

Biochemical and Spectroscopic Characterization of Almond and Cashew Nut Seed 11S Legumins, Amandin and Anacardein

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Native, undenatured amandin and anacardein secondary structures were estimated to be, respectively, 56.4 and 49% β -sheet, 14 and 23.7% α -helix, and 29.6 and 27.4% random coil. Circular dichroic (CD) and fluorescence spectroscopy were used to assess structural changes in amandin and anacardein subjected to denaturing treatments that included heat (100 °C, 5 min), guanidium HCl (GuHCl), urea, sodium dodecyl sulfate (SDS), and reducing agent, 2% v/v β -mercaptoethanol (β ME) + heat. Mouse monoclonal antibodies (mAbs) 4C10 and 4F10 directed against amandin and 1F5 and 4C3 directed against anacardein were used to assess the influence of denaturing treatments on the immunoreactivity of amandin and anacardein. Among the denaturing treatments investigated, SDS and β -ME caused a significant reduction in the immunoreactivity of amandin and anacardein when probed with mAb 4C10 and 4C3, respectively.

KEYWORDS: Amandin; anacardein; immunoreactivity; protein structure; fluorescence; denaturation; antibodies

INTRODUCTION

Edible tree nut seeds are used as snack foods or as ingredients in a variety of processed foods. Although safely enjoyed by most, sensitive individuals cannot tolerate tree nuts as these individuals suffer from mild, moderate, or severe adverse reactions known as food allergies. Type I food allergies are often triggered by food proteins. In the United States, eight food groups, cow's milk, egg, wheat, soybeans, fish, crustaceans, peanuts, and tree nuts, together account for >90% of food allergies. Peanut (PN) and tree nut (TN) induced allergies are of special concern as their prevalence in the United States has either remained steady or increased over the past several years. An ongoing U.S. survey (1) reported that the rate of PN and/or TN allergy among adults did not exhibit major changes from 2002 to 2008. However, the survey reported a significant increase in PN and/or TN induced (from 1.2 to 2.1%) and TN induced (0.5 to 1.1%) allergies among children during the same period. The survey of 118 households reporting one or more subjects with an allergy to PN, TN, or both found that the numbers of subjects reporting TN allergies were as follows: walnut, 41; cashew, 29; pecan, 26; almond, 25; pistachio, 19; Brazil nut, 19; hazelnut, 17; macadamia nut, 17; and pine nut, 11. The United States continues to be the largest producer and exporter of almonds and the largest importer of cashew nuts (2).

Many allergenic proteins have been identified in 13 tree nuts (3–7). Edible tree nut seeds are high in lipids and proteins (8).

The type and amount of protein in an individual tree nut may differ significantly. For example, the globulins are dominant in almond (9), Brazil nut (10, 11), and pistachio (12). In cashew nut, the globulins and albumins are in equal proportion (13), whereas in walnut (14) and pecan (15) the glutelins dominate the seed protein composition.

Seed globulins can be grouped on the basis of their sedimentation coefficients as either 7–8S or 11–12S proteins (16). The 11–12S group, referred to as legumins, constitutes the hexameric proteins of ~275 000–450 000 M_r . Each monomer subunit of the hexamer is made of an acidic and a basic polypeptide with molecular masses ~40 000 and ~20 000 M_r , respectively (16). One acidic polypeptide is linked to one basic polypeptide by disulfide bond(s). The resulting polypeptide trimerizes, and two such trimers constitute the native hexameric legumins. Legumin allergens have been identified in almond (Pru du 6) (17, 18), Brazil nut (Ber e 2) (19), cashew (Ana o 2) (20), peanut (Ara h 3) (21), hazelnut (Cor a 9) (22), pecan (Car i 4, unpublished), pistachio (Pis v 2) (3), and walnut (Jug r 4) (6) as well as other plants (23, 24).

The major storage globulin, an 11S legumin, in almond is known as amandin or almond major protein (9), whereas the corresponding globulin in cashew nut is known as anacardein or cashew major protein (25). In food allergen nomenclature, amandin and anacardein are respectively termed Pru du 6 and Ana o 2. In this paper these two proteins will be referred to as amandin and anacardein, respectively. Native amandin (9) and anacardein (25) have been purified to homogeneity from the respective defatted seed flours. When probed with the respective rabbit anti-whole nut seed protein polyclonal antibodies,

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amandin and anacardein retained their immunoreactivity even after the nut seeds had been exposed to several food-processing treatments that included γ -irradiation and several thermal treatments such as microwave heating, blanching, autoclaving, and dry and oil roasting (26–28).

In most type I food allergies the allergenic protein cross-links the patient serum immunoglobulin E (IgE) on the surface of the basophils/mast cells. Such IgE cross-linking is an obligatory step for the induction and propagation of an allergic response. The portion of the allergenic molecule that recognizes and binds to the IgE molecule is known as the epitope. There are two types of known epitopes, linear (contiguous stretches of amino acids) and conformational (discontinuous amino acid stretches). The contribution of the protein structure in food allergenicity has been recognized (29–31). Recently, the presence of an IgE-reactive conformational epitope in cashew legumin Ana o 2 has been demonstrated (32, 33). By some accounts, conformational epitopes are considered to be more immunogenic than their linear counterparts (34). Because foods may be eaten raw unprocessed or in processed forms, understanding the immunoreactivity of native as well as denatured epitopes is important.

