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Spectroscopic and Biochemical Characterization of Almond (Prunus Dulcis L.) and Cashew (Anacardium Occidentale L.) Nut Seed Legumins

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SPECTROSCOPIC AND BIOCHEMICAL CHARACTERIZATION OF
ALMOND (*Prunus dulcis* L.) and CASHEW (*Anacardium occidentale* L.)
NUT SEED LEGUMINS

By

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**LIST OF TERMS AND ABBREVIATIONS**

**Ana o 1/ Jug r 1 etc.:** Allergen nomenclature formed by the Allergen Nomenclature Subcommittee of the International Union of Immunological Societies using first three letters of genus and first letter of the species of the allergen’s source (in italics) followed by a roman numeral to indicate the allergen in chronological order of purification.

**ANOVA:** Analysis of variance

**βME:** β-Mercaptoethanol

**BT:** Abbreviations used for buckwheat allergens

**Epitope:** Specific amino acid sequence or conformational motif of an antigen recognized by an antibody.

**CBB:** Coomassie brilliant blue R

**CM:** Abbreviations used for wheat allergens

**ELISA:** Enzyme-linked immunosorbant assay

**GF:** Gel filtration

**GuHCl:** Guanidine hydrochloride

**IgE:** Immunoglobulin E

**λ<sub>max</sub>** Wavelength maximum

**LSD:** Least significant difference

**mAb:** Monoclonal antibody

**Mr:** Relative molecular mass

**NCBI:** National centers for biological information

**NDND PAGE:** Nondenaturing nondissociating polyacrylamide gel electrophoresis

**nsLTP:** Nonspecific lipid transfer proteins

**pI:** Isoelectric point

**PR:** Pathogenesis-related

**SDS-PAGE:** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

**SPT:** Skin-prick test

**T<sub>m</sub>** Transition temperature in calorimetry
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ABSTRACT

Many legumin-like seed proteins, although known to cause human allergies, remain poorly defined—especially with respect to their molecular properties, including immunoreactivity. The legumin-like proteins are complex oligomeric proteins and are major storage proteins in several tree nut seeds including almond, cashew, pecan, and walnut. These legumin-like proteins retain immunoreactivity even after subjecting the seeds to a variety of food processing treatments, and therefore may serve as useful markers when developing detection assays to determine presence of trace quantities of the corresponding tree nut residues in food and feed.

The current investigation focused on three specific aims: (i) to purify legumin-like proteins from almond, cashew nut, walnut, pistachio and hazelnut seeds, (ii) to characterize structural properties of tree nut legumins in purified, native states, and (iii) to investigate stability of purified, native legumin allergens subjected to select chemical treatments.

Amandin and anacardein were purified according to the methods established by Sathe et al., 2002 and Sathe et al., 1997 respectively. Purification of legumin-like proteins from walnut, pistachio, and hazelnuts was attempted by chromatographic techniques. Polypeptide composition of legumin preparations was determined using SDS PAGE and 2D PAGE under reducing conditions. Two-dimensional PAGE was used to investigate presence of isoforms. Secondary structures of purified legumins were estimated by far-UV Circular Dichroic (CD) spectroscopy while fluorescence spectroscopy was used to study tertiary structure. Effects of chemical treatments on protein structures and on immunoreactivity of the legumins were assessed.

Chromatographically purified amandin and anacardein preparations contained three proteins each, constituting over 90% of the protein. Two of these three proteins were 11S globulin isoforms as judged by their closely matching polypeptide composition. The third
protein, in both amandin and anacardein, exhibited polypeptide composition and properties similar to vicilin-like proteins.

When amandin and anacardein preparations were subjected to chemical treatments and studied with CD spectroscopy, SDS treatments caused higher $\alpha$-helical structures and perturbation in the surface structure in amandin and anacardein. Gradual loss in specific mAb immunoreactivity was detected with increasing concentration of SDS treatments of amandin and anacardein. Specific mAb immunoreactivity appeared to be influenced by location of mAb-reactive peptide stretches on amandin and anacardein structures generated by homology modeling. GuHCl treatment resulted in disruption of amandin and anacardein structures. The loss of secondary and surface structure was more gradual (0 to 2M GuHCl) in amandin than in anacardein (>0.2 M GuHCl), perhaps owing to higher random coil structure in amandin. Specific mAb immunoreactivity of GuHCl-treated amandin and anacardein did not change significantly compared to their corresponding controls. Amandin conformational structure in up to 2 M urea appeared to alter only marginally compared to amandin control. More significant changes were observed in anacardein when treated with urea: major loss of $\alpha$-helical structure in >0.2 M urea, and gradual disruption of surface structure at higher urea concentrations. Specific mAb immunoreactivity of both amandin and anacardein in 2.5 M urea did not change significantly relative to the corresponding controls. Reduction ($\beta$ME) and heat denaturation of amandin and anacardein resulted in fractional loss of surface structure, and significant loss of immunoreactivity in 3 of the 4 mAbs used for probing the proteins.

Key Words: Amandin, anacardein, immunoreactivity, protein, isoforms, fluorescence, denaturation, structure, antibodies.
CHAPTER 1

INTRODUCTION

Rising human population and changing lifestyles necessitate constant availability of nutritious, inexpensive, convenient and safe food products. Proteins are indispensable components of foods not only as nutrients but also as functional ingredients that add value to processed foods. For example, egg proteins, due to their foaming property, are vital to whipped cream or meringue. Proteins are often used for multiple applications in food products. For example, soy protein isolate may act as a nutrient, a thickener and an emulsifier in a single product (e.g. a fruit smoothie). Physicochemical (Tolstoguzov, 1998) and molecular biology-related (Day, 1996) techniques have expanded the possibilities of modification of food proteins in order to enhance their functional qualities. Some examples include an antimicrobial peptide from a tree frog Phyllomedusa bicolor engineered into potatoes to increase the resistance of potatoes to fungal infections (Osusky et al., 2005), engineering metabolic pathways to increase production of flavonoids in plants (Schijlen et al., 2004), and production of cholera toxin in plants (Arakawa et al., 1999). To take advantage of the possibilities offered by physicochemical and genetic techniques, it is essential to overcome certain limitations to facilitate wider application of protein in foods. These limitations include incompatibilities with other protein or non-protein components in food system (Razumovsky and Damodaran, 2001), organoleptic attributes such as generation of bitter peptides in enzymatic hydrolyzates of milk and soy proteins (Saha and Hayashi, 2001), nutritional inadequacies (e.g., insufficient amounts of essential amino acids) and IgE mediated food allergies.

Up to 3.7% adults and about 6% children (Sicherer and Sampson, 2006) are affected by food allergies that are characterized by an IgE-mediated defensive response in an individual to otherwise innocuous food proteins. The response can range from mild itching to fatal anaphylactic shock. Every year food allergies result in about 200 fatalities
(FAAN, 2004) and may cause economic setbacks because of food recalls (Hefle and Taylor, 2004, FDA, 2008). A cure for food allergies remains unavailable, leaving avoidance of the offensive food item and/or ingredient as the best option for food allergy sufferers.

Ninety per cent of documented food allergies have been reported to be traceable to eight food groups (the “Big 8”) i.e. eggs, cow’s milk, fish, shellfish, wheat, peanuts, soy, and tree nuts (Sampson, 2004). Probability of unwarranted exposure to these eight food groups for a sensitive individual is high due to their ubiquitous presence in foods. As absolute avoidance of allergen is the only option available to a sensitive individual, ability to detect food allergens in foods, packaging material or the environment before exposure is critical. Hence, food allergen detection methods are being developed (Besler, 2001, Poms and Anklam, 2004, Sathe et al., 2005, Weber et al., 2006). At the same time, means to develop hypoallergenic food proteins are being investigated (Herman et al., 2003, Leung et al., 2004).

Some food allergies (e.g., milk allergies) may be outgrown while others (e.g., peanut and tree nut allergies) are typically considered to be permanent. Nonetheless, ~9% of pediatric patients may outgrow tree nut allergies (Fleisher et al., 2005). Tree nut allergic individuals account for 0.2% of US population (Sicherer and Sampson, 2006). Of these, 70% are traceable to almonds, cashew nuts, hazelnuts, pistachio and walnuts (Sicherer et al., 1999; 2003). Almonds, pecans, and walnuts, in that order, are the most widely consumed tree nuts in the US (Lin et al., 2005). Furthermore, almond, hazelnut, pistachio and walnut together account for close to 94% of US annual tree nut production (Pollack and Perez, 2005), while the US continues to be the largest importer of cashew nuts (36.77% of global imports in 2004, FAO Statistical Database, 2008). Tree nuts are popular due to their unique nutty flavors and crunchy texture. Additionally, tree nuts offer a variety of nutrients such as proteins, fatty acids, fiber and certain vitamins (e.g., Vitamin E in almonds) and minerals (e.g., Mg, Zn, Se, P, K) (ARS/USDA Nutrient Database, 2006). However, incidences of allergic reactions may limit wider applications of tree nuts in food products.
So far, 27 allergenic proteins have been identified in 13 tree nuts (Sathe et al. 2005, Tawde et al., 2006, Benito et al., 2007, Tawde et al., 2007). Several classification strategies have been used to better understand the properties of allergenic plant proteins, including those from tree nuts. One of them is Osborne fractionation (Osborne, 1924), which utilizes differences in solubility of plant proteins. Thus, plant proteins can be classified as albumins (water-soluble), globulins (soluble in dilute salt solutions), glutelins (soluble in dilute aqueous acid or alkali) or prolamins (soluble in aqueous alcohol). Perhaps owing to their diverse botanical sources, tree nuts differ from each other considerably in their protein compositions, when subjected to Osborne fractionation. Globulins are dominant in pistachios (Shokraii and Esen, 1988). In cashew nut, globulins are about equal to albumins (Sathe, 1994) while glutelins account for the major portion of walnut (Sze-Tao and Sathe, 2000) and pecan (Venkatachalam et al., 2004) proteins.

The majority of globulins can be grouped based on their sedimentation coefficients as either 7S-8S or 11S-12S proteins (Dunwell, 1998). The 7S-8S group, also referred to as vicilins, are typically trimeric proteins with molecular mass ~150,000-190,000-Mr (Dunwell, 1998). Each subunit of the trimer ranges ~40,000-80,000-Mr. Vicilins are devoid of disulfide bonds (Garcia et al., 2005) and are often glycosylated (Mills et al., 2004). Vicilin allergens have been identified in cashew (Ana o 1, Wang et al., 2002), hazelnut (Cor a 11, Lauer et al., 2004) and walnut (Jug r 2, Teuber et al., 1999). The 11S-12S group, referred to as legumins, constitutes hexameric proteins of ~275,000-450,000-Mr. Each monomer subunit of the hexamer is made of an acidic and a basic polypeptide with molecular masses ~40,000-Mr and ~20,000-Mr respectively (Dunwell, 1998). Disulfide bond(s) link the acidic and basic subunits in a single monomeric subunit and also, two trimers forming a hexamer. Legumin allergens have been identified in almond (Roux et al., 1999), cashew (Ana o 2, Wang et al., 2003), peanut (Ara h 3, Rabjohn et al., 1999), hazelnut (Cor a 9, Beyer et al., 2002) and walnut (Jug r 4, Wallowitz et al., 2006) as well as other plants (Malley et al., 1975, Teuber and Peterson, 1999). Of these, amandin (Sathe et al., 2002), and anacardein (Sathe et al., 1997) have been purified to homogeneity from seeds and investigated in detail with regards to biochemical properties.
such as Mr, polypeptide composition, pI etc. Amandin has been shown to be a major human allergen of legumin class (Sathe et al., 2001, 2002). Allergenicity of amandin survives food processing treatments and γ-irradiation (Su et al., 2004). Anacardein (major human allergen Ana o 2), is an abundant 11S globulin (47-54% of total protein) found in cashew nuts (Sathe et al., 1997). Anacardein also retains its immunoreactivity after food processing treatments as well as γ-irradiation (Su et al., 2004) but is susceptible to proteolysis (Sathe et al., 1997), although effect of proteolysis on allergenicity of anacardein has not been investigated. Reports on purification and characterization of other legumin allergens from tree nuts are still lacking.

Through genetic engineering of plant genes in prokaryotic hosts, recombinant allergens have been expressed for almond (Garcia-Mas et al., 1995), cashew (Wang et al., 2003) and walnut (Wallowitz et al., 2006) legumins. Generation of recombinant cDNA libraries of allergens has facilitated establishing complete amino acid sequences and identification of specific IgE binding sites, or epitopes of allergens. Twenty-two linear epitopes responsible for IgE recognition have been identified on Ana o 2. Seven out of these 22 were immunodominant (Wang et al., 2003) and the majority (68% overall and 83% of most strongly reactive) were located on the acidic polypeptide. Further, critical amino acid residues in the immunodominant epitopes of Ana o 2 (Tawde et al., 2004) were also recently identified. These studies suggest that with suitable epitope modification, one may be able to design appropriate immunotherapy candidate peptides and/or genetically alter the targeted epitopes in an effort to produce hypo- or non-allergenic proteins.

