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ARTICLE

Structural Stability of Amandin, a Major Allergen from Almond (*Prunus dulcis*), and Its Acidic and Basic Polypeptides

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Information relating to the resistance of food allergens to thermal and/or chemical denaturation is critical if a reduction in protein allergenicity is to be achieved through food-processing means. This study examined the changes in the secondary structure of an almond allergen, amandin, and its acidic and basic polypeptides as a result of thermal and chemical denaturation. Amandin (~370 kDa) was purified by cryoprecipitation followed by gel filtration chromatography and subjected to thermal (13−96 °C) and chemical (urea and dithiothreitol) treatments. Changes in the secondary structure of the protein were followed using circular dichroism spectroscopy. The secondary structure of the hexameric amandin did not undergo remarkable changes at temperatures up to 90 °C, although protein aggregation was observed. In the presence of a reducing agent, irreversible denaturation occurred with the following experimental values: T_m = 72.53 °C (transition temperature), ΔH = 87.40 kcal/mol (unfolding enthalpy), and C_p = 2.48 kcal/(mol °C) (heat capacity). The concentration of urea needed to achieve 50% denaturation was 2.59 M, and the Gibbs free energy of chemical denaturation was calculated to be ΔG = 3.82 kcal/mol. The basic and acidic polypeptides of amandin had lower thermal stabilities than the multimeric protein.

KEYWORDS: Amandin; circular dichroism (CD); almond proteins; prunin; secondary structure

INTRODUCTION

Food allergy affects as many as 6% of young children and 3−4% of adults (*1*). During the past decade, concern over avoidable mortality associated with allergic reactions to nuts has increased (*2*). A clear indication of the seriousness of tree nut allergies is the fact that a large percentage of fatal allergic reactions to foods for individuals over age 6 reported to a U.S. national registry were caused by tree nuts (*3*). In recent years, an increase in tree nut and peanut allergies has been reported in both Europe and the United States as the production and consumption have also increased considerably. For example, according to the statistics of the Food and Agriculture Organization (FAO), almond production grew by 5% annually over the period 1993 to 2005, with the United States and Spain as the main producers (43.88 and 12.85%, respectively, for 2005) (*4*). Of the tree nut allergens, almond ranks third (15% reactive) behind walnut and cashew nuts in the Food Allergy and Anaphylaxis Network (FAAN) self-reporting survey of tree nut allergies (*5*). Nevertheless, the consumption of almonds is higher than that for walnuts and cashew nuts, as almonds are frequently used as ingredients in many uncooked and processed foods.

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Almond is known to contain as many as 188 different proteins as determined by two-dimensional sodium dodecyl sulfate−polyacrylamide gel electrophoresis (2D SDS-PAGE) (*6*) of which amandin is the major protein and constitutes up to ~65% of the total soluble fraction (*7*). Amandin has been identified as a major allergen (*8*). The allergenicity of amandin measured by the capacity to bind human IgE has been demonstrated for multiple almond species and cultivars (*9*).

The biochemical properties of amandin have been studied, and it was characterized with a sedimentation value of 14S (*7*). Structurally, it is an oligomeric protein that contains prunin monomers. We have initiated an X-ray crystallographic study of amandin, and so far, the resulting electron density map allowed modeling of most of the main-chain atoms for all monomers. The three protomers are related by a noncrystallographic 3-fold axis to form a doughnut-shaped trimer, and two amandin trimers form a homohexamer similar to the glycinin hexamer (*10*). The prunin monomers are formed by a 40 or 42 kDa acidic α-chain and a 20 kDa basic β-chain (*10*) that seem to be expressed from different genes (*11*), whose translational products are proteolytically processed by removal of the signal peptide and further cleaved post-translationally by a unique asparaginyl endopeptidase after the formation of an interchain disulfide bond between the N-terminal acidic and the C-terminal basic polypeptides (*12*, *13*).

Amandin is known to be a legumin seed storage protein that belongs to the cupin superfamily (*14*). This superfamily

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comprises the allergenic 7S and 11−13S globulin storage proteins from peanut, soybean, and tree nuts, which are also known as heat stable proteins that can form immunonogenicity-enhancing aggregates (*15*). Interestingly, most plant food allergens belong to very few protein families and superfamilies, indicating that conserved structures and biological activities may play a role in determining or promoting allergenic properties (*16*, *17*).

In general, the legumin-like seed globulins have a high degree of thermostability, requiring temperatures in excess of 70 °C for denaturation, and also a high resistance to proteolysis. It seems that such exceptional conditions may play a role in determining the allergenic activity of globulin storage proteins (*18*). These proteins also have a propensity to form large aggregates upon heating (*19*) and still retain, to a large degree, their native secondary structure (*20*, *21*).

Because of the fact that the functionality and immunoreactivity of a protein is closely linked to its conformation, the study of the structural changes of a protein induced by thermal and/or chemical denaturation would provide important information regarding its global stability and may help explain changes in allergenicity that occur as a result of food processing.