The relationship or the lack thereof between molecular structure and immunoreactivity of amandin and anacardein, however, remains unexplored. Here we report our findings on the structure and immunoreactivity of amandin and anacardein subjected to several denaturing treatments.

MATERIALS AND METHODS

Materials. Sources of certain reagents are indicated under Methods. All remaining chemicals, of reagent or better grade, were purchased from Fisher Scientific Co., Orlando, FL.

Methods. *Preparation of Legumins from Defatted Nut Seed Flours.* Raw almond and cashew seeds were ground (Osterizer blender, Jaden Consumer Solutions, Boca Raton, FL), sifted through a 40-mesh sieve, and extracted with petroleum ether (boiling point range 39–53.8 °C) at a flour/solvent ratio of 1:10 (w/v) in a Soxhlet apparatus for 8 h. Defatted flours were then treated with 100% acetone, to ensure removal of tannins. The powders were then dried overnight in a fume hood, ground, passed through a 40-mesh sieve, and stored in sealed containers at –20 °C until further use. Amandin (9) and anacardein (25) were purified as described earlier.

Sample Preparation. Optically clear protein solutions, typically 50–100 $\mu\text{g/mL}$, in 0.02 M sodium phosphate buffer (pH 7.5) were used as control for spectroscopic and immunochemical measurements. In the case of β -mercaptoethanol (β ME)-treated samples, protein solutions in the aforementioned buffer were added with β ME to a concentration of 2% v/v, then boiled at 100 °C for 10 min, and cooled to room temperature before measurements. In the case of protein samples treated with sodium dodecyl sulfate (SDS), guanidinium HCl (GuHCl), and urea, the denaturant stock solution was prepared in the same buffer on the day of the experiment. Aliquots of the concentrated stock were added to the protein solution, before measurements, to achieve the desired final denaturant concentration in the final solution, mixed thoroughly, and immediately used for spectroscopic and immunochemical analyses.

Circular Dichroic (CD) Spectroscopy. Optically clear protein solutions, typically 50–100 $\mu\text{g/mL}$, in 0.02 M sodium phosphate buffer (pH 7.5) were used for CD spectroscopy. The CD spectra were recorded with an AVIV spectropolarimeter at a wavelength range of 260–190 nm. (1R)-(–)-10-Camphorsulfonic acid was used as a standard for calibration of the CD instrument. Molar ellipticity per amino acid residue (θ) was calculated from the collected data (number of amino acids was calculated from the c-DNA derived amino acid sequences of the proteins, available at NCBI website). All of the spectra were later smoothed with third-order polynomial curve fitting using computer software Origin (Origin Lab. Corp., Northampton, MA; version 8). Ratios of θ of denatured and undenatured protein (control) (θ/θ_0) were plotted against denaturant concentration. The computer program CDPro (<http://lamar.colostate.edu/~sreeram/CDPro/main.html>) was used to determine secondary structure (helices, β -sheets, β -turns, and random coil) of the selected samples.

Fluorescence Spectroscopy. Fluorescence spectra of the protein solutions 50 $\mu\text{g/mL}$ in 0.02 M sodium phosphate (pH 7.5) were collected at an excitation wavelength 295 nm and an emission wavelength range of 280–400 nm at 25 °C (constant temperature water bath) in a Perkin-Elmer fluorometer (model LS 50B, Perkin-Elmer Corp., Wellesley, MA). Excitation and emission slits were set at 5 nm each, and the scan speed was set at 100 nm/min. Protein solution was incubated for 1 h in the dark with the desired concentration of a denaturant and buffer in a final volume of 2 mL. Fluorescence spectra for appropriate blanks were run simultaneously. Protein fluorescence spectra were corrected for the signal contributions by buffers and denaturants. All of the spectra were processed as described above. Fluorescence emission intensities of intrinsic tryptophan residues at wavelength of maximum fluorescence emission (λ_{max}) of denatured proteins and undenatured protein control (F_0/F_0) were plotted against denaturant concentration.

Fluorescence Quenching. A Perkin-Elmer LS 50B luminescence spectrometer was used for acrylamide and iodide quenching experiments with excitation wavelength at 295 nm and the λ_{max} emission wavelengths of 346 and 338 nm for amandin and anacardein, respectively, under constant stirring and at 25 °C. Protein, quencher, and denaturant solutions were made fresh on the day of experiment. Working protein concentration was 50 $\mu\text{g/mL}$. Fluorescence intensity values were corrected for dilution effect as well as for contribution by blanks. Iodide quenching was carried out using 0.1 mM sodium thiosulfate in 0.02 M sodium phosphate (pH 7.5). The Stern–Volmer equation (eq 1) and the modified Stern–Volmer equation (eq 2) were used to fit and interpret the fluorescence quenching data (35).

$$F_0/F = 1 + K_{\text{SV}}[Q] \quad (1)$$

F_0 is the fluorescence intensity of the protein solution in the absence of the quenching agent, F is the fluorescence intensity of the same protein solution in the presence of the desired amount of the quenching agent, and $[Q]$ is the quenching agent concentration. The Stern–Volmer quenching constant (K_{SV} , M^{-1}) is determined from the slope of the F_0/F plot. For a homogeneous population of fluorophores the plots are linear. Deviation from linearity indicates a heterogeneous population that can be modeled as a mixture of accessible and inaccessible (buried) fluorophores. In such cases, the modified Stern–Volmer equation and plots are used to account for the absence of the quenching of the buried fluorophores.

$$F_0/(F_0 - F) = (1/f_a) + (1/f_a K_q [Q]) \quad (2)$$

f_a is fractional accessibility, and K_q is the effective quenching constant of only the accessible fluorophore population.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE (36) was performed as described by Sathe (37). Typically, samples were electrophoresed on an 8–25% linear monomer acrylamide gradient separating gel (14.5 cm $\times$ 16.5 cm $\times$ 1.5 mm) and a 4% monomer acrylamide stacking gel (1.0 cm $\times$ 16.5 cm $\times$ 1.5 mm).