1.1 Statement of the problem

Because 65% of known food allergens can be categorized based on their conserved structures into only four groups (structural “families”), it has been argued that structure may contribute to allergenicity of food allergens (Jenkins et al., 2005). In plant-based food allergens, experimental evidence supporting the argument has demonstrated the influence of conformational structure on allergenicity. Some examples include preservation of IgE reactive epitopes of peanut allergen Ara h 1 at locations of monomer-
monomer interaction involved in the formation of Ara h 1 trimer (Maleki et al., 2000), reduction in allergenicity with concurrent loss of $\alpha$-helical structure due to thermal treatments in soybean allergen P34 (Wilson and Gonzalez de Mejia, 2005) and loss of conformational epitopes due to irreversible changes in tertiary structure during thermal, carbohydrate-mediated degradation as well as during polyphenol oxidation in Pru av 1 from cherry (Gruber et al., 2004). In tree nut allergens, existence of IgE-reactive sites governed by conformational structure has been noted in walnut albumin Jug r 1 (Robotham et al., 2002) and cashew legumin Ana o 2 (Robotham, 2006).

In plants, protein polymorphism (expression of variant forms of a single protein) is believed to originate from expression of multiple alleles of a single gene or from families of related genes (Lagares et al., 2002). In plant-based allergens, polymorphism may result in isoforms of a single allergen differing slightly in primary sequences (Robotham et al., 2005, Sancho et al., 2006) but can have significantly different immunological properties (Becker et al., 1995, Hales et al., 2004). Existence of protein isoforms has been reported for a few tree nut proteins including amandin (Sathe et al., 2001), cashew 2S albumin (Teuber et al., 2002) and hazelnut 11S acidic subunit (Beyer et al., 2002b).

Certain molecular properties of plant proteins such as hydrophobicity (Seong and Matzinger, 2004, Furmonaviciene et al., 2005), compactness (Pantoja-Uceda et al., 2002) and affinity towards lipophilic substances (Pastorello et al., 2004a) have been linked to allergenicity. Plant legumins display certain typical molecular properties such as (a) sedimentation coefficients close to 11S, (b) high molecular weight (Sathe et al., 1997, 2002), (c) stability against thermal denaturation ($T_m$ > 90 °C, Renkema et al., 2000, Mills et al., 2003) (d) susceptibility to common proteases such a pepsin and trypsin (Sathe et al., 1997, Sze-Tao and Sathe, 2000) and (e) a rather large amount of disordered secondary surface structure rich in acidic amino acids (Adachi et al., 2003, Mills et al., 2003).

Eleven-S globulins show mixed secondary structure (helices + sheets), which is the core structure of symmetrical acidic and basic subunits (Adachi et al., 2003). Secondary structure of 11S globulins stabilizes higher orders (3$^\circ$ and 4$^\circ$) of protein structure and is
largely preserved after denaturing proteolytic, thermal or chemical (Mills et al., 2002) treatments that are detrimental to tertiary and quaternary structures (Lakemond et al., 2000, Mills et al., 2002). Tertiary and quaternary surface structures have been found to be determinants of allergenicity in Ara h 1 (Shin et al., 1998) and Ana o 2 (Robotham, 2006) respectively. Alteration of surface structure through genetic modifications, limited proteolytic or chemical treatments is likely to have potential to disrupt IgE binding locations on the molecules without destroying the core structure. Defining these structural modifications that affect IgE reactivity in native legumins known to be human allergens could offer clues towards engineering hypoallergenic legumins. As IgE-binding linear epitopes and conformational motifs have been defined and mapped in Ana o 2, anacardein could be used as a model protein for such investigation.

Although the preponderance of legumins in seed proteins is documented (Sun et al., 1987, Shokraii and Esen, 1988, Sathe, 1993, Sathe et al., 1994) and several legumins have been demonstrated to be major human allergens (Roux et al., 1999, Beyer et al., 2002, Wang et al., 2003, Wallowitz et al., 2006, Teuber and Peterson, 1999), the relationship between protein conformation and immunoreactivity remains largely under-investigated. The current investigation focused on amandin (Sathe et al., 2002) and anacardein (Sathe et al., 1997), the two 11S globulins. Following separation of amandin and anacardein isoforms, the separated isoforms were investigated for their structure using spectroscopic techniques and immunoreactivity using rabbit polyclonal and select mouse monoclonal antibodies to discern the relationship between structure and immunoreactivity of the isoform. Additionally, attempts were made to purify hazelnut, pistachio and walnut legumin-like proteins using the protocols used for amandin and anacardein purification.

The specific aims of the proposed investigation were:

I. Isolation and purification of legumin-like proteins from almond, cashew nut, walnut, pistachio and hazelnut seeds

1. Amandin (Sathe et al., 2002) and anacardein (Sathe et al., 1997) were purified using the published protocols.
2. Attempts were made to isolate legumin-like proteins from walnut, pistachio, and hazelnuts.

II. Characterization of amandin and anacardein structures

1. Polypeptide composition was studied with SDS PAGE and with 2D PAGE under reducing conditions.
2. Existence of isoforms was investigated with a 2D PAGE procedure.
3. Secondary structures were estimated with far-UV Circular Dichroic (CD) spectroscopy.
4. Tertiary structures were studied using fluorescence spectroscopy and fluorescence quenching.

III. Characterization of stability of amandin and anacardein against chemical treatments

1. Stability against chemical denaturation: SDS, urea, guanidine HCl and βME were used to investigate changes in protein structures with CD and fluorescence spectroscopies. Changes in immunoreactivity were followed using immunochemical techniques (dot blots and inhibition ELISA).
1.2 Research hypotheses

1. Preparation of native legumins to homogeneity:
   a. Based on the typical SDS PAGE results of legumin-like proteins from tree nuts (Sathe et al., 1997, Sathe et al., 2002), analysis of purified legumins with SDS-PAGE under reducing conditions is anticipated to reveal: (a) a cluster of bands at molecular weight ~40,000-Mr, corresponding to the acidic subunit of legumins, (b) a second cluster of bands at molecular weight ~20,000-Mr, corresponding to the basic legumin subunit and (c) several minor polypeptides between molecular masses 10,000-60,000-Mr corresponding to post-translational proteolytic products of the major polypeptides.
   b. N-terminal sequencing of some of the protein bands observed on SDS PAGE will reveal sequence matches to tree nut legumins.
   c. Microheterogeneity of purified legumin preparations will be evident with 2-D PAGE.
   d. Coomassie blue R staining of NDND PAGE gels will reveal the protein preparations to be at least 90% pure.

2. Investigation of structures of the purified, native legumins is anticipated to reveal:
   a. Stokes’ radii of legumins purified from hazelnut, pistachio and walnut to be in the range of 50-60 Å.
   b. CD spectroscopic analysis of purified legumins to indicate closely related, mixed secondary structures predominant in β-strands and turns.
   c. Cashew and pistachio being from the same botanical family (Anacardiaceae), structures of anaacardein and pistachio legumin expected to be closely related.
   d. Predominantly electronegative surface charge to result in high Stern-Volmer constants in iodide quenching experiments.

3. Legumin stability:
   a. Legumins will exhibit high thermal stability.
   b. Effect of chemical denaturation on protein structure:
i. Treatment with SDS will result in increase in random structure.

ii. Treatment with chaotropic agents such as guanidine HCl and urea will cause a significant unfolding of native molecules resulting in improved exposure of buried tryptophan residues. βME treatments will result in significant loss of ordered structure with a concomitant gain in random structure as a result of disulfide bond breakage.

1.3 Significance of the study

1. The proposed investigation is the first study of its kind.
2. Two-dimensional PAGE investigations, structural complexity of tree nut 11S globulin preparations was revealed. Native 11S globulins exhibited isoforms.
3. NDND PAGE facilitated isoform separation.
4. Secondary structures of amandin and anacardein revealed mixed secondary structures in amandin and anacardein.
5. In both amandin and anacardein, upon treatment with chemical agents, α-helices decreased with an increase in the β-sheets and turns.
6. Immunoreactivity of amandin and anacardein appeared to be sensitive to SDS and βME.

1.4 Limitation of the study

Only two legumins, amandin and anacardein, and their selected isoforms were investigated in detail. Other tree nut legumins may not be similar to amandin and anacardein and therefore the findings of the current investigation must not be automatically applied to legumins from other tree nut seeds.
CHAPTER 2

REVIEW OF LITERATURE

2.1 Food allergies

Abnormal physiological response to an otherwise innocuous food item constitutes food hypersensitivity reactions. Hypersensitivity reactions that are mediated by antigen-specific immunoglobulin-E (IgE) are referred to as type-I food allergies. From recent estimates, IgE-mediated, type-I (or class I) food allergies currently affect 3.5-4% Americans (Sicherer and Sampson, 2006). Exposure to the offensive allergenic agent first sensitizes the immune system to generate allergen-specific IgEs. Subsequent exposure, either conscious or accidental, to even trace amounts of the same allergen provokes a quicker and more aggressive allergic response. Allergic reaction is a complex set of immunological and biochemical reactions initiated by a cross-linking event. At least two allergen-specific IgE molecules cross-link with an allergen molecule on the surface of mast cells and basophils. This event activates the cells to release biochemical agents such as histamine that provoke multiple physiological responses such as hypotension, edema, irritation and swelling of the oro-nasal mucosa. In extreme cases, allergic reaction can result in anaphylactic shock and sudden death (Bock et al., 2001, Sutherland et al., 2000). Most of the reported food allergic reactions result due to consumption of non-packaged food. However, accidental exposure to minute amounts of undeclared or undetected contaminant allergen is also important to consider. Accidental contamination has resulted in expensive recalls of processed food products (Vierk et al., 2002, Hefle and Taylor, 2004). In addition to gastrointestinal and/or respiratory symptoms, food allergies may increase the risk of allergic individuals developing asthma (Berns et al. 2007) or developing symptoms for several other related disorders such as anxiety and depression (Patten and Williams, 2007).
2.2 Food allergens

Food allergens, generally proteins in nature, are spread across plant and animal kingdoms. Food groups that are most frequently implicated in type-I allergic reactions, in the descending order of frequency of implication, are shellfish, peanuts, tree nuts, fish, milk and eggs (Sampson, 2004). Prevalence of allergic reactions is most frequent during earlier years of life. Some allergies e.g., cow-milk allergy may be outgrown as the individual matures. Other allergies e.g., peanut and tree nut allergies are more often than not, lifelong. Of these, peanut allergies are on the rise, even in adult population.

After ingestion, food items interact with a large surface area of oral, esophageal and gastrointestinal mucosa. Still, only a limited number of food proteins exhibit the ability to sensitize the immune system to produce IgEs, thus acting as food allergens. Thus, a certain set of molecular characteristics of allergenic proteins must predispose them to become food allergens. In food allergy literature, major, class I food allergens have been generalized as water-soluble 10,000-70,000-Mr glycoproteins that are stable to heat, acid and proteases (Lehrer et al., 1996, Sicherer and Sampson, 2006). As more information about structure-allergenicity relationships in food allergens becomes available, quite a few exceptions to the aforementioned generalization have been stated. For example, legumin-like allergens of the cupin superfamily (such as Ara h 3, Ana o 2, Jug r 4, etc.) are not readily soluble in water but need dilute salt solutions as solvents, are not glycoproteins and have much larger Mr (250,000-350,000) (Sathe et al., 2005). Thus, food allergens are likely to have a much larger array of features, some of which could influence allergenicity. The current literature review is therefore aimed at providing a brief overview of structure-allergenicity relationship of plant-based food allergens.

2.3 Allergens from plant sources

2.3.1 Allergens from seeds (oil seeds, legumes and tree nuts)

2.3.1.1 2S albumins. The low molecular weight, cysteine-rich 2S albumin allergens belong to prolamin superfamily of allergens. Two-S albumins are generally composed of
two different polypeptide chains linked by two disulphide bridges, an exception being sunflower 2S albumin SFA-8, which consists of a single polypeptide (Pantoja-Uceda et al., 2004). Existence of several isoforms of the two constituent polypeptides has been noted in native 2S albumins (Hsiao et al., 2006, van Boxtel et al., 2006).

In legumes such as peanut (Ara h 2, 6, 7, Burks et al., 1992, Koppelman et al., 2004), soybean (Ogawa et al., 1991), tree nuts such as almond (Poltronieri et al., 2002), cashew (Ana o 3, Robotham et al., 2005), walnut (Jug r 1, Teuber et al., 1998), brazil nut (Ber e 1, Bartolome et al., 1997), hazelnut (Cor a 11, Lauer et al., 2004), pecan (Car i 1, Hu et al., 2003) and oil seeds such as sesame (Ses i 1, Tai et al., 1999, Pastorello et al., 2001c), rape (Puumalainen et al. 2006), sunflower (SFA-8, Burnett et al., 2002) and castor bean (Ric c 1, 3, Bashir et al., 1998), 2S albumins have been identified as major allergens.

Certain members of 2S albumin family such as those from soybeans (Lin et al., 2006) and sunflower seed SFA8 (Pantoja-Uceda et al. 2004, Tengel et al. 2005), are relatively less allergenic. Although sunflower seed 2S albumins are IgE-reactive (Kelly et al., 2000, Kelly and Hefle., 2000), the immune response induced by SFA8 is predominantly Th1 than Th2 type (Kean et al., 2006), which makes SFA8 a weak allergen.