Circular dichroism (CD) spectroscopy has been frequently used to study the secondary (far-UV) and tertiary structures (near-UV) of proteins. The structural stability of some food allergens such as recombinant or natural Ara h 1, Ara h 2, and Ara h 6 from peanut (*22*−*24*) or Ber e 1 from Brazil nut (*25*), Pru av 3, the lipid transfer protein from cherry (*26*), and β-lactoglobulin (*27*) has been studied by using CD. By analyzing the far-CD spectrum, predictions of the relative contents of α-helix, β-pleated sheet, and random coil structures in a protein can be obtained (*28*). Differences in the secondary structure of the protein in the presence of a denaturant (i.e., urea or guanidine hydrochloride) or a reducing agent [i.e., dithiothreitol (DTT) or β-ME] can be determined using CD. The irreversibility of the denaturation process can also be assessed.

In the present work, the thermal stability of amandin with and without a reducing agent was determined by CD. The denaturation temperature, the unfolding enthalpy, and the heat capacity of amandin in the native and reduced forms were calculated. At the same time, the stability of amandin toward chemical denaturation by urea was analyzed. Finally, the thermal stability of the acidic and basic polypeptides of amandin was examined. The results obtained would help to increase the understanding of the stability of the allergenic proteins belonging to the cupin superfamily.

MATERIALS AND METHODS

Materials. Raw whole Nonpareil Supreme almonds were purchased from VineTreeOrchards (Burbank, CA). Tris(hydroxymethyl)aminomethane was from Bio-Rad Laboratories (Richmond, CA). Methanol, hexane, glacial acetic acid, and Coomassie Brilliant Blue R-250 of reagent or better grade were obtained from Fisher Scientific (Pittsburgh, PA). Urea (minimum purity of 99.5%), sodium chloride, sodium azide, β-mercaptoethanol (β-ME), DTT, and phosphate-buffered saline (PBS), pH 7.4, were from Sigma Chemical Co. (St. Louis, MO). See Blue Pre-Stained molecular marker, Novex 10−20% Tricine Pre-Cast Mini gels (1.0 mm × 15 well), and all electrophoresis reagents were from Invitrogen Corp. (Carlsbad, CA). HiPrep 16/60 Sephacryl S-300 HR columns were from GE Healthcare Bio-Sciences Corp. (Piscataway, NJ). The Prep Cell (model 491), a preparative electrophoresis system, was from Bio-Rad Laboratories.

Preparation of Defatted Almond Flours. Almonds were ground in an analytical mill (Tekmar, Germany) and defatted as described elsewhere (*8*). The almond slurry was air-dried overnight in a fume hood at room temperature, and the dried material was homogenized in a mortar with a pestle. The resulting defatted flour was stored at −20 °C in airtight plastic bottles until used.

Amandin Purification. Amandin was purified by a combination of two methods by Sathe et al. (*7*, *29*) with minor modifications. Defatted almond flour was extracted at room temperature for 1 h with deionized distilled water containing 0.02% NaN_3 (flour/H_2O ratio 1:50). The mixture was constantly stirred using an orbital shaker with vortexing at 10 min intervals. After centrifugation (6000*g* for 10 min), the residue was re-extracted once again using the same conditions. The supernatants were pooled and filtered through a 0.45 μm cellulose filter (Millex, Millipore Corp., Bedford, MA). The clear filtrate was refrigerated (4 °C) overnight (12−14 h), and the milky precipitate was recovered after centrifugation (12000*g* for 20 min, at 4 °C). The precipitate containing mostly amandin was then resuspended in 0.01 M PBS (pH 7.4) and dialyzed against distilled water (24 h, 4 °C, three changes), frozen, and stored at −20 °C in airtight plastic bottles until required. The cryoprecipitated amandin was further purified by gel filtration using a Sephacryl S-300 HR column. The gel filtration column was equilibrated with 0.02 M Tris-HCl buffer (pH 8.1) containing 0.1 M NaCl and 0.001 M NaN_3 prior to loading the protein. The protein was eluted with the same equilibration buffer. Fractions (5 min per fraction; flow rate, 0.9 mL/min) containing amandin were collected, concentrated using Amicon concentrator tubes (MWCO 30000) (Millipore Corp.), dialyzed against distilled water (24 h, at 4 °C, 4 changes), lyophilized using a Labconco Freeze-Dry System (Kansas City, MO), and stored frozen at −20 °C until use.