Immunoassays. (1) *ELISA.* ELISA was performed as described earlier (38). Briefly, 96-well Costar (Corning Inc., Corning, NY) plates were coated with appropriate dilutions of the antigen-capturing polyclonal antibody (either anti-whole almond or anti-whole cashew, both raised in separate rabbits) in citrate/phosphate coating buffer (pH 5, 48.5% 0.1 M citric acid, 51.5% 0.2 M Na_2HPO_4), incubated at 37 °C for 1 h. After incubation, coated plates were washed three times with Tris-buffered saline (TBS-T; 10 mM Tris, 0.9% w/v NaCl, 0.05% v/v Tween 20, pH 7.6), blocked with 100 μL of 5% w/v nonfat dry milk (NFD) in 0.05% Tween 20 and 1 mM ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS; 10 mM, pH 7.2). Aliquots of either amandin or anacardein solutions, at appropriate concentration, were then applied to the top well of the plates and serially diluted (typically, 10-fold) in the next six rows. The plates were then incubated at 37 °C for 1 h. After the plates had been washed three times with TBS-T, suitable dilutions of specific mouse monoclonal antibodies (mAbs) (for amandin, mAbs 4C10 and 4F10, and for anacardein, mAbs 4C3 and 1F5) were added to the plates, and the plates were then incubated again at 37 °C for 1 h. Following the three washes with TBS-T, alkaline phosphatase labeled secondary antibody (anti-mouse rabbit antibody, 1:5000 v/v dilution) was used for the detection of bound mAbs, and *p*-nitrophenyl phosphate (50 μL of 1 mg/mL solution)

Table 1. Effect of Denaturants on Amandin and Anacardein Fluorescence^a

treatment	amandin				anacardein			
	acrylamide		iodide		acrylamide		iodide	
	% $f_a \pm \text{SEM}$	$K_q (\text{M}^{-1}) \pm \text{SEM}$	% $f_a \pm \text{SEM}$	$K_q (\text{M}^{-1}) \pm \text{SEM}$	% $f_a \pm \text{SEM}$	$K_q (\text{M}^{-1}) \pm \text{SEM}$	% $f_a \pm \text{SEM}$	$K_q (\text{M}^{-1}) \pm \text{SEM}$
control	67.4 ± 1.3	1.9 ± 0.1	48.4 ± 0.6	2.8 ± 0.3	73.5 ± 0.8	8.7 ± 0.6	64.1 ± 0.5	10.9 ± 0.2
boiled (100 °C, 10 min)	105.6 ± 2.8	4.4 ± 0.5	75.0 ± 10.5	4.1 ± 1.0	84.1 ± 0.5	12.2 ± 0.8	66.6 ± 4.0	10.2 ± 1.6
β ME (2% v/v) with heat (100 °C, 10 min)	72.2 ± 0.8	4.8 ± 0.2	55.8 ± 11.2	2.9 ± 0.8	80.5 ± 5.9	7.4 ± 2.7	76.0 ± 36.9	7.0 ± 5.3
GuHCl (6 M)	no fit obtained		101.0 ± 12.8	4.4 ± 0.9	91.1 ± 0.1	7.7 ± 0.1	90.3 ± 1.0	8.7 ± 0.1
SDS (10 mM)	92.5 ± 1.0	9.3 ± 0.8			89.2 ± 0.4	12.8 ± 0.6		
urea (6 M)	91.4 ± 1.3	9.1 ± 1.0	75.2 ± 0.3	4.5 ± 0	88.4 ± 0.2	12.0 ± 0.2	83.2 ± 2.8	5.8 ± 4.1
LSD, $N = 3$, $p = 0.05$	17.9	2.1	26.6	0.9	14.1	1.3	49.2	2.6

^a Differences between two means within the same column exceeding the LSD value ($p = 0.05$) are significant.

was used as a substrate for color development. Color development was terminated by adding 50 μL of 3.0 M NaOH to each well. Antigenic reactivity of the test sample was expressed as immunoreactivity relative to the control, which was arbitrarily assigned a value of 1.0.