Compactness of the core structure provides 2S albumins with an exceptional stability towards thermal denaturation (up to 100°C) (Pantoja-Uceda et al., 2002, Lehmann et al., 2003) as well as proteolytic and chemical degradation (Murtagh et al., 2003). Such stability perhaps confers 2S proteins the ability to cross gastrointestinal barrier intact (Moreno et al., 2006) that may contribute to strong allergenicity. Chemical processing (reduction and alkylation) was shown to destroy the β-sheet structure, and consequently the globular fold of Ber e 1, while retaining its α-helical structure (Koppelman et al., 2005). Reduced and alkylated Ber e 1 was proteolyzed by pepsin within 30 s, compared to native Ber e 1, which was not completely proteolyzed even after 1 h exposure to pepsin.
Similarly, solution structures of recombinant Ric c 3, Bran 1 (black mustard 2S albumin allergen) and SFA-8 revealed a common structure consisting of 5 $\alpha$-helices folded in a compact structure of right handed superhelix (Pantoja-Uceda et al., 2003).

Attempts to map IgE binding sites on peanut 2S albumin structure have shown that linear as well as conformational motifs might be the determinants of allergenicity (Sen et al., 2002). As both allergenic (e.g. Ric c 3, Bran 1 and Ber e 1) and nonallergenic (e.g. SFA-8) 2S albumins display a common secondary structure consisting of 5 $\alpha$-helices folded in a compact structure of right handed superhelix (Pantoja-Uceda et al., 2003), it was recently argued that tertiary rather than secondary protein structure may be the determinant of allergenicity in 2S albumins (Tengel et al. 2005). Structural comparison between Ric c 3 and SFA-8 showed that SFA8 has a relatively inaccessible hypervariable loop region, which may contribute to lower allergenicity of SFA8 (Pantoja-Uceda 2004). Along with the two linear IgE-reactive epitopes, helix-turn-helix structure was suggested to be the conformational determinant of allergenicity in Ber e 1 (Alcocer et al., 2004), while in case of Jug r 1, a discontinuous glutamate residue influences IgE binding to the sole linear epitope (Robotham et al., 2002). In cashew 2S albumin allergen Ana o 3, sixteen linear IgE binding sites were found (Robotham et al., 2005), of which only two were recognized by all the patient sera tested. Disulfide bonds were found to be critical for structural stability of peanut allergen Ara h 2 and reduced allergen was more susceptible to proteolytic degradation than native allergen molecule (Magni et al. 2005). However, these investigators did not test IgE reactivity of reduced Ara h 2.

2.3.1.2 Cupin superfamily. The name “cupin” comes from Latin word “cupa” meaning barrel (Dunwell, 1998). Proteins of the cupin superfamily contain a double stranded $\alpha$-helix structure (cupins) that might be duplicated in some proteins (bicupins). Seven-S and 11S seed storage proteins are members of the cupin superfamily (Dunwell, 1998).

Vicilin-like proteins. First identified allergen from peanuts was initially described as Peanut-1 (Barnett et al., 1986) and subsequently as Ara h 1 (Burks et al.
Ara h 1 is a 7S globulin with a monomeric molecular weight of 63,000-Mr and an isoelectric point of 4.55 (Burks et al. 1991). In its purified, native state, Ara h 1 is a trimetric protein that is stabilized by interactions among hydrophobic amino acids at the distal ends of monomers. As majority of the total 23 IgE-binding epitopes (Burks et al. 1997) are located at points of monomer-monomer interactions, IgE epitopes are protected in the native protein from proteolysis (Maleki et al. 2000). In the native state, Ara h 1 has a very stable structure that is high in β-sheets with comparable α-helices (Koppelmann et al. 1999). Distinct differences have been noted in molecular make up of native versus recombinant Ara h 1. Depending on the isoform, either 78 or 84 amino acids at the N-terminal are cleaved off during post-translational maturation of Ara h 1. The cleaved off peptide hosts three IgE-reactive epitopes, two of which are immunodominant (Wichers et al., 2004).

In almonds, allergen conglutin-γ is a vicilin with subunit molecular weight of 45,000-Mr (Poltronieri et al., 2002). Ana o 1 is a vicilin allergen in cashews. Ana o 1 was identified and sequenced through expression of cashew cDNA libraries generated in E. coli. Eleven linear IgE-reactive epitopes were identified on Ana o 1 sequence with three being immunodominant (Wang et al., 2002). A vicilin-like protein in pistachio was recently identified as a human allergen, with cross-reactivity to Ana o 1 (Wilson et al., 2008). In hazelnut, vicilin allergen Cor a 11 has been identified and purified in recombinant form. In spite of being glycosylated, the glycan moiety is not essential for IgE-reactivity of Cor a 11 (Lauer et al., 2004). The glycan portion of peanut Ara h 1 (van der Veen et al., 1997) and soy Gly m Bd 28k (Hiemori et al., 2000) vicilins have been shown to be essential to IgE recognition of the allergen. Cor a 11 has been shown to be cross-reactive with Ara h 1. Walnut vicilin allergen Jug r 2 has been identified and purified in recombinant form (Teuber et al., 1999), but structure-allergenicity relationship has not been investigated. Len c 1 is a trimeric 7S globulin from lentils with a monomer molecular weight of 48,000-49,000-Mr (Lopez-Torrejon et al., 2003). Allergenicity of Len c 1 survives boiling (Sanchez-Monge et al., 2004). A cross-reactivity between Len c 1 and pea vicilin, Pis s 1 has been noted. Sequence homology based molecular modeling of peanut, soybean and pea vicilins showed a molecular surface with predominantly
electronegatively charged areas (Barre et al., 2005). Pea Vicilin, Pis s 1, has a molecular weight of 44,000-Mr (Sanchez-Monge et al., 2004, Salcedo, 2004). Allergenicity of Ara h 1 is thermostable and resistant to pepsin digestion for up to 3 h. Minor changes take place in Ara h 1 structure in 6 M urea (Burks et al., 1997, Koppelmann et al., 1999, Maleki and Hurlburt, 2004). β-conglycinin, a soybean vicilin, is thermostable up to 75 °C with minor loss of structure. All subunits of β-conglycinin (α, α’ and β) contain IgE-reactive sites (Krishnan et al., 2009). Oligomeric β-conglycinin resists proteolysis (Mills et al., 2001). Len c 1 and Ara h 1 have over 50% sequence homology with β-conglycinin.

IgE-reactive vicilin-like proteins have also been identified in coconut (Benito et al., 2007) and tomato (Bässler et al., 2009). Both proteins were found to be cross-reactive to walnut Jug r 2. Coconut 7S globulin was identified as Mr-29,000 band in SDS PAGE under reducing conditions and was susceptible to pepsin proteolysis (Benito et al., 2007). Additionally, in its native form, native purified coconut 7S globulin is a Mr-156,000 protein without any glycation (Garcia et al., 2005).

**Legumin-like proteins.** Legumin-like proteins are characterized by an oligomeric structure consisting of a large acidic and a small basic subunit linked by disulfide bond. Six of such acidic-basic subunit pairs come together to form the mature hexamer. The sequence of events that lead to formation of mature 11S globulin hexamers is known (reviewed in Jung et al., 1997). The prepropeptides are synthesized by polysomes associated with membranes and are then translocated to endoplasmic reticulum (ER). The components of ER are responsible for removal of translocation signal sequence, folding and assembly of the propeptides into 9S trimers. These trimers are then transported to the protein storage vacuoles and cleaved at the conserved asparagine-glycine bond into acidic and basic polypeptides. The 11S globulins contain two pairs of highly conserved cysteine residues that are involved in one interchain disulfide bond between the acidic and basic subunits and one intrachain disulfide bond within the acidic subunit. The oxidative redox state (i. e. presence of an oxidizing agent) in endoplasmic reticulum is essential for trimer formation (Jung et al., 1997); because it promotes interchain disulfide bond formation in legumin monomers. Also, cleavage of a subunit
propeptide at a conserved asparagine-glycine bond into acidic and basic polypeptides is essential for hexamer formation (Jung et al., 1998).

Crystal structure of soybean glycinin exhibits significant similarities between core structures of the acidic and basic subunits. Hydrophobic interactions predominate in the interaction of acidic and basic subunits. The subunit structure is rich in strands with helices at the termini. Six disordered regions were found in the crystal structure of glycinin corresponding to the five variable regions proposed by sequence alignments of various legumins. Soybean glycinin Gly m 2 and peanut glycinins Ara h 3 and Ara h 4 are homologous proteins. The acidic chain of soy glycinin G1 was found to share an IgE epitope with Ara h 3 (Beardslee et al., 2000).

L’Hocine et al. (2007) investigated influence of pH and ionic strength on soybean glycinin immunoreactivity. The pH values tested were 2.2, 2.8, 6.1, and 7.2. It may be noted that pH values in the range 3-6 were not used in this investigation due to poor solubility of 11S as its pI is ~ pH 4.8. When pH of the solution was adjusted to pH 6.1, near-pI value (5.67) (a) FTIR spectra indicated glycinin aggregation and secondary structure dependence on ionic strength (e.g. at acid pH = 2.2 and high ionic strength - I =0.5 more unfolded state was observed) and (b) significant change in immunoreactivity (IgG binding) was noted as a function of change in pH and ionic strength. For example, at pH 2.8 (at ionic strengths = 0.01, 0.2, and 0.5; p < 0.001) a significant loss in immunoreactivity occurred when compared to that at pH 2.2. Due to oligomeric nature of glycinin, intermolecular β–sheet formation in a single glycinin molecule could result in compactness due to inter-subunit contact (L’Hocine et al., 2007). When several glycinin molecules are present in a solution environment, intermolecular β–sheet formation upon heat treatment has also been interpreted as aggregation (Mills et al., 2003). In glycinin solution, both increased compactness and aggregation are likely to occur upon intermolecular β–sheet formation, either of which may result in decreased immunoreactivity due to shielding of epitopes.
Polypeptides corresponding to Legumin-like protein in peanut were first identified as \( \sim 14000\text{-Mr} \) potential human allergens (Eigenmann et al., 1996). Later, cloning the Ara \( h \) 3 gene in a microbial host revealed a protein product approximately \( 60,000\text{-Mr} \) (Rabjohn et al., 1999). The apparent difference in molecular mass between native and recombinant Ara \( h \) 3 was explained when Ara \( h \) 3 was purified from peanuts by anion exchange chromatography (Koppelmann et al., 2003). SDS-PAGE of purified, native Ara \( h \) 3 followed by MALDI-TOF sequencing of in-gel tryptic digest bands revealed that although the basic legumin subunit exists as a single polypeptide of \( \sim 23,000\text{-Mr} \), the acidic subunit is truncated at several locations on the primary sequence. These peptide products of proteolytic processing exist with the acidic and basic subunit in oligomeric native state, making the native protein much more complex than its recombinant counterpart (Piersma et al., 2005). Meanwhile, Kleber-Janke et al. (2001) described an identical peanut allergen, and the official name given by the IUIS Allergen Nomenclature Sub-Committee was Ara \( h \) 4. Ara \( h \) 3 and Ara \( h \) 4 are considered to be the same allergen. Heat treatment followed by 10 min of \textit{in vitro} proteolysis disrupts IgE-reactivity of Ara \( h \) 3 (van Boxtel et al., 2008). An Ara \( h \) 3 isoform Ara \( h \)-3im, lacking IgE binding property has been recently reported (Kang et al., 2007). Critical amino acids (EYEYDEEDRRRG) of an immunodominant Ara \( h \) 3 IgE epitope (E4) were found to be missing in Ara \( h \) 3 im amino acid sequence. These missing amino acids were linked to changed secondary structure and lack of IgE binding in Ara \( h \) 3 im.

Amandin is an abundant legumin-like protein in almonds. IgE from almond-allergic patients’ sera recognize amandin polypeptides (Sathe et al., 2001). Amandin is also synonymous (Sathe et al., 2002) with the major storage proteins in almonds, prunin-1 and prunin-2, obtained through the cDNA libraries (Garcia-Mas et al., 1995). A recent X-ray study indicates prunin-1 protomers forming a donut shaped heaxamer in mature protein (Albillos et al., 2008). Native amandin contains two major classes of polypeptides: 42,000-46,000-Mr (acidic polypeptides) and 20,000-22,000-Mr (basic polypeptides) linked by disulfide bonds. Several minor polypeptides were also noted in the range of 16,000-68,000-Mr (Sathe et al., 2002). Amandin is stable against heat denaturation encountered in common food processing techniques such as roasting, blanching,
autoclaving, microwaving (Roux et al., 2001, Venkatachalam et al., 2002) and against γ-
irradiation (Su et al., 2004).

Major storage globulin anacardein in cashew nut seeds exhibits SDS-PAGE profile
typical of legumins (Sathe et al., 1994). Isolation of anacardein was reported earlier
(Osborne and Campbell, 1896). However, native anacardein purification was reported
only recently (Sathe et al., 1997). Anacardein is an 11S globulin with ~275,000-Mr. The
monomeric subunit of the mature hexameric molecule has a Mr of 53,000-Mr with the
light chains Mr- 18,000-24,000 linked to the heavy chains Mr-30-37,000 via disulfide
bonds. Recombinant anacardein (Ana o 2) is expressed, mapped for immunodominant
epitopes (Wang et al., 2003), and critical amino acid residues in the immunodominant
epitopes have been identified (Wang et al., 2003, Tawde et al., 2004).