Amandin Polypeptides Purification. The acidic (40 and 42 kDa) and basic (22 kDa) polypeptides of amandin were purified by preparative SDS-PAGE electrophoresis with the Prep Cell system (model 491) using the protocol developed by Ayuso et al. (*30*) with some modifications. Two gels were cast in the tube assembly, a preparative gel (T = 14%), and a stacking gel (T = 4%). The separation gel buffer was 1.5 M Tris HCl, pH 8.8, and the stacking gel buffer was 0.5 Tris HCl, pH 6.8. Both buffers were filtered and degassed prior to use. Six liters of 1× electrode buffer were prepared by dilution of 10× Tris/glycine/SDS buffer (Bio-Rad). All samples (∼20 mg of lyophilized protein in 1.5 M Tris HCl buffer, pH 8) were reduced with Laemmli buffer and boiled for 10 min, before being loaded onto the gel. The unit was assembled, and a flow rate of 1 mL/min was applied using a peristaltic pump. The power supply was set at a constant rate of 12 W (200−300 V and 60−70 mA). The elution time was around 6.5 h before the dye front disappeared at the bottom of the separation gel. At this point, fractions were collected for 3 h at a rate of 4 mL/fraction with a fraction collector (Gilson, Middleton, WI). The fractions collected were analyzed using vertical Pre-Cast Mini gels (10−20% Tricine), and those fractions containing the 22, 40, and 42 kDa fragments were individually pooled, concentrated using Amicon tubes MWCO 10000 or 30000 (Millipore Corp.), frozen, and stored at −20 °C until needed. Prior to the CD experiments, the SDS was removed from the samples by using the ProteoSpin Detergent Clean-up Kit (Norgen Biotek Corp., Thorold, ON, Canada).

Protein Determination. Soluble total protein was determined in triplicate using the BCA (bicinchoninic acid) assay (*31*) from Pierce Biotechnology, Inc. (Rockford, IL). Appropriate blanks were included in all of the determinations. Bovine serum albumin (BSA) was used as the protein standard.

Electrophoresis. SDS-PAGE was performed according to the method of Fling and Gregerson (*32*). In all cases, Invitrogen's XCell Sure Lock unit was used with 10−20% Tricine gels. For reduced samples, 2× Laemmli buffer was added to the samples (1:1 v/v) followed by 10 min of boiling. For nonreduced samples, a buffer of the same composition as Laemmli buffer but without β-ME was freshly prepared for each use, and no boiling treatment was applied (in both cases, 15 μL/well were loaded). The gels were stained using Coomassie Brilliant Blue R-250 for at least 2 h followed by destaining overnight. Prestained molecular mass marker See Blue was used as a reference.

CD Measurements. Far-UV CD spectra were measured with a JASCO spectropolarimeter, model J-710 (JASCO International Co., Tokyo, Japan). Quartz cuvettes with light path lengths of 0.1 and 0.4 cm were used. Single point temperature scans were performed and recorded as an average of 16 spectra at a scan rate of 100 nm/min and a time constant of 1 s. The spectra were smoothed with the Spectra Manager software (JASCO Corp.) using the Means-Movement filter with a value of 5 for the convolution width.

For thermal measurements, temperature regulation was carried out using a Peltier temperature controller PTC423S thermocouple (JASCO Corp.). Comparison of the actual temperature in the cell with the temperature set by the Peltier element showed that the deviation of the actual temperature was < 0.1 °C. PBS at pH 7.4 was used in all samples. The scans were recorded at temperatures ranging from 20 to 90−96 ± 0.5 °C at intervals of 1−2 °C. The heating rate was 1 °C/min at a scanning speed of 5 nm/min, with a spectral width of 1 nm, response of 1 s, and resolution of 1 nm. Spectra were always corrected for a protein-free spectrum obtained under identical conditions.

CD Spectral Analysis. The relative percentage of each of the structural elements (e.g., α-helix, β-sheet, β-turns, or unordered) in the protein was determined from the measured CD curves using the CDPro package (*33*−*35*) by taking the average of CDSSTR, CONTINLL, and SELCON3 determinations and using several reference sets (SP43, CLSTR, SMP56, and SDP48) for each sample. This package combines variable selection principle and cluster analysis with neural network, ridge regression, and self-consistent methods to obtain a structure prediction.

Urea Denaturation. A stock solution of urea (8 M) was prepared in PBS. For each sample, 30 μL of protein solution in PBS (1.14 and 2.16 mg/mL as determined by BCA assay) was mixed with various volumes of PBS and urea stock solution (up to a total volume of 240 μL) to achieve the desired concentrations of urea. The samples were thoroughly mixed and left overnight at room temperature. The CD analyses were performed the following day. The free Gibbs energy of denaturation was calculated using two different methods according to the thermodynamics involved in the unfolding process of proteins (*36*). Origin Pro7 software was used for curve fitting and for statistical calculations (OriginLab Corp., Northampton, MA).

RESULTS AND DISCUSSION

CD Analysis of Native Amandin. The CD spectrum of amandin at 13 °C (**Figure 1**) displayed two minima around 222 and 208 nm, indicative of helical content, and another signal at 216 nm, indicative of a β-sheet structure.