(2) *Dot Blot Assays.* Nitrocellulose membranes (0.2 μm , Schleicher and Schuell Biosciences Inc., Keene, NH) were blotted with amandin or anacardein solution in sodium phosphate buffer (0.02 M, pH 7.5) using a Bio-Dot Microfiltration Apparatus (Bio-Rad Laboratories, Inc., Hercules, CA). Typically, each nitrocellulose membrane was blotted with 100 μL of protein control and test solutions, following the manufacturer's directions. Protein control was blotted, in triplicate, in the range of 1000–0.97 ng, in 2-fold serial dilutions. Typically, 1 mL solutions in 20 mM sodium phosphate buffer (pH 7.5) were incubated for 1 h at room temperature with the desired concentration of denaturant. One hundred microliters of the solution was blotted in quadruplicate (two dots of two replicate solutions). After blotting, the unbound sites on the membrane were blocked with 5% w/v NFDM in TBS-T buffer at room temperature for 1 h and then with appropriate mAb diluted in TBS-T overnight at 4 °C. After incubation with the primary antibody, the membrane was washed three times (5 min each) with TBS-T and incubated for 1 h at room temperature with 10000-fold diluted HRP-labeled rabbit anti-mouse (secondary) antibody (Sigma-Aldrich Co., St. Louis, MO). After 1 h, the membranes were washed with TBS-T, as described before. Reactive spots on the membrane were visualized using the luminol/ p -coumaric acid substrate system and exposed to Kodak Biomax XAR Film (Eastman Kodak Co., Rochester, NY). Bio-Rad Gel Doc 2000 gel documentation system (Bio-Rad Laboratories) was used to quantify the immunoreactivity. A standard curve was developed by plotting the dot density (intensity/ mm^2) against blotted control protein amount (ng). With the straight-line equation obtained from the standard curve, effective antigen amount (ng) in the test samples was determined. The ratio of antigen amount obtained from the standard curve to the protein amount blotted on to the membrane was expressed as the relative immunoreactivity.

Statistical Analysis. Fluorescence spectra were recorded in duplicate (two spectra each for two samples), CD spectra were recorded in triplicate (three spectra each for two samples), and averages were reported. Data were analyzed for significance (one-way ANOVA) using SPSS 9.0 (SPSS Inc., Chicago, IL). Fisher's protected LSD ($p = 0.05$) values were calculated for appropriate data.

RESULTS AND DISCUSSION

Amandin and Anacardein Controls. In the native state, both amandin and anacardein were estimated to have comparable secondary structures composed of β -sheets and turns (56.4 and 49% respectively), α -helices (14 and 23.7%), and random coil (29.6 and 27.4%). Secondary structure estimates obtained in the present investigation are in agreement with those previously reported for amandin (39) and for soybean glycinin, a legumin (40, 41), but somewhat different from the recent reports by Albilos et al. (42), who found higher helix (34.3%) and lower β -structure (37.8%) in an isolated amandin.

In addition to recording secondary structures, fluorescence spectroscopy was used to investigate the local changes in the

microenvironment of tryptophan residues in amandin and anacardein. In amandin, three tryptophan residues are located at positions 56, 211, and 431 in the prunin sequence (NCBI accession CAA55009). In anacardein, seven tryptophan residues are located at positions 19, 49, 145, 266, 315, 335, and 392 on the amino acid sequence (NCBI accession AAN76862). On the basis of previous studies on a well-characterized 11S globulin, soybean glycinin (40, 43), some of these tryptophan residues in the native amandin and anacardein are likely to be surface-accessible, whereas others may be buried. Therefore, two different fluorescence quenchers, anionic iodide and nonpolar acrylamide, were used to differentiate between the exposed and buried tryptophan residues, respectively. For each protein, effective quenching (Table 1) obtained using acrylamide and iodide was comparable, suggesting that tryptophanyl fluorophores in both proteins are in a neutral environment. The fractions of buried fluorophores, $(1 - f_a)$ were comparable in amandin and anacardein; however, K_q (acrylamide) for anacardein was about 4 times higher than for amandin. The accessible fluorophores are quenched with differing efficiencies in the two proteins, reflecting possible differences in their microenvironment as a result of differences in their respective tertiary and quaternary structures.

Through a combination of the results of CD and fluorescence spectroscopy, both amandin and anacardein appear to have a large proportion of unordered and open structures. In amandin, unordered structure has been suggested to arise due to insertion of multiple stretches of glutamine residues in the acidic subunit (39). Glutamate stretches are known to promote random coil structures (44, 45). Glutamate stretches form an important part of 11S globulin bioactivity as they are involved in intermolecular associations such as hydrogen bond formation (46, 47) and IgE reactivity (20, 48).

Effect of SDS. When amandin was exposed to SDS, a loss in CD signal (expressed as molar ellipticity per unit amino acid residue) was noted at 208 nm in up to 0.5 mM SDS. In > 1 mM SDS, a steady gain in CD signal at 208 nm was observed (Figure 1A). The CD signal at 208 nm is believed to arise predominantly from α -helical structure with negligible contributions from other (β -sheets and turns) fractions to the total intensity (49). Thus, SDS treatments at up to 1 mM appeared to result in loss of helical structure, followed by a gain in helical structure at > 1 mM SDS concentration. When SDS-treated amandin was subjected to fluorescence spectroscopy, intrinsic fluorescence of amandin tryptophan residues did not change significantly in up to 1.2 mM SDS and then was lost gradually at higher SDS concentrations (Figure 1B). Together, results from CD and fluorescence spectroscopy of SDS-treated amandin suggested major changes in amandin conformational structure at > 1 mM SDS concentration. At 10 mM SDS, the accessibility