Legumin allergen from hazelnut, Cor a 9, was detected by probing hazelnut cDNA
library with two oligonucleotides prepared according to two internal sequences obtained
from a 40,000-Mr band on 2D PAGE of hazelnut proteins (Beyer et al., 2002).
Purification of legumin-like protein from hazelnut seeds was recently reported (Rigby et
al., 2008). The amino acid sequence of Cor a 9 thus obtained contains the fractional N-
terminus amino acid sequence of the 35,000-Mr band identified previously as human
allergen (Pastorello et al., 2002). These two investigations demonstrate the possible
complex polypeptide makeup of natural Cor a 9, as in other legumin allergens.

2.3.2 **Allergens from cereals.** With the exception of oat, rice and buckwheat allergens,
most allergenic proteins found in cereals belong to the prolamin superfamily of proteins.
Two allergenic vicilin-type proteins and three type-1 potato serine protease inhibitors
(Belozersky et al., 1995, Pandya et al., 1996) have been identified as human allergens in
buckwheat. In rice, glyoxalase, α-amylase and trypsin inhibitor proteins have been
identified as human allergens (Yasaki et al., 1991, Usui et al., 2001). In oats, globulins
have been shown to be dominant allergens (Shewry, 1999, Shotwell, 1999).
Prolamin superfamily members are characterized by up to eight highly conserved cysteine residues found in a characteristic pattern Cys-Cys and Cys-X-Cys (Kreis et al., 1985). Though prolamin superfamily members do not share a high sequence identity, their three-dimensional structures are strikingly similar—rich in α-helices stabilized by disulfide bonds (Shewry et al., 2002). The prolamin superfamily is sub-divided into several families, which are, the 2S albumins, non-specific lipid transfer proteins (nsLTPs), and inhibitors of α-amylase, trypsin, or both. Of these, the 2S albumins are seed storage proteins that are found much more frequently in seeds, legumes and tree nuts than in cereals.

Cereal Prolamins. Prolamins are characterized by solubility in aqueous alcohols and an unusually high content of proline and glutamine (Shewry et al., 2002). Gliadins (Weichel et al., 2006) and glutenins (Pastorello et al., 2007) from wheat, secalins from rye (Rocher et al., 1996, Palosuo et al., 2001), hordein from barley and zeins from corn (Frisner et al., 2000) are prolamin allergens from cereals. Cereal prolamins are stable towards heat processing and proteolytic degradation (Tatham et al., 1993, Palosuo et al., 2001).

Non-specific lipid transfer proteins (nsLTPs). Though common among cereals, nsLTPs are also found in fruits (e.g. peaches and other rosaceae fruits, Zuidmeer et al., 2005) tree nuts (hazelnut, Pastorello et al., 2002, Schocker et al., 2004, walnut, Pastorello et al., 2004b) and oil seeds (castor bean, Takishima et al., 1988). Because of such wide distribution in plant kingdom, nsLTPs have been referred to as pan-allergens (Sanchez-Monge et al., 1999). Certain members of this group (e.g., LTPs in fruits) might be involved in plant defense mechanisms in vivo and hence are together called group 14 of the pathogenesis-related (PR) proteins (van Loon and van Strien, 1999). These allergens represent a group comprising of small molecular weight proteins capable of non-specific transfer of phospholipids in vitro between natural and artificial membranes (Kader, 1996). In Arabidopsis, nsLTPs localize in cell walls (Thoma et al., 1993). Cereal nsLTPs from wheat (Battaïs et al., 2004), barley (Sorensen et al., 1993) and rice show a high degree of sequence homology (Mills et al., 2004), which is also evident from their three-
dimensional structure. Major human allergen in maize (corn) has also been identified as a lipid transfer protein (Pastorello et al., 2001a).

Monomeric in nature, nsLTPs have eight conserved cysteine residues in their sequence that form four intrachain disulfide bonds. 3-D structure shows a hydrophobic tunnel in nsLTPs that may be located in the C-terminal region and changes its structure upon complexing with lipid molecules. Up to 40% of total secondary structure in nsLTP is composed of \(\alpha\)-helices that are linked to each other by flexible loops. The helical segments are also connected to each other by disulfide bridges (Kager, 1996). Cross-reactivity between nsLTP allergens from cereals and those from fruits has been observed, emphasizing the role of similar structural properties, rather than botanical origin in human allergenicity of nsLTPs (Pastorello et al., 2001b, Asero et al., 2007).

Though no direct link between structural features and allergenic stability has been established in case of nsLTPs, indirect evidence that secondary structure may influence allergenicity is available. Cherry allergen Pru av 3, an nsLTP, has been reported to retain secondary structure and allergenicity after thermal treatments (Scheurer et al., 2004). Resistance of carrot and broccoli nsLTP to proteolysis (Asero et al., 2000) and chemical denaturation (Lindorff-Larsen and Winther, 2001) has been demonstrated, but no clues have been offered about structural changes as a result of these treatments.

Four vegetables- carrots, melons, potatoes and bananas- fail to evoke allergic response in LTP allergic patients, and appear safe for consumption by LTP-allergic patients (Asero et al., 2007).

**Inhibitors of \(\alpha\)-amylase and trypsin.** These allergens inhibit the activity of digestive enzymes in insects and mammals as a part of plant’s defense mechanism against destruction. Allergens of this class are generally larger in molecular weight than nsLTPs and exist in monomeric (rice allergenic protein, RAP, Matsuda et al., 1988) to tetrameric (wheat CM16, Tsuji et al., 2001) forms. Oligomeric structures of these inhibitors appear to be stabilized by noncovalent forces although, as in other classes of
prolamin superfamily, eight to ten conserved cysteine residues and up to five disulfide bonds are commonly found in these enzyme inhibitors. Glycosylated subunits of tetrameric CM16* in wheat and CMb* in barley (Sanchez-Monge et al., 1992), Sec c1 from rye (Garcia-Casado et al., 1995) and rice allergenic protein (RAP) (Nakase et al., 1998) are some examples of cereal allergens with α-amylase and protease inhibitory activity. Allergenicity of CM16* and RAP can be reduced or eliminated after heat processing (Carbonero & Garcia-Olmedo, 1999, Maeda et al., 1983, Matsuda et al., 1988). On the other hand, trypsin inhibitors of buckwheat, BTI-1/-2, and BWI-1c/-2/-2b/-2c have been shown to retain allergenicity after thermal and chemical denaturation (Belozersky et al., 1995, Pandya et al., 1996).

Pastorello et al. (2007) recently reported that wheat α-amylase inhibitors are present in albumin, globulin, gliadin, and glutenin fractions of wheat protein extract and are the most important wheat allergens due to their presence and thermal stability in all wheat protein fractions tested.

2.3.3 Allergens from fruits and vegetables. Majority of literature on allergens from fruits and vegetables describes proteins of plant defense system (pathogenesis-related (PR) proteins), though other classes such as prolamin (bell pepper allergen Cap p 2, Jensen-Jarolim et al., 1998) and LTP (carrot LTP allergen, Meijer et al., 1993) have also been identified. Seventeen different classes have been identified among PR proteins (Mills et al., 2004). Of these, following classes have allergenic members.

**PR-2 proteins: β-1, 3-glucanases.** These enzymes exhibit the potential to degrade β-1, 3-glucan fibers of the fungal cell walls. Allergens from this class are found in banana, avocado, fig, kiwi, potato and tomato (Brehler et al., 1997). Due to homology of these allergens with allergens from the latex products, they are also implicated in latex-fruit allergy syndrome.

**PR-3 proteins: class-I chitinases.** Enzymes of this class have an ability to degrade chitin, which is a major structural component of the exoskeleton of insects and
cell wall of certain pathogenic fungi. These proteins are composed of two distinct domains, one of which- the N-terminal domain, exhibits chitinase activity and structural homology to latex allergen hevein (Diaz-Perales et al., 1998). PR-3 family allergens are found in chestnut, avocado and banana, and are all cross-reactive with hevein. Avocado allergen Pers a 1 did not exhibit any resistance to proteolysis in simulated gastric fluid digestion, but the digest showed allergenic activity in both ELISA and SPT (Diaz-Perales et al., 2003).

**PR-4 proteins: wound-induced (Win) chitinases.** Major turnip allergen belongs to this class and shows high similarities to Win proteins from potato and tomato. This 18,700-Mr allergen also cross-reacts with prohevein from natural rubber latex (Hanninen et al., 1999).

**PR-5 proteins: thaumatin-like proteins (TLPs).** Allergens from apple (Mal d 2), corn (zeamatin, Malehorn et al., 1994), sweet cherry (Pru av 2, Fils-Lycaon et al., 1994), bell pepper (Cap a 1, Jensen-Jarolim et al., 1998) and kiwi (Act c 2, Gavrovic-Jankulovic et al., 2002) that exhibit sequence similarity to thaumatin, a sweet tasting protein from *Thaumatococcus danielli* and show antifungal activity have been grouped into this class. IgE-reactivity of thaumatin-like proteins from apple and kiwi is not susceptible to heat processing (Oberhuber et al., 2008, Bublin et al., 2008).

**PR-10 proteins: Bet v 1 homologues.** IgE from individuals allergic to major birch pollen allergen Bet v 1 cross-reacts with Bet v 1-homologous proteins from several fruits and vegetables. This syndrome is named oral allergy syndrome (OAS) and the allergens Bet v 1 homologs. Bet v 1 homologs are wide spread in plant kingdom. Common allergens from this class are found in soybean (SMA-22, Kleine-Tebbe et al., 2002) apple (Mal d 1, Vanek-Krebitz et al., 1995), sweet cherry (Pru av 1, Scheurer et al., 1997), celery (Api g 1, Breiteneder et al., 1995) and carrot (Ballmer-Weber et al., 2001).

**PR-14 proteins: lipid transfer proteins (LTPs).** These allergens have ~9000-Mr and most have eight conserved cysteine residues in their primary structure, responsible
for four disulfide bonds and thus exceptional stability against thermal, chemical or proteolytic destruction (Kader, 1996). Apple allergen Mal d 3, peach allergen Pru p 3, allergens from apricot, plum and cherry belong to this class. Recently, an immunodominant T-cell epitope was identified in Pru p 3, (amino acid #65-80), as a potential vaccine candidate against reactions associated with LTP allergens from Rosaceae fruits (Tordesillas et al., 2009).

2.3.4 **Kunitz-type trypsin inhibitors.** The cross-reactive Soybean Kunitz-type Trypsin Inhibitor, SKTI (Koide et al., 1973, Burks et al., 1994) and potato Sol t 2, 3, 4 (Seppala et al., 2001) belong to this family of proteins. These are 16-21000-Mr, glycosylated (Seppala et al., 2001) proteins that are characterized by a conformational structure rich in antiparallel β-strands and resistant to denaturation by heat and chemicals (Roychoudhuri et al., 2004).

2.3.5 **Profilins.** Profilins are 12-16000-Mr actin-binding proteins that mediate between signal transduction and actin microfilaments of the cytoskeleton during cell differentiation. Profilins are present in wide range of eukaryotes. Profilins from distantly related species share a low (<25%) sequence homology (Schlüter et al., 1997), while those from related species can show a very high (80-85%) sequence homology (Rihs et al., 1999). However, profilins have similar tertiary structures (comprising of α-helices and β-sheets) and biochemical properties (Schlüter et al., 1997). Profilins from peanut and soybeans show 83% sequence homology. Gly m 3 from soybeans (Rihs et al., 1999), Ara h 5 from peanuts (Kleber-Janke et al., 1999), Cor a 1 from hazelnut (Lüttkopf et al., 2002) Pru av 4 from cherry (Scheurer et al., 2001), Api g 4 from celery (Vallier et al., 1992), Pyr c 4 from pear (Ebner et al., 2001) Mal d 4 from apples (van Ree et al., 1995) have been identified as food allergens of profilin class. Conformational structure of rGly m 3, rather than any linear stretches of Gly m 3 amino acid sequences were found to be critical for IgE binding (Rihs et al., 1999). Profilins from pollen and food sources are frequently cross-reactive (van Ree et al., 1992). Allergenicity of Ara h 5 and Api g 4 survives heat treatments (Kleber-Janke et al., 2001 and Jankiewicz et al., 1996.
respectively). However, celery profilin 

\textit{Api g 4} is susceptible to destruction by peptic and pancreatic enzymes (Jankiewicz et al., 1997).

\section*{2.4 Protein Isoforms}

Protein isoforms have been defined as different forms of a protein that may be produced from different genes, or from the same gene by alternative splicing (National Library of Medicine, 2008). As the name implies, isoforms may differ at the primary sequence level by as little as one (Tadokoro et al., 2005) to as many as 147 amino acids (Nakao et al., 2005). Amino acid sequence differences may translate to differences in structures (Daley et al., 1998, Campos et al, 2004), function (Conboy et al, 1988, Griffin et al., 1999), cellular localization (Nakao et al., 2005) or a combination thereof (Daley et al., 1998) among protein isoforms. Post-translational alternative splicing of mRNA at multiple locations seems to be a dominant mechanism of generation of protein isoforms (Nakao et al, 2005, Blencowe, 2006, Kim et al., 2008). Although, involvement of other mechanisms such as phosphorylation (Hoode and Clark, 2007, Stamm, 2008), proteolysis (Shimada et al, 2003) has been noted.

