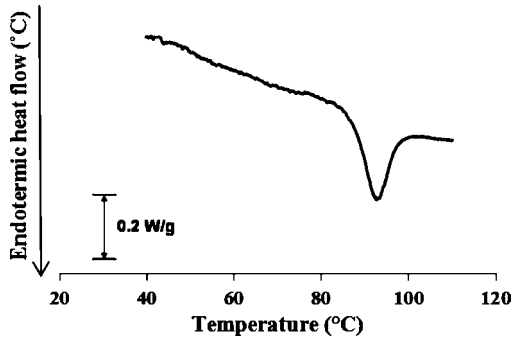

Figure 4. DSC thermogram of Ara h1 protein. The scanning was performed at a heating rate of 10 °C/min. Heat flow was recorded as watts per gram.

DSC thermogram of Ara h1 protein scanned at a heating rate of 10 °C/min. The values of the maximum peak temperature of denaturation increased with the heating rate (Table 2), and they fit a straight line with a correlation coefficient of 0.987. This finding suggests that the denaturation process of Ara h1 is kinetically determined. The value of this parameter, obtained by extrapolation to a scan rate of 0 °C/min, was 90.22 °C. Values of the maximum peak temperature obtained in this work are higher than that of 87 °C reported by Koppelman et al.[7] using a scan rate of 0.5 °C/min.

The calorimetric enthalpy change was also affected by the heating rate, obtaining a negative relationship with a correlation coefficient of 0.537. The value of the enthalpy change of the trimer protein estimated by extrapolation to a scan rate of 0 °C/min was 1574 kJ/mol. The high value of the enthalpy change obtained for Ara h1 suggests that an important part of the protein undergoes a conformational change upon heat denaturation. Values of the enthalpy change estimated in this work for Ara h1 are similar to those indicated for other proteins of the same family, such as soy β-conglycinin,[23,24] but higher than that reported for the same protein by Koppelman et al.[7]

It was also observed that, upon cooling of the sample, no transition was observed in a second heating scan, as previously found,[7] demonstrating that the denaturation process was complete and not reversible (results not shown).

The relationship between the calorimetric enthalpy change obtained from the thermogram and the van't Hoff enthalpy ($\Delta H_{ap}/\Delta H_{VH}$) has also been calculated. This ratio is in all cases higher than 1, indicating the presence of intermediates in the denaturation process. Normally, for small single-domain globular proteins, the denaturation implies a cooperative transition and the calorimetric enthalpy change is equal to the van't Hoff enthalpy, the ratio $\Delta H_{ap}/\Delta H_{VH}$ being around 1.[14] However, in proteins in which the conformation is stabilized by interactions among several domains or subunits, the ratio $\Delta H_{ap}/\Delta H_{VH}$ is greater than 1, as is the case for Ara h1 protein.

The activation energy for the thermal denaturation of Ara h1 was calculated by the Kissinger method.[15] As shown in Figure 5, the Kissinger plot for Ara h1 adjusts very close to a straight line, with a correlation coefficient of 0.976. The activation

Table 2. Thermodynamic Parameters of the Denaturation of Ara h1 by DSC at Different Heating Rates and Extrapolated to 0 °C/min (Confidence Interval 95%)[a]

heating rate (°C/min)	T_i (°C)	T_p (°C)	ΔH_{ap} (kJ/mol)	ΔH_{VH} (kJ/mol)	$\Delta H_{ap}/\Delta H_{VH}$
0	87.05	90.22	1574		
3	88.27 ± 0.76	90.84 ± 0.30	1525 ± 112	1154 ± 118	1.23 ± 0.29
5	87.37 ± 1.06	91.81 ± 0.43	1410 ± 125	952 ± 101	1.51 ± 0.29
10	89.48 ± 0.11	93.56 ± 0.13	1500 ± 122	960 ± 28	1.56 ± 0.11
15	90.50 ± 0.10	94.58 ± 0.04	1004 ± 159	817 ± 45	1.30 ± 0.46
20	91.44 ± 0.52	96.12 ± 0.45	1219 ± 182	620 ± 42	1.94 ± 0.21
		E_a = 401.12 kJ/mol			

[a] T_i = onset temperature, T_p = maximum peak temperature, ΔH_{ap} = enthalpy change, ΔH_{VH} = van't Hoff enthalpy, and E_a = activation energy. Values are the mean ± SD of at least four replicates.

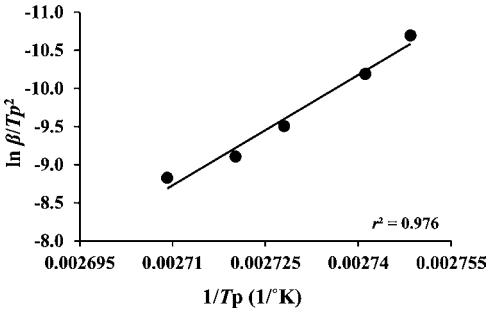

Figure 5. Kissinger plot for heat denaturation of Ara h1 protein. T_p is the maximum peak temperature (K), and β is the scanning rate.

energy value was calculated from the slope of the straight line and found to be 401.12 kJ/mol.