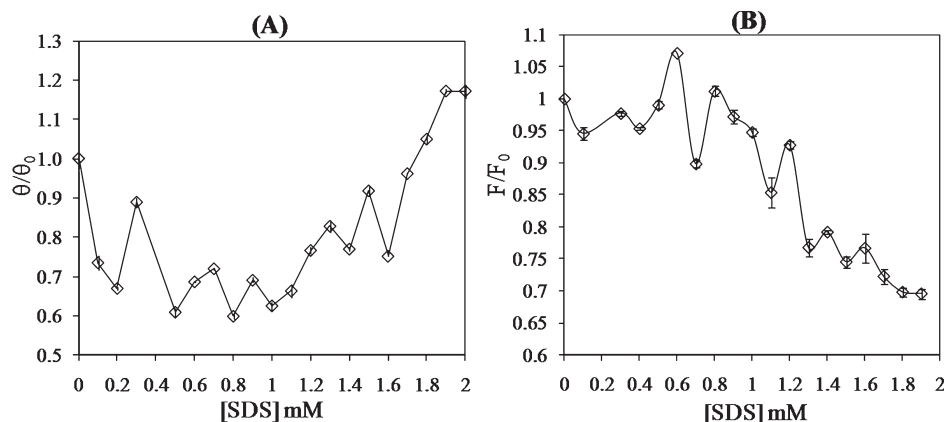


Figure 1. (A) Changes in molar ellipticity per unit amino acid residue (θ) of SDS-treated amandin relative to that in the amandin control (θ_0) at 208 nm plotted against [SDS]. (B) Changes in fluorescence intensity of intrinsic tryptophan residues (F) of SDS-treated amandin relative to that in the amandin control (F_0) at $\lambda_{\max}$ plotted against [SDS].

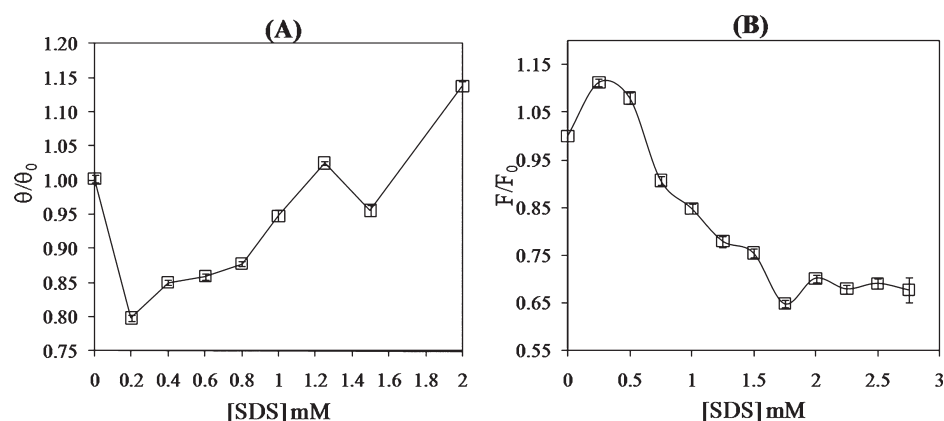


Figure 2. (A) Changes in molar ellipticity per unit amino acid residue (θ) of SDS-treated anacardein relative to that in the anacardein control (θ_0) at 208 nm plotted against [SDS]. (B) Changes in fluorescence intensity of intrinsic tryptophan residues (F) of SDS-treated anacardein relative to that in the anacardein control (F_0) at $\lambda_{\max}$ plotted against [SDS].

of tryptophan fluorophores to acrylamide was >92% as compared with 67% in the native state, indicating that amandin conformation unfolds to a more open structure (Table 1).

Gradual but constant changes in anacardein CD spectra were observed at 208 nm in ≥ 0.2 mM SDS solution (Figure 2A), indicating a gradual but constant gain in α -helical structure in anacardein. Tryptophan intrinsic fluorescence in anacardein was lost gradually in 0–1.75 mM SDS (Figure 2B). Overall, SDS treatments caused higher α -helical structures and perturbation in the surface structure of anacardein. SDS-induced anacardein unfolding was indicated by increased accessibility (from 73 to 89%) of tryptophan residues to acrylamide (Table 1).

To assess the effects of SDS-induced unfolding on immunoreactivity, 100 ng of the desired protein samples was exposed to 0–3 mM SDS and subsequently probed with specific monoclonal antibodies in a dot blot assay format. Exposing amandin to 2.5 mM SDS resulted in loss of immunoreactivity of amandin when probed with mAbs 4C10 and 4F10 (Table 2). Loss of immunoreactivity was more gradual when probed with 4F10 as compared to 4C10 (Figure 3A). Anacardein-specific monoclonal antibody 4C3 could detect anacardein treated with up to 1 mM SDS, whereas 1F5 could detect anacardein treated with up to 1.5 mM SDS (Figure 3B). Thus, loss of immunoreactivity appeared to be dependent not only on the SDS concentration but also on the mAb used to probe the molecule as well.

In 1 mM SDS >60% soybean 11S globulin (glycinin) dissociates to a 2S form, and at ~ 3.5 mM SDS most of the 11S form

is dissociated to the 2S protein (50). SDS is known to bind proteins with high affinity at ≥ 0.5 mM (51). It is possible that with increasing SDS concentration, SDS binding to amandin induced its dissociation from the 11S to 2S form. Simultaneously, dissociating subunits may have been coated with SDS, which at least partially may render the protein surface unavailable for interaction. Simultaneous interaction of any antigen with the nitrocellulose membrane and with probing antibody is essential for signal generation in dot blot assays. Thus, lack of signal may be due to ineffective interaction of amandin with the nitrocellulose membrane, the antibody, or both. Lack of amandin–nitrocellulose membrane interaction is an unlikely reason for loss of signal as 3.4 mM SDS is typically used in Western blotting (52).