Estimations of the relative percentage of secondary structure elements were done using CDPro package (*33*, *34*). The combination of CDSSTR, CONTINLL, and SELCON3 provides a good estimation of α-helix, β-sheet, β-turns, and unordered structures (*35*). For the native conformation of amandin at pH 7.4, values of 34.3% α-helix, 25.0% β-sheet, 12.8% β-turns, and 27.2% of unordered structures were calculated.

After running the Cluster program for classification of the protein class (CDPro), amandin was classified into the α + β class. The characteristic of α + β structures is that α- and β-elements are not intimately associated, suggesting that the α-helixes are separated from the β-sheets when the protein is folded. This was confirmed by the preliminary X-ray crystallographic data of prunin-1 (Pru1), a component of amandin, which consists of monomers with a high percentage of β-sheet conformation and also several clusters of α-helix (*10*), in agreement with the classification of amandin as a member of the cupin superfamily of proteins characterized by having the so-called β-barrel conformation.

Thermal Stability of Amandin. The thermal stability of amandin was studied by following the changes in ellipticity of the protein (monitored by CD) as a function of temperature (**Figure 1**). A temperature range of 13−91 °C was covered. The concentration used for this experiment was 0.8 mg/mL, and aggregates of protein were observed after the thermal study. This indicates that the protein was irreversibly denatured. Separate experiments to determine the solubility of amandin indicated that a solution of 40 mg/mL of amandin formed a sticky gel when the protein was heated to 80 °C, although some aggregates became visible even at lower temperatures.

The spectra obtained for each temperature over the interval indicated that no major changes in the secondary structure of the protein were discernible from 13 to 77 °C. When the temperature reached 77 °C, there was an increase in the CD negative values

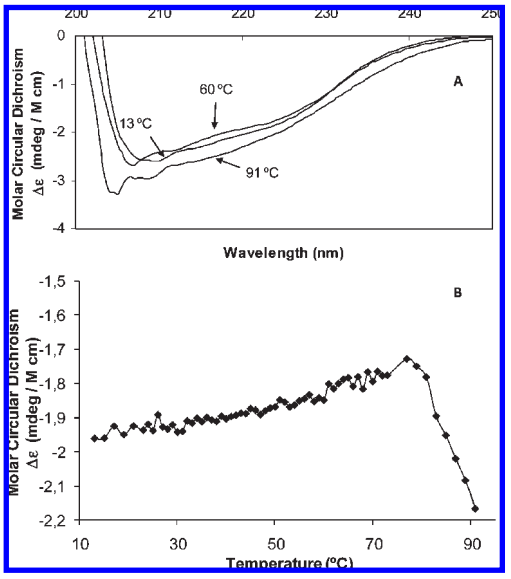

Figure 1. (**A**) Effect of heat treatment on molar CD (Δε) values of purified amandin from 13 to 91 °C. The protein concentration was 0.8 mg/mL. The heating rate was 1 °C/min. Only scans at 13, 60, and 91 °C are shown for clarity. (**B**) Molar CD value at 222 nm vs temperature.

(**Figure 1B**). This may be caused by interference in the spectroscopic reading of the aggregates that are formed or the release of some of the monomers from the hexameric structure of the protein with a consequent increase in the concentration of the protein in solution. The minimal changes in the secondary structure observed (**Figure 1**) suggested that the destabilization process does not occur by protein unfolding but through aggregation. This phenomenon has also been observed during thermal denaturation of a peanut allergen, Ara h 1 (*37*). An increase in the negative ellipticity was observed when Ara h 1 was heated at high temperatures (50−80 °C). It was found that the increase in ellipticity was not due to changes in the secondary structure content of the protein but rather the overall increase in optical intensity possibly caused by the dissociation of the quaternary complex of the protein. Above 80 °C, a decrease in the negative ellipticity was accompanied by an increased level of optical density as a result of extensive aggregation of the protein. Recent studies on soy protein glycinin (11S) indicated that above 80 °C, a negative change in the ellipticity at 190−220 nm was observed as compared to 25 °C, indicating irreversible structural changes (*38*). Some authors suggested a heat-set gel network for glycinin with highly aggregated protein after heating with loss of α-helical domains and more limited loss of the β-sheet structure (*39*) or the collapse of tertiary structure in a configuration where some secondary structure would be maintained (*40*). It has been reported by Fourier transform infrared spectroscopy that 11S soy protein treated at 95 °C not only retained much of the intramolecular β-sheet structure but also formed newly intermolecular β-sheet structures (*39*). Calorimetric analysis of Ara h 3, an 11S allergen from peanut, indicated that in general, Ara h 3 was more stable to heat processing than glycinin and solely formed insoluble aggregates as opposed to glycinin that formed some type of soluble aggregates along with dissociated polypeptides (*41*).