Existence of protein isoforms has been identified as a key feature in several biological processes from normal cellular growth (Chronis and Krishnan, 2004), maintenance, and defense (Gomes et al., 2000, Garcia-Boleno et al., 2004). Understanding the involvement of protein isoforms in maintaining health has led to treatments that focus on isoforms (Gomes et al., 2000, Garcia-Boleno et al., 2004, Kozaci et al., 2007).

In biomedical sciences, proteins are widely used as diagnostic markers of diseases, diagnostic reagents etc. Isoforms of proteins critical in disease management can influence accurate diagnosis. For example, peanut seed lectin isoforms exhibit differing affinities to cell surface glycoprotein antigens (Ortiz et al., 2000) and may thus be applied to diagnosis of different tumors. Isoforms of functional enzymes, isozymes may exhibit differing substrate specificities, which may influence the biochemical process being catalyzed by the enzyme, influencing their practical applications (Osuji et al., 2003, Pelloux et al., 2007).
2.4.1 Isoforms of plant proteins. In plant kingdom, several reports show specific protein isoforms being expressed in certain plant parts (Hlousek-Radojcic et al., 1992), at a certain stage of maturation (Chiera and Grabau, 2007, Odaira et al., 1997), and in response to specific stresses (Onishi et al., 2006). Several seed storage proteins exhibit isoforms. Examples include proteins in lentil nsLTP (Finkina et al., 2007), soybean (Wadahama et al., 2007, Rainaldi et al., 2007), tepary bean protease inhibitor (Campos et al., 2004), mung bean storage proteins (Odaira et al., 1997), barley thioredoxin h (Finnie et al., 2006), adzuki bean vicilin (Fukuda et al., 2007), sesame 11S globulin and 2S albumin (Hsiao et al., 2006).

2.4.2 Importance of protein isoforms in human allergies. Isoforms of proteins relevant to human allergies have been identified in Peanut Ara h 2 (Chatel et al. 2003, Hales et al., 2004), Ara h 6 (Bernard et al., 2007), Ara h 8 (Riecken et al., 2008), and Brazil nut Ber e 1 (van Boxtel et al., 2006). Allergen isoforms have been shown to differ in immunoreactivity, with human IgE (Hales et al., 2004, Wangorsch et al., 2007) and with rabbit or mouse IgG (Grima-Pettenati et al., 1994). Among 11S globulins, existence of isoforms has been noted in sesame 11S globulin (Hsiao et al., 2006, Navuluri et al., 2006) and anacardein (Sathe et al., 1997).

2.5 Concluding remarks

Overall, plant-based food allergens have a wide range of molecular masses (8,000-350,000-Mr), characteristic protein folding (Aalberse, 2000) and a variety of roles in plant physiology. Over the past decade, significant advances have been made in identification and characterization of linear epitopes in several plant allergens. However, relationship between structure, especially protein conformation and allergenicity for a large number of plant allergens remains to be defined.
CHAPTER 3

MATERIALS AND METHODS

Materials
Raw, unprocessed nut seeds were obtained from sources indicated below:
Almonds and walnuts: Blue Diamond Growers, San Francisco, CA.
Cashews and hazelnuts: New Leaf Market, Tallahassee, FL.
Pistachios: Paramount Farms, Los Angeles, CA.

Sources of certain reagents are indicated in the methods section. 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 almonds, cashews, pistachios and walnuts seeds were ground (Osterizer blender, Jarden Consumer Solutions, Boca Raton, FL), sifted through 40-mesh sieve and extracted with petroleum ether (boiling point range 39-53.8 °C) at flour: solvent ratio of 1:10 in a Soxhlet apparatus for 8 h. Defatted flours were then treated with acetone, to ensure removal of tannins. The powders were then dried overnight in fume hood, ground, passed through a 40-mesh sieve, and stored in sealed containers at -20 °C until further use.

Amandin (Sathe et al., 2002) and anacardein (Sathe et al., 1997) were purified as described earlier. For purification of legumins from hazelnut, pistachio and walnut, following techniques were used in combination.

Preparation of Hazelnut and Pistachio legumins
Ground, defatted pistachio seed flour (40-mesh) was extracted with ten volumes of 2 M NaCl in 0.02 M Tris HCl, pH 8.5 for 2 h at 4 °C with constant stirring. Following extraction, the supernatant, containing the solubilized proteins, was separated using
centrifugation (Beckman Model J-21 centrifuge, 15,300×g_{max} at 4 °C for 30 min), glass-wool filtration and finally, vacuum filtration (Whatman filter paper No. 4, Whatman International Ltd, Maidstone, England). Clarified extract was then subjected to gel filtration chromatography followed by anion exchange chromatography.

**Gel filtration chromatography**

Sephacryl S-300HR (Amersham Biosciences, Piscataway, NJ) was used as a separation matrix in a Pharmacia XK-50 (5 cm × 50 cm) column with Gradifrac chromatography system (Amersham Biosciences, Piscataway, NJ) at a set flow-rate of 45 ml/hr. The gel filtration column was packed under gravity and then equilibrated with 0.02 M Tris-HCl, pH 8.1 containing 0.1 M NaCl and 0.001 M NaN₃. After loading the sample solution (up to 2 % of the total bed volume), fractions were collected at 20 min intervals. Eluate absorbance at 280 nm was plotted against corresponding eluted fraction to generate elution profiles.

**Anion exchange chromatography**

Protein extracts in anion exchange equilibration buffer (Tris-HCl (0.02 M), pH 8.1) were loaded on to an activated DEAE Sepharose DE-53 anion exchange resin column (2.6 cm × 23 cm) (Whatman International Ltd, Maidstone, England) at a flow rate set at 30 ml/h. Typically, sample elution was performed with a 0-0.4M linear NaCl gradient in the column equilibration buffer spread over five column volumes. After completion of the linear gradient, the anion exchange resin was washed with two volumes of 2M NaCl in equilibrium buffer to elute remaining proteins. Absorbance at 280 nm was plotted against the corresponding fraction numbers to generate elution profiles.

**NDND PAGE**

NDND PAGE, as described by Sathe (1993), was used to evaluate composition of protein preparations in non-denatured states. Typically, 3-30 % linear acrylamide gradient separating gels with 3 % acrylamide stacking gels (acrylamide: bis ratio of 37:1) of desired thickness were used. Running buffer composition was: 0.09 M Tris base, 0.08 M boric acid, 0.0025 M Na-EDTA, pH 8.5. Proteins were mixed NDND-PAGE sample
buffer (two volumes of 0.45 M Tris, 0.4 M boric acid, 12.5 mM Na-EDTA mixed with 1 volume of glycerol) containing 0.001 % bromophenol blue as the tracking dye. Pharmacia HMW kit proteins were used as standards. Up to 12 samples with protein concentrations ranging from 50 µg to 0.1 µg were loaded onto a single NDND PAGE gel. After staining the gel with a protein stain of known detection limit (e.g., Coomassie blue R, detection limit 1.5 µg, colloidal silver staining detection limit 0.1 µg), % purity of the desired protein was estimated.

**Isolation of amandin and anacardein isoforms**

To obtain the isoforms, amandin and anacardein samples were prepared and subjected to the NDND PAGE. Typically, NDND PAGE gels were run for 60 h at 6 mA. Then, a thin slice was cut from the side of the gel and the rest of the gel was preserved with few ml of water in a closed container in a refrigerator. The excised slice was briefly stained with CBB and destained with 40% aqueous methanol containing 10 % acetic acid (30 min each) to visualize the bands. The slice was then aligned with the rest of the gel; gel portions containing each isoform (blue-stained band) were cut, macerated in a mortar and pestle and stirred overnight at room temperature with BSB. The next day, gel macerate was filtered under vacuum (Whatman filter paper No. 4, Whatman International Ltd, Maidstone, England) and the filtrate was subjected to dialysis against deionized water at 4 ºC (48 h, 8 water changes). After extensive dialysis with deionized water, the isolated isoforms were lyophilized and stored at -20 ºC until further use.

Protein folding of the isomers after dialysis was checked with CD spectroscopy. The CD spectra of the isomers matched with the CD spectra of the corresponding native, whole protein (figure 1).

**SDS PAGE**

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Fling and Gregerson (1986) described by Sathe (1993)). Typically, samples were electrophoresed on an 8-25 % linear monomer acrylamide gradient separating gel (14.5 cm × 16.5 cm × 1.5 mm) and 4 % stacking gel (1.0 cm × 16.5 cm × 1.5 mm).
Figure 1: Comparison of CD spectra of amandin with its major isoform A2 and anacardein with its major isoform C2. CDPro estimates of secondary structure fractions are presented in inset tables.

The separating gel was prepared by mixing 8 % and 25 % acrylamide solutions (15 ml each) using a gradient maker and a peristaltic pump. Protein samples were prepared by mixing protein solutions with suitable volumes of SDS-PAGE sample buffer (0.05 M Tris-HCl, pH 6.8; 1% SDS; 0.01 % bromophenol blue as the tracking dye; and 30 % glycerol) containing 2 % βME (when reducing conditions were needed) and heated for 10 min in a boiling water bath. Typical sample solution to sample buffer ratio was 2:1. Suitable aliquots of protein samples were loaded on the gels, along with EZ run Rec Protein ladder (Catalog No. BP3600-25, Fisher Scientific Co., Orlando, FL) as molecular weight markers. Electrophoresis was carried out at a set current for each application (8-12 mA/gel) until the tracking dye reached the gel edge. Running tap water cooling (~15 °C) was provided during the gel run. All gels were stained with Coomassie Brilliant Blue R (CBBR) in 50 % v/v aqueous methanol containing 10 % acetic acid, unless otherwise indicated. After 8 h of staining, gels were destained in 50 % v/v aqueous methanol containing 10 % acetic acid, until the background was colorless.

Amandin isoforms A1, A2, A3 and anacardein isoforms C1, C2 and C3 were boiled with SDS-PAGE sample buffer with βME were subjected to SDS PAGE. SDS PAGE gels
were then stained either with CBB or with gelcode staining kit for glycoprotein identification or subjected to Western immunoblotting to be probed with specific monoclonal antibodies.

**Glycoprotein analysis**

Glycoprotein staining was done on SDS-PAGE gels using the Gelcode Glycoprotein staining (Pierce Chemical Co., Rockford, IL) procedure as per the manufacturer’s instructions with suitable modifications. Specifically, the incubation step with 1% w/v periodic acid and 3 % v/v acetic acid in water was extended to overnight. Further, the ultimate washing step with 3 % v/v acetic acid was repeated at least 8 times to ensure proper development.

**2D electrophoresis**

Proteins separated in the first dimension (NDND PAGE) were subjected to SDS PAGE under reducing condition in the second dimension. Typically, NDND PAGE strips were heated with SDS PAGE sample buffer with 2 % v/v βME at 100 °C for 5 min, cooled, and lowered into SDS PAGE gel assembly. To ensure constant contact between the strip and the SDS PAGE gel, the strip was sealed to the gel with 0.5 % w/v agarose solution. After SDS PAGE the gels were stained either with CBB or colloidal silver staining.

**Immunoassays**

**ELISA**

ELISA was performed as described by Wei et al. (2003). Briefly, 96-well Costar (Corning Inc, Corning, NY) plates were coated with appropriate dilutions of appropriate 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 Na₂HPO₄)), incubated at 37 °C for 1 h. After incubation, coated plates were blocked with 100 µl of 5 %w/v nonfat dry milk (NFDM) in 0.05 % Tween 20 and 1 mM ethylene diamine tetraacetic acid (EDTA) in phosphate-buffered saline (PBS; 10 mM, pH 7.2). Aliquots at appropriate concentration of either amandin or anacardein solutions 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. Next, suitable dilutions of appropriate specific mouse monoclonal antibodies [for amandin, mAbs 4C10 and 4F10 (Roux et al., 2001), and for anacardein, mAbs 4C3 and 1F5 (Robotham, 2006)] was added to the plates, and plates were then incubated again at 37 °C for 1 h. Following the incubation with monoclonal antibodies, alkaline phosphatase labeled secondary antibody (anti-mouse rabbit antibody, 1:5000 v/v dilution) was used for detection of bound mAbs. p-Nitrophenyl phosphate (50 µl of 1 mg/ml solution) 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.

**Immunoblotting**

**Western immunoblotting**

Proteins (~30 µg/lane) were subjected to (SDS-PAGE; 8–25 % linear monomer acrylamide gradient) and transferred onto 0.2 µm nitrocellulose (NC) paper according to the method of Towbin et al. (1989). Unbound sites on the NC paper were blocked using 5% w/v non-fat dry milk (NFDM) in Tris-buffered saline (TBS-T; 10 mM Tris, 0.9 %w/v NaCl, 0.05 %v/v Tween 20) for 3 h. The blots were washed twice, 5 min each, in TBS-T and then incubated with appropriate antibody in TBS-T for 1 h. NC sheets were rinsed twice with TBS-T and then washed once for 15 min followed by three 5 min washes with TBS-T. The blots were then incubated for 1 h with appropriate horseradish peroxidase (HRP)-labeled secondary antibody (anti-rabbit) raised in goat. The blots were washed as before and developed using a luminol/p-coumaric acid system and Kodak X-O-MAT photo film (Eastman Kodak Company, Rochester, NY).