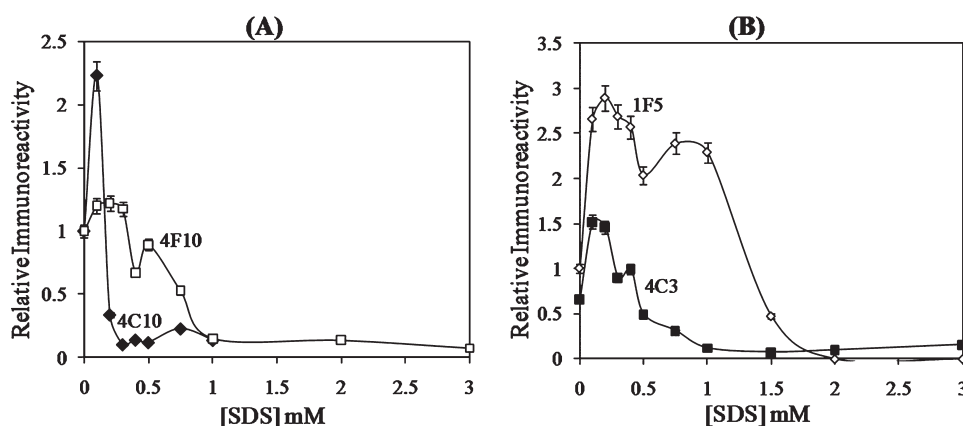
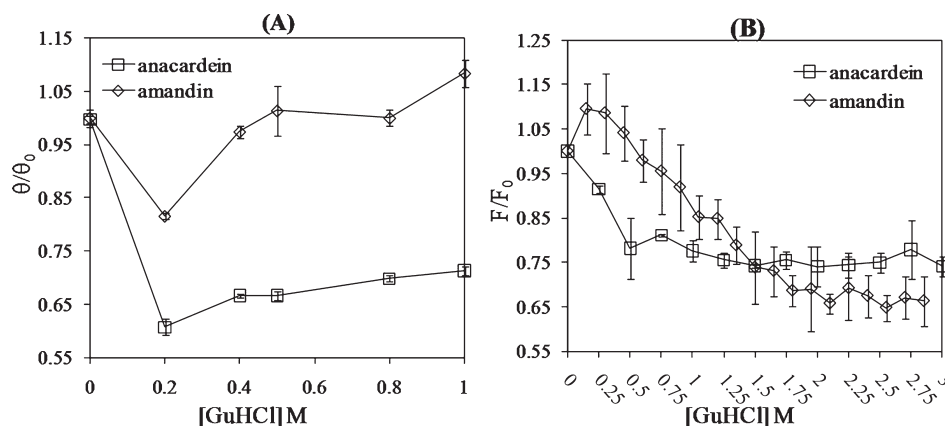
To further investigate the nature of the epitopes targeted by the mAbs, the polypeptides recognized by the mAbs were separated on the SDS-PAGE gels, transferred onto PVDF membranes, and then subjected to the N-terminal sequencing. The N-terminal sequence of the 4C10 reactive polypeptide was ARQSQLSPQN. This amino sequence matched the amino acid sequence 1–10 of the prunin chain A (NCBI accession 3EHK_A) and the amino acid sequence stretch 21–30 in the prunin sequence (NCBI accession CAA55009.1). The N-terminal sequence for 1F5 reactive polypeptide in anacardein, YEAGTVEAWDPNHEQ, matched the amino acid residues 41–55 in the Ana o 2 sequence (NCBI accession AAN76862.1).

Effect of GuHCl. Amandin treated with 0.2 M GuHCl exhibited a significantly lower α -helical content, as indicated by the

Table 2. Effect of Denaturants on Immunoreactivity of Amandin and Anacardein Probed with Corresponding Monoclonal Antibodies and Estimated by Dot Blot Immunoassays^a

treatment	% immunoreactivity (av $\pm$ SEM)			
	amandin		anacardein	
	4C10	4F10	1F5	4C3
control	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0
β ME (2% v/v) with heat (100 °C, 10 min)	8.2 $\pm$ 1.5	80.3 $\pm$ 4.9	108.1 $\pm$ 0.5	20.6 $\pm$ 10.9
GuHCl (2.5 M)	101.2 $\pm$ 5.2	93.8 $\pm$ 2.9	103.6 $\pm$ 2.9	83.5 $\pm$ 21.3
heat (100 °C, 10 min)	94.5 $\pm$ 2.3	98.4 $\pm$ 4.5	104.5 $\pm$ 1.2	90.4 $\pm$ 33.8
SDS (2.5 mM)	0.46 $\pm$ 0.41	0	64.7 $\pm$ 13.4	0.1 $\pm$ 0.1
urea (2.5 M)	100.89 $\pm$ 1.77	99.5 $\pm$ 4.1	100.2 $\pm$ 3.2	129.0 $\pm$ 24.8
LSD, $N = 3$, $p = 0.05$	7.69	6.76	60.73	17.73

^a Differences between two means within the same column exceeding the LSD values ($p = 0.05$) are significant.