The impact of the food matrix on allergenic behavior and thermal stability of amandin should be taken into consideration. However, for establishing the underlying thermodynamic properties of these important allergens by means of CD, purified proteins are needed. Other methodologies such as Western blotting analyses with rabbit antisera and sera from almond-allergic patients demonstrated that amandin and its polypeptides

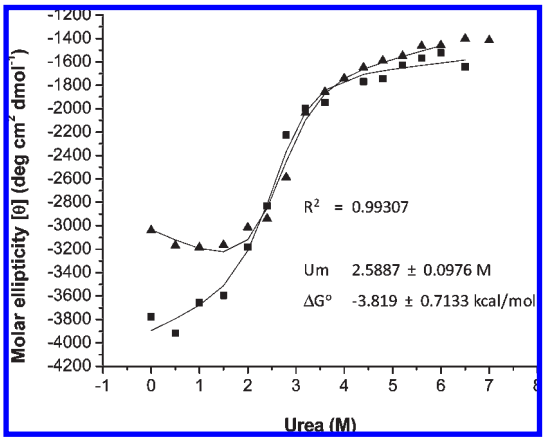

Figure 2. Denaturation curve of amandin in the presence of urea. The data were fitted according to the equations shown in the text. The calculated urea concentration to achieve 50% of denaturation (U_m) = 2.59 M. The free Gibbs energy value ($\Delta G°$) = 3.82 kcal/mol. Denaturation was measured by CD at 222 nm of wavelength. The experimental temperature was 22 °C. Samples were measured after 24 h of incubation with urea at room temperature. Amandin concentration: ▲, 2.16 mg/mL; and ■, 1.14 mg/mL.

retained both antigenic and allergenic potential even after harsh food-processing conditions, such as roasting, blanching, autoclaving, and microwaving, confirming the general stability of the various peptides in almond and almond-containing foods (*8*, *42*). Similarly, heating of Ara h 3 and glycinin did not cause a decrease in the IgE binding capacity of these allergens (*41*).

Chemical Denaturation of Amandin by Urea. Amandin stability was studied by the addition of urea at several concentrations. Chemical denaturation is one of the primary ways to assess protein stability, the effects of mutations on stability, and protein unfolding. In the current study, a denaturation plot, mean of two experiments, was obtained for amandin in the presence of various concentrations of urea (**Figure 2**), and the free energy of unfolding for this protein was calculated according to the method described by Pace (*36*). The unfolding process was followed by measuring the far-UV CD spectra of amandin at 25 °C. The changes in ellipticity revealed a single-step process in which the secondary structure is lost in a cooperative manner. The sigmoidal shape of the curve indicates that the denaturation of amandin could be easily explained by a two-stage equation between native and unfolded states without intermediates (N ↔ U). The concentration of urea (U_m) needed to achieve 50% of unfolding was 2.59 ± 0.09 M, and when the concentration of the denaturant was increased to 5 M, amandin was totally denatured. Similar results were obtained for soybean glycinin. With ≥3 M urea and ≥2.5 M guanidine hydrochloride, the protein underwent denaturation also in a two-step fashion (*43*). In the present study, the free Gibbs energy of denaturation for amandin ($\Delta G_D^{H_2O}$) was calculated using the following equations for curve fitting (*44*):

$$\Delta G_D = \Delta G_D^{H_2O} \times \left(1 - \frac{x}{U_m}\right)$$

$$k = e^{-\Delta G_D/(RT)}$$

where x is the molar concentration of urea and k is the equilibrium constant. The curve obtained is shown in **Figure 2**, and the value for $\Delta G_D^{H_2O}$ was 3.82 ± 0.71 kcal/mol.

The analysis of the structural energetics (i.e., the energetics of protein folding and binding reactions as a function of temperature) of globular proteins indicates that there is a correlation between the number of structural domains in small globular proteins and their size (*45*), and as the length of the polypeptide chain increases, it becomes energetically unfavorable to form one large domain. Thus, globular proteins with molecular masses over 30 kDa tend to form multidomain structures with different degrees of interdomain interactions. In some cases, the interactions between domains can be so strong that the domains can form a single unit that unfolds under denaturing conditions as one cooperative block (*46*−*48*). For amandin, a cooperative system for chemical denaturation was observed that can be explained in terms of a multidomain structure in which the conformation is that of a hexamer with covalent disulfide bonds inter and intra the acidic and basic polypeptides. It should be noted that no aggregates of the protein were detected visually when it was denatured in the presence of urea or when a nondenaturing nondissociating PAGE gel was run (results not shown), suggesting that the protein is stabilized in solution by solvation.

A study of the molecular basis for the chemical denaturation of chymotripsin inhibitor 2 (CI2) suggests that urea can cause unfolding by both indirect and direct mechanisms (*49*). In a direct interaction, urea binds through its hydrogen atoms to the polar moieties of a protein, especially peptide groups, leading to masking of intramolecular hydrogen bonds. Solvation of the hydrophobic core of a protein may initially proceed via the influx of water molecules and then urea. On the other hand, in an indirect manner, urea can alter the water structure and dynamics, thereby diminishing the hydrophobic effect and facilitating the exposure of the hydrophobic core residues thus encouraging solvation.