**Dot blot assays**

Protran nitrocellulose membranes (0.2 µm, Scheicher and Schuell Biosciences Inc., Keene, NH) were blotted with amandin or anacardein solution in sodium phosphate buffer (0.02 M), pH 7.5 using Bio-Dot Microfiltration Apparatus (Bio-Rad Laboratories, Inc., Hercules, CA). Typically, each nitrocellulose membrane was blotted with 100 µl of anacardein control and test solutions, following the manufacturer’s directions.
Anacardein control was blotted, in triplicate, in the range of 1000-0.97 ng, in two-fold serial dilutions. Typically, 1 ml solutions in 20 mM sodium phosphate buffer, pH 7.5 were incubated for 1 h at RT with desired concentration of denaturant. A hundred-µl of the solution was blotted in quadruplicate (two dots of two replicate solutions). After blotting, the unbound sites on the membrane were blocked with nonfat dry milk (5 %w/v) in TBS-T (10 mM Tris, 0.9 %w/v NaCl, 0.05 %v/v Tween-20, and pH 7.6) buffer at RT for 1h and then with appropriate primary monoclonal antibody (culture supernatant) 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. After washing, the membranes were incubated for 1 h at RT with 10,000-fold diluted with HRP-labeled anti-mouse (secondary) antibody, raised in rabbit (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 then visualized using the luminol/p-coumaric acid substrate system and exposed Kodak Biomax XAR Film (Eastman Kodak Company, Rochester, NY). Bio-Rad Gel Doc 2000 gel documentation system (Bio-Rad Laboratories, Hercules, CA) was used to quantify immunoreactivity. A standard curve was developed by plotting dot density (intensity/mm²) against blotted protein amount (ng) in control anacardein. 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 relative immunoreactivity.

**CD spectroscopy**

Optically clear protein solutions, typically 50-100 µg/ml, in 0.02 M sodium phosphate buffer (pH 7.5) were used for CD spectroscopy. The CD spectra were recorded with AVIV spectropolarimeter at wavelength range 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 collected data (number of amino acids was calculated from c-DNA derived amino acid sequences of the proteins, available at NCBI website). All the spectra were later smoothed with third order polynomial curve fitting.
using computer software Origin (Origin Lab. Corp., Northampton, MA, and version 8). Smoothed data were copied into MS Excel. Ratios of \( \theta \) of denatured and undenatured protein (control) \( (\theta/\theta_0) \) were plotted against denaturant concentration. Computer program CDPro (http://lamar.colostate.edu/~sreeram/CDPro/main.html) was used to predict overall secondary structure (helices, \( \beta \)-sheets, \( \beta \)-turns and random coil) of select samples.

**Fluorescence Spectroscopy**

Fluorescence spectra of protein solutions 50 \( \mu \)g/ml in 0.02 M sodium phosphate (pH 7.5) were collected at excitation wavelength 295 nm and 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 scan speed was set at 100 nm/min. Protein solution was incubated with desired concentration of denaturant and buffer in a final volume of 2 ml for 1 h in the dark. Fluorescence spectra for appropriate blanks were run simultaneously. Protein fluorescence spectra were corrected for contributions by buffers and denaturants. All the spectra were later smoothed with third order polynomial curve fitting using computer software Origin (Origin Lab. Corp., Northampton, MA, version 8). Smoothed data were copied into MS Excel. Fluorescence emission intensities of intrinsic tryptophan residues at wavelength of maximum fluorescence emission \( (\lambda_{\text{max}}) \) of denatured proteins and undenatured protein control \( (F/F_0) \) were plotted against denaturant concentration.

**Fluorescence Quenching**

Perkin Elmer LS50B Luminescence Spectrometer was used for acrylamide and iodide quenching experiments with excitation wavelength 295 nm and emission wavelengths 346 nm 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 \( \mu \)g/ml. In denaturation experiments, proteins were incubated with the desired concentration of denaturing agent. In a quartz cuvette aliquots of protein, denaturant, quencher stock solution, and phosphate buffer
were added and fluorescence intensity was noted. 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 solvent.
Stern-Volmer equation (Equation 1) and Modified Stern-Volmer equation (equation 2)
were used to fit and interpret fluorescence quenching data (Lakowicz, 1999).

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

Where \(F_0\) is the fluorescence intensity of protein solution without any quenching agent; \(F\)
is the fluorescence intensity of same protein solution with a specific amount of quenching
agent. \([Q]\) is quenching agent concentration. When a ratio of \(F_0\) over \(F\) is plotted against
\([Q]\), resultant plot is called Stern-Volmer plot. The slope of Stern-Volmer plot is referred
to as Stern-Volmer quenching constant (\(K_{SV}, \text{M}^{-1}\)), which is used to express quenching
efficiency. Stern-Volmer plots for a single fluorophore population are linear, whereas
curved Stern-Volmer plots indicate at least two different populations of fluorophores:
accessible and inaccessible, to the quencher at quencher concentrations tested. In such
cases, Modified Stern Volmer equation and plots are used to account for quenching
behavior of inaccessible fluorophores population.

\[
\frac{F_0}{(F_0 - F)} = \left(\frac{1}{f_a}\right) + \left(\frac{1}{f_aK_q}[Q]\right) \quad \text{(Equation 2)}
\]

In modified Stern-Volmer equation, \(F_0/(F_0-F)\) represents reciprocal of the fluorescence of
accessible fluorophores population. When \(F_0/(F_0-F)\) is plotted against the inverse of \([Q]\), a
straight line results. Y-intercept of the line, referred to as \(f_a\) (fractional accessibility)
represents a fraction of accessible fluorescence at infinite \([Q]\) (as \(1/\text{infinity} = 0\)). Slope of
the line is a reciprocal of \(f_a\) and \(K_q\). \(K_q\) is called the effective quenching constant as it
takes into account fluorescence of only the accessible fluorophore population. In the
present investigation, \(K_{SV}\) and \(f_a\) were used for data interpretation.

**Statistical Analysis**

Fluorescence spectra were recorded in duplicates (two spectra each for two samples), CD
spectra were recorded in triplicates (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.
CHAPTER 4

RESULTS AND DISCUSSION

To establish structure-immunoreactivity relationships of tree nut 11S globulins, it is essential to obtain homogenous protein preparations. Since purification procedures for hazelnut, pistachio and walnut 11S globulins were not available, attempts were made to purify these globulins using a combination of ammonium sulfate precipitation, gel filtration and anion exchange chromatography.

Purification of hazelnut 11S globulin
Defatted hazelnut flour was extracted with 2 M NaCl to solubilize total globulins. Gel filtration (Sephacryl S-300HR) chromatography of crude hazelnut globulin resulted in a single major peak (figure 2A, tubes 40-60). Anion exchange (Sepharose DEAE-DE53) chromatography resolved the gel filtration peak into two distinct fractions, H1 and H2 at 0.18-0.30 M and 2.0 M NaCl respectively (figure 2B). H1 behavior was similar to that reported for amandin (Sathe et al., 2002) and anacardein (Sathe et al., 1997) with respect to NaCl concentrations at which amandin (0.17-0.21 M) and anacardein (0.18–0.29 M) eluted off the anion exchange column.

Polypeptide composition of H1 and H2 represented a typical SDS-PAGE profile of 11S globulins that contain acidic subunits in the range 30,000-45,000-Mr and basic subunits in the range 15,000-25,000-Mr. Similarity of polypeptide composition of H1 and H2 (Figure 2B, inset) was suggestive of H1 and H2 to be isoforms. Polypeptide ~31,000-Mr (indicated by arrowhead in Figure 2B, inset) observed in H1 and H2 has been previously reported to be a part of Cor a 9 (11S globulin), a major human allergen in hazelnut (Beyer et al., 2002).
(Figure 2; continued)
Purification of pistachio 11S globulin

Upon gel filtration (Sephacryl S-300HR), BSB extracted pistachio proteins were resolved into two peaks (P1 and P2, figure 3A). Subsequent anion exchange chromatography resolved P1 into two peaks: P1a and P1b (figure 3B); while P2 still eluted as a single peak (figure 3C). P1a and P2 exhibited polypeptide profiles consistent with that of 11S globulins and were mainly composed of two polypeptide types: 24,000-28,000-Mr and 32,000-48,000-Mr (Figure 3B and 3C insets). Again, similarity of polypeptide composition suggests P1a and P2 to be isoforms. P1b on the other hand contained a major polypeptide at ~49,000-Mr, suggestive of a 7S globulin. NaCl concentration needed to elute P1a and P2 (Figure 3B and 3C) was in the range of 0.08-0.17 M and 0.05-0.08 M, respectively. Based on elution off the anion exchange columns, compared to amandin and anacardein, native pistachio 11S globulins appears to have less net negative charge.

(Figure 3-Continued)
Figure 3: (A) Gel filtration (S-300HR) column (5 × 42.7 cm) elution profile for pistachio crude globulin extract. Solid lines indicate pooled tubes. Inset: NDND-PAGE and SDS-PAGE results are shown. (B) Anion exchange (DEAE Sepharose DE-53) column (2.6 × 28.5 cm) elution profile for pooled peak P1 (tubes 49-83) off gel filtration column (horizontal line shown in A). (C) Anion exchange (DEAE Sepharose DE-53) column (2.6 × 28.5 cm) elution profile for pooled peak P2 (tubes 84-110) off gel filtration column (horizontal line shown in A).
**Purification of walnut 11S globulin**

Ammonium sulfate fractionation of BSB extracted proteins from defatted walnut flour followed by SDS PAGE separation of polypeptides (figure 4) suggested that the fraction precipitated at 20-40% ammonium sulfate saturation was an 11S globulin. The fraction was characterized by 30,000-35,000-Mr and 20,000-25,000-Mr polypeptide clusters with additional polypeptides in the range 48,000-52,000-Mr and 1,000-5,000-Mr. Poor solubility of the fraction in aqueous buffers (data not shown) precluded further biochemical investigation of the fraction.

![Figure 4](image.png)

Figure 4: Ammonium sulfate fractionation of walnut protein extract in BSB. S: Fisher molecular weight markers and Sigma-Aldrich peptide markers (1:1 mixture), molecular weights indicated on the left hand side. OE: walnut BSB extract. Top labels on the lanes indicate either precipitates (PPT) or supernatants (SPT) collected after ammonium sulfate fractionation.

**Purification of amandin**

A summary of amandin purification (Sathe et al., 2002) is presented in Table 2.

**Purification of anacardein**

A summary of anacardein purification (Sathe et al., 1997) is presented in Table 3.
Table 2: Summary of amandin purification.

<table>
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<tr>
<th>Prep #</th>
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<th>Anion exchange chromatography (DEAE Sepharose DE-53, volume 946 ml)</th>
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<td>Crude extract powder (g)</td>
<td>Concentration (Bradford, mg/ml)</td>
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<th>Loading Volume (ml)</th>
<th>Total loaded protein (g)</th>
<th>Flow rate (ml/hr)</th>
<th>Ve (ml)</th>
<th>Fractions (ml/ 20min)</th>
<th>Tubes pooled (#)</th>
<th>Total volume (ml)</th>
<th>Concentration (Bradford, mg/ml)</th>
<th>Total eluted protein (g)</th>
<th>% Recovery</th>
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<td>Prep II</td>
<td>53.75</td>
<td>18</td>
<td>0.97</td>
<td>406</td>
<td>27-36 (10)</td>
<td>140</td>
<td>2.72</td>
<td>0.381</td>
<td>39.36</td>
<td>14.14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prep III</td>
<td>57.19</td>
<td>34</td>
<td>1.94</td>
<td>476</td>
<td>28-39 (12)</td>
<td>168</td>
<td>2.64</td>
<td>0.444</td>
<td>22.81</td>
<td>10.25</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3: Summary of anacardein purification.

<table>
<thead>
<tr>
<th>Prep#</th>
<th>Sample preparation</th>
<th>Gel filtration chromatography (Sephacryl S300HR, volume 898 ml)</th>
<th>Pooling information</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Crude extract powder (g)</td>
<td>Buffer</td>
</tr>
<tr>
<td>Prep I</td>
<td>1.6</td>
<td>20mM Tris-Hcl and 100mM NaCl, pH 8.1</td>
<td>46.16</td>
</tr>
<tr>
<td>Prep II</td>
<td>1.5</td>
<td></td>
<td>30.2</td>
</tr>
<tr>
<td>Prep III</td>
<td>1.5</td>
<td></td>
<td>46.37</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Prep #</th>
<th>Sample preparation</th>
<th>Anion Exchange Chromatography (DEAE Sepharose DE-53, volume 946ml)</th>
<th>Pooling Information</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Equilibration Buffer</td>
<td>Concetration (Bradford, mg/ml)</td>
</tr>
<tr>
<td>Prep I</td>
<td>0.02 M Tris-HCl and 0.05 M NaCl, pH 8.1</td>
<td>1.28</td>
<td>228</td>
</tr>
<tr>
<td>Prep II</td>
<td>1.13</td>
<td>180</td>
<td>203.40</td>
</tr>
<tr>
<td>Prep III</td>
<td>1.32</td>
<td>280</td>
<td>369.60</td>
</tr>
</tbody>
</table>
Although linear IgE-reactive epitopes have been mapped in anacardein (Ana o 2), the role of conformational epitopes in immunoreactivity of anacardein has not been investigated. In case of amandin, neither linear nor conformational IgE-reactive epitopes are known. Overall IgE-reactivity of a protein is believed to be a mixture of linear and conformational determinants (epitopes) (Alberse, 2000). Conformation partly controls IgE reactivity of linear epitopes. Therefore, it is of interest to investigate the influence of conformational characteristics of amandin and anacardein and their possible correlation with their immunoreactivity. Judicious exposure to chemical denaturants permits controlled unfolding of proteins, thereby facilitating careful investigations into relationships between protein structure and protein biological activity. Amandin and anacardein were therefore subjected to controlled denaturation using SDS, GuHCl, urea and βME. Resultant protein unfolding was monitored with CD and fluorescence spectroscopies and fluorescence quenching. Immunoreactivity of native and denatured amandin and anacardein was simultaneously determined using immunoassays.