**Figure 3.** Effect of SDS on immunoreactivity amandin (A) and anacardein (B). The control (no SDS exposure) protein immunoreactivity was arbitrarily assigned a value of 1.0.**Figure 4.** (A) Changes in molar ellipticity per unit amino acid residue (θ) of GuHCl-treated amandin (diamonds) and anacardein (squares) relative to those in the respective control samples (θ_0) at 208 nm plotted against [GuHCl]. (B) Changes in fluorescence intensity of intrinsic tryptophan residues (F) of GuHCl-treated amandin (diamonds) and anacardein (squares) relative to those in the respective control samples (F_0) at $\lambda_{\max}$ plotted against [GuHCl].

lower CD signal at 208 nm. This initial decrease was found to be statistically significant and reproducible. Increasing the denaturant concentration resulted in the recovery of helical content (Figure 4A, diamonds). Fluorescence intensity at the $\lambda_{\max}$ of intrinsic tryptophan residues of amandin decreased in up to 2 M GuHCl (Figure 4B, diamonds). In 6 M GuHCl, the wavelength of fluorescence emission of GuHCl-treated amandin samples was 353 nm, indicating complete exposure of tryptophan residues. Tryptophan accessibility of GuHCl-treated amandin samples to both acrylamide and iodide was 100% (Table 1). Complete unfolding of proteins in >2 M GuHCl has been observed for

other proteins, for example, κ -immunoglobulin (53) and lactate dehydrogenase (54), and in >4 M GuHCl for papain (55).

Similar to amandin, anacardein exposure to 0.2 M GuHCl caused a significant drop in α -helical content (Figure 4A, squares). However, unlike amandin, no recovery at higher GuHCl concentrations was observed. The tryptophan fluorescence was following the same pattern as in amandin (Figure 4B, squares) including the final change of the fluorescence emission maximum to 353 nm, indicating complete exposure of tryptophan residues. Tryptophan accessibility of GuHCl-treated anacardein samples to both acrylamide and iodide was $>90\%$ (Table 1).

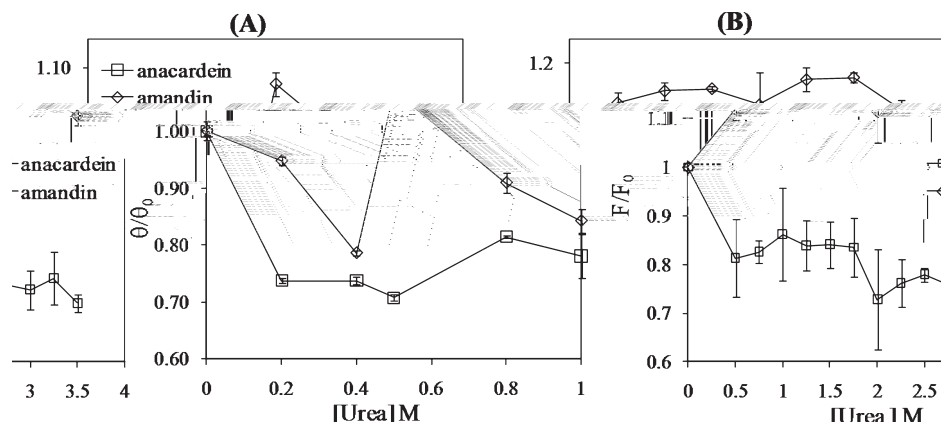


Figure 5. (A) Changes in molar ellipticity per unit amino acid residue (θ) of urea-treated amandin (diamonds) and anacardein (squares) relative to those in the respective control samples (θ_0) at 208 nm plotted against [urea]. (B) Changes in fluorescence intensity of intrinsic tryptophan residues (F) of urea-treated amandin (diamonds) and anacardein (squares) relative to those in the respective control samples (F_0) at $\lambda_{\max}$ plotted against [urea].

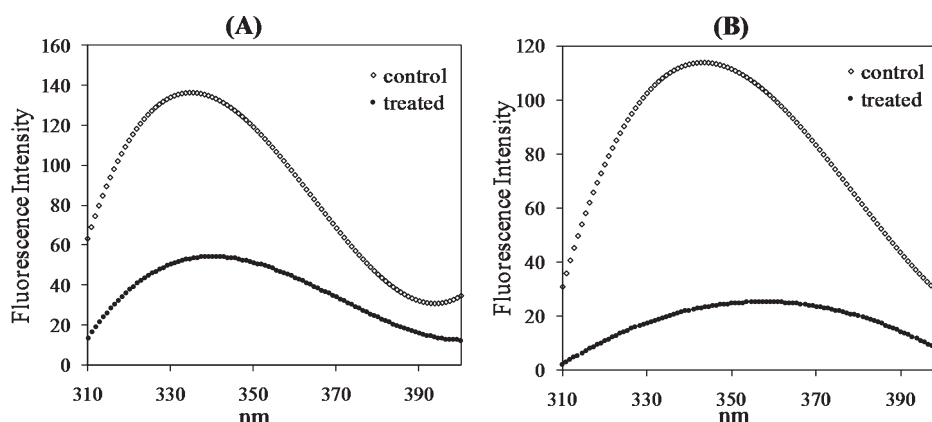


Figure 6. Fluorescence emission spectra of amandin (A) and anacardein (B) control samples (open diamonds) as compared to the respective samples treated with 2% v/v β ME and boiling (solid circles).