The $\Delta G_D^{H_2O}$ value obtained for amandin was similar to those of other globulins (around 3.8 kcal/mol) according to the ProTherm Database (BioInfo Bank, Kyushu Institute of Technology, Fukuoka, Japan), which consists of a collection of ∼18900 entries of numerical data about the thermodynamic parameters for wild-type and mutant proteins (*49*). For globular proteins with a hydrophobic core, and members of the cupin superfamily among them, it has been postulated that the free Gibbs energy reflects the energy of unfolding of this core of the protein and that the variations in stability observed among different proteins are due to the remaining structures protecting this core (*50*).

Thermal Denaturation of Amandin in the Presence of a Reducing Agent. As amandin is known to be an oligomeric protein (a hexamer) with the acidic and basic chains linked by disulfide bonds (*11*), it was of interest to study the thermal stability of the protein after the disulfide bonds had been reductively cleaved. To that end, experiments were performed using 2% β-ME or 5 mM DTT, both of which gave very similar results.

Under reducing conditions, amandin had a midpoint temperature of denaturation (T_m) of 72.53 ± 0.61 °C when subjected to thermal treatment (**Figure 3**). The variation of the enthalpy for this thermal denaturation process was calculated (ΔH_{cal}) using the following equations (*44*):

$$\Delta G_D = \Delta H_{cal} - x \times \frac{\Delta H_{cal}}{T_m} + C_p \times \left[x - T_m - x \times \ln\left(\frac{x}{T_m}\right)\right]$$

$$k = e^{-\Delta G_D/(RT)}$$

where x is the temperature at each point on the curve, T_m is the medium or midpoint temperature of unfolding, ΔH_{cal} is the enthalpy of denaturation, ΔG_D is the Gibbs free energy of denaturation, and C_p is the heat capacity. The calculated values for these parameters are shown in the inset of **Figure 3**. The ΔH_{cal} for the amandin in the presence of the reducing agent (DTT) was 87.40 ± 6.14 kcal/mol, which is close to those of some other globular proteins such as bovine α-lactalbumin (84.30 kcal/mol),

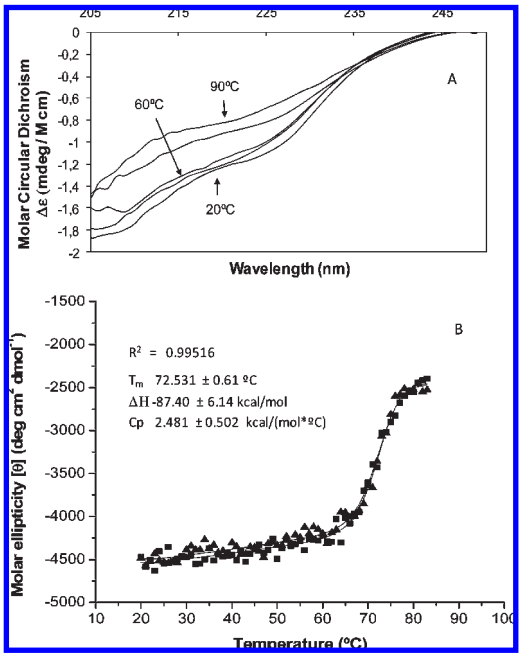

Figure 3. (**A**) Effect of heat treatment on the molar CD value ($\Delta\varepsilon$) of purified amandin in the presence of a reducing agent (5 mM DTT) from 20 to 90 °C. The protein concentration was 0.35 mg/mL. The heating rate was 1 °C/min. Only scans obtained at 20, 40, 60, 80, and 90 °C are shown for clarity. (**B**) Thermal denaturation curve of amandin with DTT at 222 nm. The data were fitted according to the equations shown in the text. The calculated values of T_m, ΔH, and heat capacity (C_p) and regression parameters are shown in the graph. Amandin concentration: ▲, 1.12 mg/mL; and ■, 1.09 mg/mL.

chicken parvalbumin (81 kcal/mol), and human serum albumin (88.95 kcal/mol) (data retrieved from the ProTherm database).

The temperature of denaturation observed here can be considered high when compared with other proteins included in the ProTherm database of similar ΔH_{cal} values, confirming that the reduced amandin was quite stable under the experimental conditions. The protein showed only a slight variation in the percentage of calculated secondary structure from 20 to 60 °C with average values of 10.26% α-helix, 31.58% β-sheet, 20.06% β-turns, and 37.19% unordered. At 90 °C, the percentages of the secondary structures were calculated to be 4.65% α-helix, 22.71% β-sheet, 13.77% β-turns, and 52.18% unordered, indicating that the thermal treatment increased the percentage of the unordered structure by losing mainly the α-helix contents and some β-sheet structure. Formation of a precipitate in the cuvette was observed, indicating that the process of denaturation was irreversible and that the protein had aggregated. It is very significant to note that the amandin in its native conformation formed aggregates when heated in solution, but no major changes in the secondary structure were observed (**Figure 1**). When amandin was exposed to reducing conditions (**Figure 3**), a distinct cooperative transition was detected, indicating the importance of the disulfide bonds in stabilizing the oligomeric protein. In several studies of proteins from thermophilic bacteria, it was shown that disulfide bonds increase both the thermostability (*51*, *52*) and the conformational stability of proteins at ambient temperatures (*53*). This type of bond has also been noted as a contributing factor leading to the conformational stability of plant food proteins (*54*). It is also considered responsible for the higher resistance to denaturation during food processing and to digestion in the gastrointestinal tract seen in certain food allergens with a possible correlation to allergenicity (*55*).