Measurement of Surface Hydrophobicity and Free Sulfhydryl Groups. Modifications in surface hydrophobicity of Ara h1 protein were also studied after exposure to different heat treatments using a fluorescence quenching method and acrylamide as a quencher.[25] Protein unfolding usually causes a red shift in the fluorescence emission spectrum. This red shift is due to the fact that tryptophan has moved from an apolar environment to a more polar region. Consequently, in the unfolded state, the solvent exposure of the tryptophans is quenched by acrylamide with a higher efficiency than in the native state of the proteins.[25] The results obtained indicate that the apparent hydrophobicity constants (K_{ap}) increase with the intensity of heat treatment applied, the value for untreated protein being 6.41 M^{-1} and about 36% higher for the protein treated at 90 °C for 10 min (Table 3). The low increase in

Table 3. Hydrophobicity Constant (K_{ap}) of Ara h1 Protein Subjected to Heat Treatments of Different Intensities[a]

heat treatment (°C)	15 s	10 min
72	7.26 ± 0.04	7.33 ± 1.80
80	8.12 ± 1.41	8.35 ± 0.44
95	8.43 ± 0.68	8.71 ± 1.90

[a] The K_{ap} of untreated Ara h1 protein was 6.41 ± 0.31 M^{-1}. Samples were assayed in three independent experiments.

hydrophobicity could be explained by the position of the tryptophans in the native structure, which are located relatively outside the protein.[7] Furthermore, the increase of K_{ap} of Ara h1 is indicative of a protein structure that is more flexible and/or a higher concentration of unfolded molecules.[26] These results are in agreement with those previously reported for heat denaturation of Ara h1 using near-UV CD, which indicated that the domains where the tryptophan residues are located (N-terminal) have a lower stability and can gain more flexibility when the protein is heated above 80 °C.[7]

As can be observed, Ara h1 protein subjected to different heat treatments was also analyzed by SDS–PAGE (Figure 6).

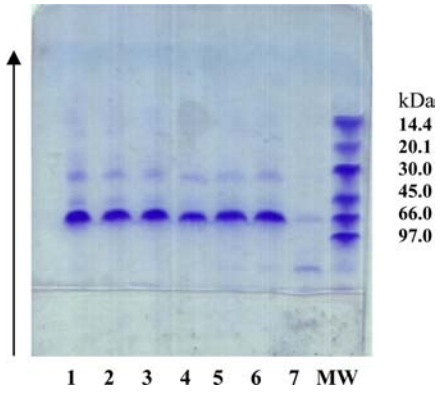

Figure 6. SDS–PAGE (8–25%) under nonreducing conditions corresponding to Ara h1 protein subjected to different heat treatments: (1) untreated, (2) 72 °C, 15 s, (3) 72 °C, 10 min, (4) 85 °C, 15 s, (5) 85 °C, 10 min, (6) 90 °C, 15 s, (7) 90 °C, 10 min, (MW) molecular mass marker (97.0 kDa, phosphorylase b; 66.0 kDa, albumin; 45.0 kDa, ovalbumin; 30.0 kDa, carbonic anhydrase; 20.1 kDa, trypsin inhibitor; 14.4 kDa, α-lactalbumin).

As can be observed, the unheated sample shows a single band around 66 kDa corresponding to the monomer form of the protein. After treatment at 85 °C for 10 min and at 90 °C for 15 s, a faint band of high molecular mass was observed. The treatment at 90 °C for 10 min produced a marked decrease in the intensity of the monomer band that was accompanied by the appearance of a band of high molecular mass aggregates. These aggregates were not observed when the sample was analyzed by SDS–PAGE under reducing conditions, indicating that they are bound by disulfide bridges (result not shown).

In addition, untreated Ara h1 did not react with DTNB, a reagent that binds to the free sulfhydryl groups of the proteins. This result suggests that the free sulfhydryl group of Ara h1 is masked within the native protein conformation, as has been reported for other proteins such as β-lactoglobulin.[27] Similar results were observed for heat-treated Ara h1 except when it was heated at 90 °C for 10 min, treatment that produced the release of two sulfhydryl groups (result not shown). These groups seem to participate in the formation of protein aggregates bound by intermolecular disulfide bridges, as was found when that sample was analyzed by electrophoresis.

The results obtained in this work show that significant changes take place in the structural conformation of this major allergenic protein upon heat treatment. However, the determination of the behavior of Ara h1 when heated in the seed or in a complex food cannot be predicted under conditions used in this work, and more studies must be conducted to know the effect of the matrix on the denaturation of this protein.

■ ASSOCIATED CONTENT

Supporting Information

Calibration curve obtained for determination of Ara h1 protein concentration by a sandwich ELISA. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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