GuHCl treatment appeared to disrupt the structure of both proteins, as observed by loss in fluorescence intensities of intrinsic tryptophan residues. Such loss occurred with anacardein in ≥ 0.5 M GuHCl. For amandin, the loss in fluorescence intensity occurred gradually between 0 and 2 M GuHCl. Immunoreactivity of both amandin and anacardein treated with 2.5 M GuHCl, however, did not change significantly, as compared to the control sample (Table 2). GuHCl treatments resulted in greater loss of α -helical structure of anacardein than of amandin. Thus, overall, amandin conformation appeared to be more stable to GuHCl treatments than anacardein conformation. The relatively higher proportion of random coil structure in amandin may partially contribute to the stability, as GuHCl has been reported to preserve the random coil distribution of native polypeptides (56).

Effect of Urea. Amandin α -helical content gradually decreased in up to 1 M urea solutions (Figure 5A, diamonds). The intrinsic tryptophan fluorescence intensity at $\lambda_{\max}$ of urea-treated samples increased relative to control at ≥ 0.5 M urea (Figure 5B, diamonds). At 6 M urea concentration, the $\lambda_{\max}$ for amandin was 352 nm, indicating complete exposure of tryptophan residues. Albilos et al. (42) have similarly reported complete unfolding of amandin in 5 M urea. Tryptophan accessibility of urea-treated amandin samples to iodide was 75%, which indicated that in 6 M urea, some fluorophores were still not completely accessible to iodide (Table 1).

Treatment of anacardein with ≥ 0.2 M urea caused a significant drop in the α -helix content (Figure 5A, squares). The α -helical structures of soy glycinin (41) and of oat bran globulin (57) have similarly been shown to decrease after urea treatment. At urea

concentrations of ≥ 0.5 M, the fluorescence intensity of anacardein tryptophan residues decreased significantly with respect to the control anacardein (Figure 5B, squares). The wavelength of maximum fluorescence emission ($\lambda_{\max}$) of urea-treated anacardein samples was 353 nm, indicating complete exposure of tryptophan residues. Tryptophan accessibility of urea-treated anacardein samples to both acrylamide and iodide was $> 80\%$ (Table 1), indicating the inaccessibility of a small yet significant proportion of tryptophan fluorophores, even at high urea concentration. Overall, urea caused significant disruption of anacardein structure.

Direct peptide–urea interaction as a possible mechanism for helix disruption (58–61) has been suggested. Urea–peptide interaction as a protein denaturation mechanism is reminiscent of the interaction of SDS micelles with peptide backbones that are known to disrupt α -helix (62). Not surprisingly, the similarity of protein denaturation upon SDS and urea is also reflected in comparable tryptophan accessibilities in amandin and anacardein to acrylamide (Table 1).

In summary, amandin conformational structure in up to 1 M urea appeared to alter only marginally relative to the control amandin and anacardein. The immunoreactivity of both amandin and anacardein in 2.5 M urea did not change significantly relative to the corresponding control (Table 2).

When results from all three denaturation treatments are compared, SDS, GuHCl, and urea appeared to alter amandin and anacardein conformation, but only SDS exposure resulted in loss of immunoreactivity. Amandin appeared to be more resistant to GuHCl and urea denaturation than anacardein. No significant

changes in the immunoreactivity were detected in amandin or anacardein after urea and GuHCl treatments, indicating that the epitopes targeted by monoclonal antibodies were not sensitive to the changes in conformation that resulted from such treatments.

Effect of β ME. The fluorescence intensity of heat-denatured (boiling water bath) amandin and anacardein in the presence of a reducing agent (2% v/v β ME) resulted in major red shifts in λ_{max} (Figure 6). In both amandin and anacardein, the red shifts were by 6 nm. This red shift was possibly due to increased tryptophan exposure to solvent resulting from the hydrogen-bonding interaction of β ME with the indole nitrogen of the tryptophan side chains (35). Although the red shift indicated the change in the tryptophan microenvironment, tryptophan accessibility to acrylamide and iodide in both proteins did not change significantly after reduction (Table 1). However, in the case of soybean glycinin, reduction and boiling extensively exposed the previously buried hydrophobic inner core to a more polar environment (40). Together, these data suggest that although amandin, anacardein, and glycinin are 11S type globulins (legumin group), their tertiary and quaternary structures may differ significantly. Whereas such structural differences in the quaternary and tertiary structures may not be critical in the global food functionality of these proteins, they may be critical in understanding the differential sensitivity of individuals allergic to these proteins. The dot blot immunoassay results exhibited significantly decreased immunoreactivity of amandin and anacardein when the reduced and heat-denatured proteins were probed with mAbs 4C10 and 4C3, respectively, indicating the epitopes targeted by these two mAbs are, at least partially, conformational. Furthermore, because the reduction of disulfide bonds and heat denaturation of the molecules did not result in signal elimination, part of the epitope targeted by 4C10 and 4C3, respectively, on amandin and anacardein appears to be a linear peptide stretch on the corresponding molecule.

Although useful, probing proteins with murine mAbs in itself is not sufficient to improve our understanding of how patient IgE may bind with the targeted protein to trigger allergic reaction. Ideally, if the three-dimensional structural motif of the allergenic protein recognized by the patient IgE and the selected mAb are the same, the selected mAb may serve as a surrogate for the patient IgE. To this end we have identified anti-amandin and anti-anacardein murine mAbs that recognize linear as well as conformational epitopes. Efforts are currently underway to identify murine mAbs that may simulate patient IgE binding with amandin and anacardein to help improve our understanding of relative contribution of conformational versus linear epitopes toward molecular immunoreactivity of the targeted allergens.

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