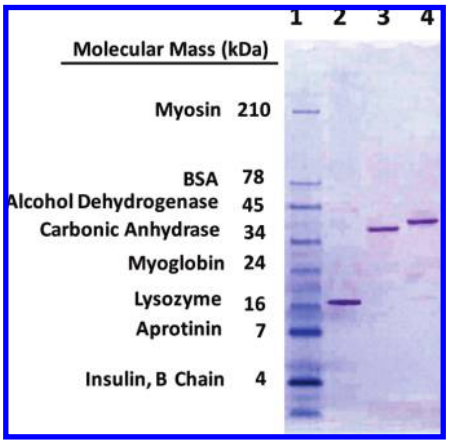

Figure 4. Purified acidic and basic polypeptides of amandin in SDS-PAGE (10−20% Tricine gel). Lane 1, molecular mass marker; lane 2, 20 kDa basic polypeptide; lane 3, 40 kDa acidic polypeptide; and lane 4, 42 kDa acidic polypeptide (protein loaded per well, 7.5 μg).

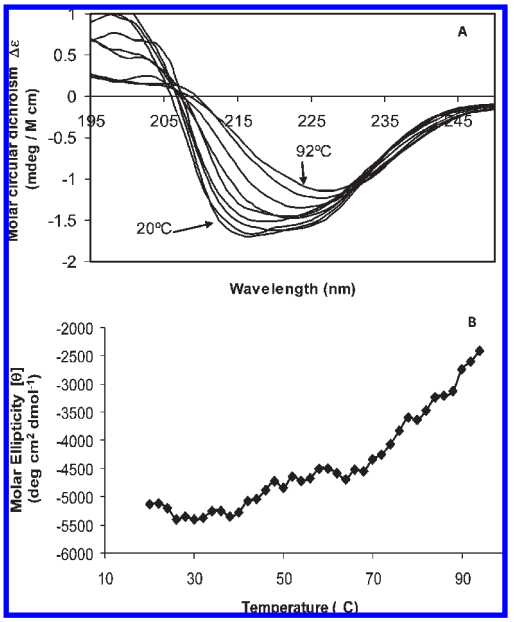

Figure 5. Thermal denaturation from 20 to 92 °C of the acidic (42 kDa) polypeptide. The protein concentration was 0.51 mg/mL. (**A**) Molar CD ($\Delta\varepsilon$). Only the spectra at 10 °C intervals are shown for clarity. The heating rate was 1 °C/min. (**B**) Protein unfolding was monitored at 218 nm.

Thermal Denaturation of the Acidic and Basic Polypeptides of Amandin. For the almond cultivar used for the present study, amandin was considered as essentially the result of the *pru1* gene and, therefore, a hexamer of Pru1 (*10*). SDS-PAGE analysis of the purified amandin revealed three main bands with MW of 22, 40, and 42 kDa, respectively. The 40 and 42 kDa bands correspond to the acidic (theoretical pI value of 5.66) polypeptides of Pru1 and the 22 kDa band to the basic (theoretical pI value of 9.25) polypeptide of Pru1 (*11*). Isolation of these protein fragments from amandin was achieved by using the PrepCell system from Bio-Rad (**Figure 4**). The purified acidic and basic polypeptides were subjected to thermal denaturation and monitored by CD. An insufficient amount of the 40 kDa SDS-free fragment was obtained, and CD analysis of this acidic polypeptide was not feasible.

The unfolding pattern of the 42 kDa polypeptide plotted in molar CD values is presented in **Figure 5**. The calculated percentages of α-helix, β-sheet, and β-turn structures indicated an overall decrease in all three structures with increasing temperature from 20 to 90 °C (**Table 1**). However, at 90 °C, the

Table 1. CDPro Predictions of Secondary Structures for the Acidic (42 kDa) Polypeptide at Various Temperatures

temp (°C)	α-helix (%)	β-sheet (%)	β-turns (%)	unordered (%)
20	5.51	37.38	24.13	32.80
30	5.47	36.81	23.73	33.73
40	6.00	35.95	24.86	33.56
50	4.35	36.58	24.04	34.75
60	4.00	35.82	23.19	36.81
70	4.67	35.86	23.39	35.76
80	4.02	35.77	22.98	37.77
90	3.72	33.88	21.98	39.26