**Spectroscopic investigations of amandin and anacardein**

**Amandin and anacardein controls**

In the native state, both amandin and anacardein were estimated to have comparable secondary structures mainly comprised of β-sheets and turns (56.4 % and 49 % respectively), followed by unordered structure (29.6 % and 27.4 % respectively), remainder being α-helix (14 % and 23.7 % respectively). Secondary structure estimates obtained in the present investigation are in good agreement with those reported for 11S globulins such as soybean glycinin (Dev et al., 1990, Sze et al., 2007). Recently, Gaur et al. (2008) reported β- structure in almond amandin to be 49.8 %, which also agrees well with results of the present investigation.

In addition to recording changes in secondary structures, fluorescence spectrometry was used to investigate subtle changes in the microenvironment of tryptophan residues in amandin and anacardein. In case of amandin, three tryptophan residues are located at positions 56, 211 and 431 in 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). Based on previous studies on a better known 11S globulin, soybean glycinin (Sze et al., 2007, Lakemond et al., 2000), some of these tryptophan residues in native amandin and anacardein are likely to be surface-accessible while others may be buried. Two different fluorescence quenchers, anionic iodide and nonpolar acrylamide were used to differentiate between exposed and buried tryptophan residues respectively. For each protein, Stern-Volmer constant (equation 1, Table 4) obtained using acrylamide and iodide were comparable, suggesting comparable quenching efficiencies for both quenchers. Comparable quenching efficiency indicates that tryptophanyl fluorophores in both proteins may not be flanked by charged amino acid side chains. The data were also fitted with modified Stern-Volmer equation to decipher the behavior of inaccessible fluorophores in both amandin and anacardein. Percent fa obtained using acrylamide were comparable in amandin and anacardein. Yet, Kq obtained using acrylamide for native anacardein was about 4-times higher than that obtained for amandin using the same quencher. The % fa obtained as the reciprocal of intercept in modified Stern-Volmer plots represents an overall accessibility of fully and partially accessible fluorophores (Lakowicz, 1999). In other words, % fa represents a weighted average of all quenched tryptophans, weighted by intensity of individual tryptophan. Therefore, in amandin and anacardein, comparable values of accessibility may arise from a sum of accessibilities of fully and partially accessible fluorophore populations. Yet, these individual fluorophore populations may be quenched with differing efficiencies, owing to differing accessibilities, resulting in different effective quenching constants. Further, although amandin and anacardein are homologous proteins, the immediate local environments of tryptophan fluorophores at comparable sites on these proteins may differ, e.g. presence of local quenchers such as Asp, Asn, Glu, Gln, His, Tyr, Cys or Lys (Chen and Barkley, 1998), leading to different effective quenching constants. Closer examination of amino acid sequences for amandin (NCBI accession CAA55009) and anacardein (NCBI accession AAN76862) revealed that although similarities exist in amino acid arrangement adjacent to tryptophan fluorophores in both proteins (e.g. amandin:VAYW(211)SYN corresponds to anacardein: VAHW(145)CYN); amandin: SPHW(431)NVN corresponds to LPHW(335)NLN), there are key differences between the proteins in the way local quencher amino acids are
present adjacent to tryptophan fluorophores (e.g. amandin: QIEW(56)NFNQ versus anacardein VEAW(49)DPN). Similar observation has been made in case of hen and human lysozymes, where, comparable accessibilities (56 % and 53 % respectively), yet different effective quenching constants (1.7 M\(^{-1}\) and 0.9 M\(^{-1}\) respectively) were recorded when proteins were adsorbed onto silica.

Combining 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 acidic subunit (Gaur et al., 2008). Glutamate stretches are known to promote random coil structures (Altschuler et al., 1997, Masino and Pastore, 2002). Glutamate stretches form an important part of 11S globulin bioactivity as they are involved in inter-molecular associations (Adachi et al., 2002, Renkema et al., 2000, L’Hocine et al., 2007) such as hydrogen bond formation (Adachi et al., 2002, Mills et al., 2003), and IgE-reactive epitopes (Helm et al., 2000, Wang et al., 2002).
Table 4: Effect of denaturants on amandin and anacardein fluorescence. Differences between the two means within the same column exceeding the LSD value \((p = 0.05)\) are significant.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Amandin</th>
<th></th>
<th>Anacardein</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K(_{SV}) (M(^{-1})) ± SEM</td>
<td>% fa ± SEM</td>
<td>K(_{q}) (M(^{-1})) ± SEM</td>
<td>% fa ± SEM</td>
</tr>
<tr>
<td>Control</td>
<td>1.14±0.012</td>
<td>67.43±1.31</td>
<td>1.90±0.05</td>
<td>48.35±0.64</td>
</tr>
<tr>
<td>Boiled</td>
<td>4.73±0.018</td>
<td>105.56±2.83</td>
<td>4.36±0.53</td>
<td>74.98±10.52</td>
</tr>
<tr>
<td>βME (2% v/v)</td>
<td>2.67±0.023</td>
<td>72.20±0.83</td>
<td>4.83±0.19</td>
<td>55.75±11.20</td>
</tr>
<tr>
<td>GuHCl (6M)</td>
<td>6.61±0.029</td>
<td>91.40±1.25</td>
<td>9.11±1.03</td>
<td>2.67±0.002</td>
</tr>
<tr>
<td>SDS (10mM)</td>
<td>7.89±0.21</td>
<td>92.47±0.96</td>
<td>9.32±0.82</td>
<td>9.34±0.079</td>
</tr>
<tr>
<td>Urea (6M)</td>
<td>7.19±0.13</td>
<td>91.40±1.25</td>
<td>9.11±1.03</td>
<td>2.67±0.002</td>
</tr>
<tr>
<td>LSD, N=3, p=0.05</td>
<td>0.28</td>
<td>17.87</td>
<td>2.13</td>
<td>0.07</td>
</tr>
</tbody>
</table>

Table 5: Effect of denaturants on immunoreactivity of amandin and anacardein probed with corresponding monoclonal antibodies and estimated by dot blot immunoassays. Differences between two means within the same column exceeding the LSD values \((p = 0.05)\) are significant.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Immunoreactivity (Average±SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amandin</td>
</tr>
<tr>
<td>Control</td>
<td>100.0±0.0</td>
</tr>
<tr>
<td>βME (2% v/v) with heat</td>
<td>8.2±1.5</td>
</tr>
<tr>
<td>GuHCl (2.5M)</td>
<td>101.2±5.2</td>
</tr>
<tr>
<td>Heat</td>
<td>94.5±2.3</td>
</tr>
<tr>
<td>SDS (2.5mM)</td>
<td>0.46±0.41</td>
</tr>
<tr>
<td>Urea (2.5M)</td>
<td>100.89±1.77</td>
</tr>
<tr>
<td>LSD, N=3, p=0.05</td>
<td>7.69</td>
</tr>
</tbody>
</table>
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 5A). 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 total intensity (Woody, 1996). Thus, SDS treatments at up to 1 mM appeared to result in loss of helical structure, followed by a gain in helical structure in >1 mM SDS. 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 lost gradually at higher SDS concentrations (Figure 5B). Together, results from CD and fluorescence spectroscopy of SDS-treated amandin suggested major changes in amandin conformational structure in >1 mM SDS. In >2 mM SDS, no significant changes in either secondary or tertiary amandin structure were observed. In 10 mM SDS, accessibility of tryptophan fluorophores to acrylamide was over 92%, indicating native-like amandin conformation unfolding to a more open structure (Table 4).

Figure 5: (A) Changes in molar ellipticity per unit amino acid residue (θ) of SDS-treated amandin relative to those in amandin control (θ₀) at 208 nm plotted against [SDS]. (B) Changes in fluorescence intensity of intrinsic tryptophan residues (F) of SDS-treated amandin relative to that in amandin control (F₀) at λ_max plotted against [SDS].
Gradual but constant changes in anacardein CD spectra were observed at 208 nm in $\geq 0.2$ mM SDS (Figure 6A) indicating a gradual but constant gain in $\alpha$-helical structure in anacardein. Intrinsic fluorescence of tryptophan residues in anacardein was gradually lost in up to 1.75 mM SDS, and changed insignificantly at higher SDS concentrations (Figure 6B). Overall, SDS treatments caused higher $\alpha$-helical structures and perturbation in the surface structure in anacardein. When treated with 10 mM SDS, accessibility of anacardein tryptophan residues to acrylamide was 89.22%, indicating marginally significant increase over tryptophan accessibility of the anacardein control (73%) (Table 4).

Figure 6: (A) Changes in molar ellipticity per unit amino acid residue ($\theta$) of SDS-treated anacardein relative to that in anacardein control ($\theta_0$) at 208 nm plotted against [SDS]. (B) Changes in fluorescence intensity of intrinsic tryptophan residues (F) of SDS-treated anacardein relative to that in anacardein control (F$_0$) at $\lambda_{\text{max}}$ plotted against [SDS].

To assess effect of SDS-induced unfolding on immunoreactivity, 100 ng of the desired protein samples were exposed to 0-3 mM SDS and then probed with specific monoclonal antibodies in a dot-blot assay format. Immunoreactivity of amandin-specific monoclonal 4C10 was lost for amandin samples treated with $>0.5$ mM SDS. Another amandin-
specific monoclonal antibody, 4F10 retained its immunoreactivity to amandin treated with up to 0.75 mM SDS (Figure 7A, Table 5). Anacardein-specific monoclonal antibody 4C3 could detect anacardein treated with up to 1 mM SDS, while 1F5 could detect anacardein treated with up to 1.5 mM SDS (Figure 7B). Thus, there appeared to be a relationship between SDS-induced unfolding and loss in immunoreactivity of amandin and anacardein.

![Figure 7. Changes in immunoreactivity with control sample as a reference (arbitrarily assigned the value of 1.0) amandin (A) and anacardein (B) as a result of SDS treatments.](image)

In 1 mM SDS over 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 (Chandra et al., 1985). SDS is known to bind to proteins with high affinity at ≥ 0.5 mM (Reynolds and Tanford, 1970). It is possible that with increasing SDS concentration, SDS-binding to amandin induced its dissociation from 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 assay. 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 (Towbin et al., 1989). Western blotting results in present investigation clearly demonstrate that amandin polypeptides retain their reactivity, when
probed with mAbs 4C10 and 4F10 (Figure 13C and 13D). Loss of amandin recognition by mAbs 4C10 and 4F10 in the dot blot assay may therefore be a result of SDS binding to amandin.

To investigate amandin-4C10 interaction further, a 4C10 reactive band in amandin (Figure 15C, indicated with an arrow) was subjected to partial N-terminal amino acid sequencing. Deduced partial N-terminal sequence, ARQSQLSPQN, matched with stretch 21-31 in the prunin sequence (NCBI Accession: CAA55009). Stretch 21-31 appears in a random coil structure at the N-terminal portion of the acidic subunit in 3D homology model (Guex and Peitsch, 1997, Schwede et al., 2003, Arnold et al., 2006) of amandin monomer (Figure 8).

Prunin sequence shares 42% identity with soybean glycinin sequence (Gaur et al., 2008). Also, amandin and glycinin have closely matching secondary and tertiary structures (Catsimpoolas et al., 1970, Sze et al., 2007, Gaur et al., 2008 and the present investigation). Based on these similarities, amandin and soybean glycinin are expected to have comparable structures. The stretch 21-31 in glycinin forms part of the unordered region actively involved in hexamer formation (Adachi et al., 2003). It appears from the present results that the native amandin conformation of the region 21-31, which may be involved in hexamer formation through hydrogen bonding, is required for 4C10 immunoreactivity. Disruption of the native conformation through SDS treatment may thus disrupt the immunoreactivity.

The monoclonal antibody 1F5 recognizes the peptide YEAGTVEAWDPNHEQ at position 17-31 in Ana o 2 sequence (Roux et al., unpublished results). Interestingly, 1F5-reactive Ana o 2 stretch has a significant positional overlap with 4C10-reactive prunin stretch. The difference in conformation, as it appears in the homology models of amandin and anacardein at that position (Figure 8) is however, notable. The stretch in anacardein is in α-helix conformation, as opposed to unordered conformation in amandin in the same region. Perhaps because of the higher stability afforded to the 1F5-reactive stretch by the
α-helix conformation, 1F5 immunoreactivity is not entirely abolished at higher SDS concentration (2 mM) (Figure 7B).