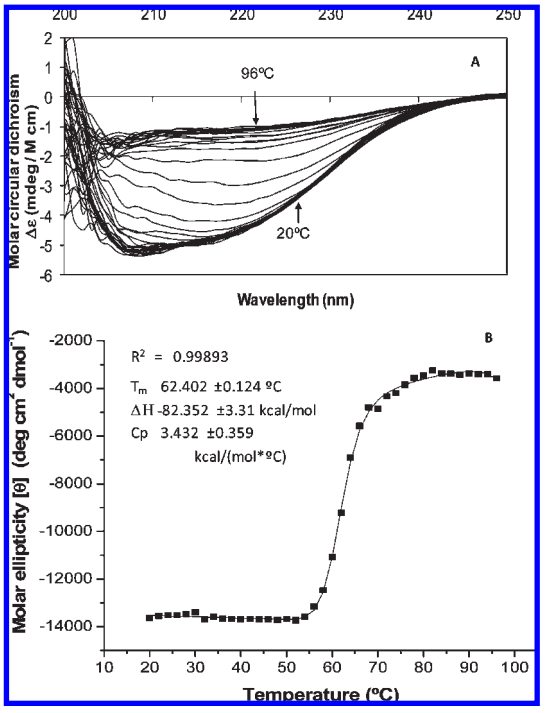

Figure 6. Thermal denaturation from 20 to 96 °C of the basic polypeptide. The protein concentration was 0.17 mg/mL. (**A**) Molar CD ($\Delta\varepsilon$). The heating rate was 1 °C/min. (**B**) Protein unfolding was monitored at 222 nm.

protein still retained a considerable percentage of orderly structures. The spectra showed a total loss of signal at 208 nm from 20 to 90 °C, while the minimum moved toward 225 nm. At the same time, the positive signal below 200 nm was lost with a shift to higher wavelengths for the crossing with the *x*-axis. This is indicative of α-helix loss, although the relative percentage of both α-helix and β-sheet was reduced. Thermodynamic calculations were not possible for this acidic fragment, as the denaturation followed a somewhat continuous unfolding pattern (**Figure 5**B) rather than the two-stage process seen previously in amandin under reducing conditions.

The thermal denaturation of the basic fragment (22 kDa) is presented in **Figure 6**. A two-stage curve was obtained from native to denatured conformation, which indicates the absence of intermediate denatured states. The structure calculations indicated that the basic polypeptide was formed in the native conformation by 20.05% α-helix, 26.68% β-sheet, 21.35% β-turns, and 32.03% unordered. A good prediction of secondary structure after heating could not be obtained as the noise level increased considerably below 200 nm. A transition temperature of 62.40 ± 0.12 °C was observed, which was considerably lower than the denaturation temperature of the intact hexameric amandin, indicating an additional gain in stability of the multimeric conformation.

Table 2. Percentages of Secondary Structures Predicted with CD Pro Software Package among Native Amandin and Its Acidic and Basic Polypeptides at 20 °C

secondary structure	native amandin	42 kDa (acidic)	20 kDa (basic)
α-helix	34.3	5.5	20.0
β-sheet	25.0	37.4	26.7
β-turn	12.8	24.1	21.3
unordered	27.2	32.8	32.0

Secondary structure estimates based upon CD signals are shown in **Table 2**. It was observed that native amandin presented a much higher content in α-helix structure as compared to the individual polypeptides. In the polypeptides, a substantial relative amount of β-sheet structure remained and, in fact, increased. This suggests that at least part of the structure underwent some sort of structural conversion during the purification process. The isolated polypeptides, in fact, contained secondary structure, as evidenced by the well-defined two-state thermal unfolding transition for the basic polypeptide, as well as the significant β-sheet composition determined by CD. On the basis of our X-ray crystallographic data (unpublished results), the relative increase in β-sheet percentage probably occurs since the polypeptides are linked together in the hexameric native structure in a hook type model through loops composed of several α-helices. Isolated α-helices are in general entropically unstable in the absence of other tertiary structure contacts, so once the polypeptides are separated, the helices presumably become destabilized and unwind. However, the β-sheet structures seemingly remained intact. The increased degree of compactness found in the whole native structure would explain the higher temperatures required for denaturation as compared to the isolated polypeptides.

In summary, this study provided some insight into the stability of amandin when subjected to thermal or chemical denaturation. The results indicated that amandin presented a higher thermal stability in its native multimeric form as compared with the state where the disulfide bonds were reduced, proving the importance of these covalent links in providing additional stability to the protein structure. Both the acidic and the basic polypeptides presented reduced thermal stability with lower denaturation temperatures than amandin as a whole.

NOTE ADDED AFTER ASAP PUBLICATION

This paper was posted ASAP on April 17, 2009, without final corrections. These corrections have been incorporated in the posting of April 29, 2009.

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