Figure 8: Homology-based models of amandin and anacardein generated using SWISSMODEL with soy glycinin A3B4 homohexamer chain A as a template. Boxed area in amandin represents the N-terminal sequence of mature amandin peptide recognized by 4C10. Similarly, boxed area in anacardein represents the 1F5-reactive anacardein peptide.

**GuHCl**

Amandin treated with 0.2 M GuHCl showed a significantly lower α-helical content, judged by lower CD signal at 208 nm. In ≥0.2 M GuHCl, however, amandin helical content was not significantly different from that of control amandin (Figure 9A, diamond markers).

Fluorescence intensity at $\lambda_{\text{max}}$ of intrinsic tryptophan residues of amandin decreased in up to 2 M GuHCl, then attaining a baseline (Figure 9B diamond markers). In 6 M GuHCl, fluorescence emission $\lambda_{\text{max}}$ 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 4). Complete unfolding of proteins in > 2 M GuHCl has been observed for κ-immunoglobulin (Chung et al.,
2005), lactate dehydrogenase (Ma and Tsu, 1991) and in > 4 M GuHCl for papain (Xiao et al., 1993).

Figure 9: (A) Changes in molar ellipticity per unit amino acid residue ($\theta$) of GuHCl-treated amandin (diamond markers) and anacardein (square markers) relative to those in respective control samples ($\theta_0$) at 208 nm plotted against [GuHCl]. (B) Changes in fluorescence intensity of intrinsic tryptophan residues (F) of GuHCl-treated amandin (diamond markers) and anacardein (square markers) relative to those in respective control samples ($F_0$) at $\lambda_{\text{max}}$ plotted against [GuHCl].

When data from acrylamide quenching of amandin in 6 M GuHCl were fitted with Stern-Volmer equation, Stern-Volmer plots with upward curvature were observed (Figure 10). Upward curvature (concave towards y-axis) in Stern-Volmer plots results from static quenching contributing to dynamic quenching. Static quenching may arise either from ground-state complex formation between acrylamide and tryptophanyl fluorophores or from quenching sphere of action. Quenching sphere of action happens when the quencher, instead of forming an actual complex with the fluorophore, is adjacent to the fluorophore at the moment of excitation. Such proximity results in immediate quenching of the excited fluorophore (Lakowicz, 1999).
Exposure to ≥ 0.2 M GuHCl caused a significant drop in α-helical content of anacardein (Figure 9A, square markers), compared to that in the control, as witnessed by a drop in signal at 208 nm.

Figure 10: Stern-Volmer plot (A) of the data obtained from acrylamide quenching of amandin in 6 M GuHCl.

Decrease in tryptophan fluorescence at $\lambda_{\text{max}}$ was noted in ≥0.5 M GuHCl (Figure 9B, square markers). Wavelength of maximum fluorescence emission ($\lambda_{\text{max}}$) of GuHCl-treated anacardein samples was 353 nm, indicating complete exposure of tryptophan residues. Tryptophan accessibility of GuHCl-treated anacardein samples to both acrylamide and iodide was over 90% (Table 4).

In summary, GuHCl treatment appeared to disrupt the surface 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 happened gradually between 0-2 M GuHCl. Also, GuHCl treatments resulted in greater loss of α-helical structure of anacardein than that of amandin. Thus, overall, amandin conformation appeared to be more stable to GuHCl treatments than anacardein conformation. Relatively higher proportion of random coil structure in amandin may partially contribute to the stability, as GuHCl has been observed to preserve the random coil distribution of native polypeptides (Plaxco et al., 1997).
Immunoreactivity of both amandin and anacardein treated with 2.5 M GuHCl, however, did not change significantly, as compared to the control sample (Table 5). This finding indicates minimal conformational change in both amandin and anacardein where the targeted epitopes are located.

**Urea**

Amandin α-helical content decreased in 0.2 M urea. In 0.1 M and 0.5 M urea, however, amandin α-helical content was comparable to that of control (Figure 11A, diamond markers). Helical structure in amandin again dropped in ≥ 1M urea. Intrinsic tryptophan fluorescence intensity at \( \lambda_{\text{max}} \) of urea-treated samples increased significantly relative to control at ≥ 0.5 M (Figure 11B, diamond markers). In 6 M urea, \( \lambda_{\text{max}} \) amandin was 352 nm, indicating complete exposure of tryptophan residues. Tryptophan accessibility of urea-treated amandin samples to iodide was 75%, which indicated that in 6 M urea, although amandin was unfolded, some fluorophores were still not completely accessible to iodide (Table 4).

Treatment of anacardein with ≥0.2 M urea caused a significant drop in α-helical content (Figure 11A, square markers). The α-helical structures of soy glycinin (Dev et al., 1988) and of oat bran globulin (Nnanna and Gupta, 1996) have similarly been shown to decrease after urea treatment. At urea concentrations ≥ 0.5 M, fluorescence intensity of anacardein tryptophan residues decreased significantly with respect to control anacardein (Figure 11B, square markers). Wavelength of maximum fluorescence emission (\( \lambda_{\text{max}} \)) 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 over 80% (Table 4), indicating inaccessibility of small yet significant proportion of tryptophan fluorophores, even at high urea concentration. Overall, urea caused significant disruption of anacardein structure.

In 11S globulins, α-helical structure is situated mainly at monomer interfaces involved in formation of a stable trimer (Adachi et al., 2003). Further, monomer interfaces in 11S
globulin hexamers have been shown to be compact, solvent-inaccessible and resistant to proteolysis (Adachi et al., 2003, Maleki et al., 2001). Solvent inaccessibility may partly obviate rearrangement of water molecules upon urea treatment, an unlikely mechanism for loss in 11S globulin helices. Rather, a direct helix-urea interaction may promote disruption of the helices. Literature appears to support the possibility of direct peptide-urea interaction as a mechanism for helix disruptions (Scholtz et al., 1995, Bennion and Daggett, 2003, Tobi et al., 2003, Itri et al., 2004). Urea-peptide interaction as a protein denaturation mechanism is reminiscent of the interaction of SDS micelles with peptide backbones, which also results in reduction of the $\alpha$-helix (Montserrett et al., 2000). Not surprisingly, the similarity of protein denaturation using SDS and urea is also reflected in comparable tryptophan accessibilities in amandin and anacardein to acrylamide (Table 4).

![Graph A](image1.png)  ![Graph B](image2.png)

Figure 11: (A) Changes in molar ellipticity per unit amino acid residue ($\theta$) of urea-treated amandin (diamond markers) and anacardein (square markers) relative to those in respective control samples ($\theta_0$) at 208 nm plotted against [urea]. (B) Changes in fluorescence intensity of intrinsic tryptophan residues (F) of urea-treated amandin (diamond markers) and anacardein (square markers) relative to those in respective control samples (F$_0$) at $\lambda_{max}$ plotted against [urea].
In summary, amandin conformational structure in up to 2 M urea appeared to alter only marginally relative to control amandin. More significant changes were observed in anacardein when treated with urea: major loss of \( \alpha \)-helical structure in >0.2 M urea, and gradual disruption of surface structure at higher urea concentrations.

The immunoreactivity of both amandin and anacardein in 2.5 M urea did not change significantly relative to the control (Table 5).

When results from all three denaturation treatments are compared, SDS appeared to alter conformational structures of amandin and anacardein and resulted in loss of immunoreactivity with increasing SDS concentrations. GuHCl and urea treatments altered conformational structures of both proteins. Amandin appeared more resistant to GuHCl and urea denaturation than anacardein. No significant changes in immunoreactivity were detected in amandin or anacardein after urea and GuHCl treatments, indicating that the epitopes targeted by monoclonal antibodies were not sensitive to changes in conformation that resulted from such treatments.

\( \beta \) ME

Fluorescence intensity of heat denatured (boiling water bath) amandin and anacardein in presence of a reducing agent (2% v/v \( \beta \) ME) resulted in major red shifts in \( \lambda_{\text{max}} \) (Figure 12 A and B). In both amandin and anacardein, the red shifts were by 6 nm, which may be due to hydrogen-bonding interaction of \( \beta \) ME with indole nitrogen of the tryptophan side chains (Lakowicz, 1999). Yet, tryptophan accessibility to acrylamide and iodide in both proteins did not change significantly (Table 4) after boiling and reduction, which suggested that core amandin and anacardein structures were conserved after boiling and reduction. These results may indicate that disulfide bonds have minimal role in maintaining conformational structures of amandin and anacardein.

Amandin and anacardein, being 11S globulins, are likely to contain two disulfide bonds: an intrachain disulfide bond in the acidic subunit, and an interchain disulfide bond linking the acidic and basic subunits. It is likely that boiling and reduction targets only the intrachain disulfide linkage, which in soybean glycinin has been shown to exist in
relatively solvent accessible area of the molecule, but not the buried interchain disulfide linkage, which is in inner core.

Figure 12: Fluorescence emission spectra of amandin (A) and anacardein (B) control samples (hollow diamond markers) along with respective samples treated with 2% v/v βME and boiling (solid, round markers).

As a result, the β-sheet structure in the vicinity of intrachain disulfide bond may be disrupted, with concomitant loss of fluorescence intensity.

When boiled and reduced amandin and anacardein were probed using specific monoclonal bodies (dot blot assay format) as described earlier, results indicated a significant loss of immunoreactitivity towards all but one antibody, anacardein-specific mAb 1F5 (Table 5). After boiling and reduction, anacardein retained immunoreactivity towards mAb 1F5. Thus mAb 1F5 seemed to be the most robust antibody for recognition of chemically denatured anacardein. Again, such retention of recognition may partially be explained by the likely conformation of anacardein peptide recognized by mAb 1F5.

To gain a better understanding of changes in amandin and anacardein structures after boiling and reduction, positions of cysteine residues and of disulfide bonds in amandin and anacardein were compared with those in glycinin crystal structure. Locations of four cysteine residues engaged in two disulfide bonds are conserved in 11S globulins (Adachi
et al., 2004). Prunin sequence shows six cysteine residues while Ana o 2 sequence shows eight. Based on known crystal structure of glycinin, residues C18 and C42 are anticipated to form an intrachain disulfide bond while residues C108 and C374 may be engaged in interchain disulfide bond. Similarly, in anacardein residues C12 and C58 are likely to form an intrachain disulfide bond while residues C101 and C278 may form an interchain disulfide linkage. From glycinin crystal structure, it was deduced that the intrachain disulfide bond is solvent accessible while interchain disulfide bond is buried.

In addition to the conformational structure, another structural property that may influence immunoreactivity in 11S globulins is their ability to exist as isoforms. Because of their abundance, existence of multiple encoding genes (Fischer and Goldberg, 1982, Nielsen et al., 1989, Beilinson et al., 2002, Chen and Fukazawa, 2000) and extensive post-translational processing (Jung et al., 1997, Jung et al., 1998), 11S globulins are likely to exist as isoforms. Depending on the extent of similarity of their complex oligomeric structures, 11S globulin isoforms are likely to have similar immunoreactivities and therefore significant cross reactivities to human as well as non-human immunoglobulins. Therefore, resolution and characterization of isoforms would be a critical step in characterization of 11S globulins as markers for detection or as targets for vaccine development. The present investigation thus focused on characterization of isoforms in amandin and anacardein.

When amandin (Sathe et al., 2002) was subjected to NDND PAGE and stained with CBB, four distinct bands could be observed at 150 μg protein load (Figure 13A). Three of these were named A1, A2 and A3, in the order of increasing negative charge, A2 being the most abundant (Figure 13B). As seen in Figure 13A, A1, A2 and A3 together accounted for over 90% of amandin. A similar profile with multiple bands was observed when anacardein (Sathe et al., 1997) was subjected to NDND PAGE, and stained with CBB. At 150 μg load, four bands could be detected (Figure 13C). Three of these were named C1, C2 and C3, with increasing electronegativity, C2 being the most prominent. When compared to the amandin bands, C1, C2 and C3 were observed to have greater relative migration from the stacking gels in comparable time of electrophoresis. Either
greater negative charge, or lower molecular weights or both may be responsible for
greater migration of C1, C2 and C3. Differing mobilities of plant protein isoforms have
been observed in the literature (Hlousek-Radojcic et al., 1992). Densitometric scanning
revealed that C1, C2 and C3 together accounted for over 99% of total anacardein loaded
in a single lane of NDND PAGE gel (Figure 13D).

![Figure 13](image)

Figure 13: (A) A CBB-stained NDND PAGE gel. Left lane; Pharmacia HMW markers,
with their molecular weights as shown on the left hand side. Right lane: amandin (Sathe
et al 2002) 150 µg, major bands named A1 through A3. (B) (1) Abundance of the three
bands, relative to the total intensity of stained amandin in a single NDND PAGE lane, as
estimated using densitometric scanning and (2) Per cent immunoreactivity of each of the
three bands with mAb 4C10 relative to whole amandin, as estimated using a sandwich
ELISA. (C) A CBB-stained NDND PAGE gel. Left lane; Pharmacia HMW markers, with
their molecular weights as shown on the left hand side. Right lane: anacardein (Sathe et al
1997) 150 µg, major bands named C1 through C3. (D) (1) Abundance of the three bands,
relative to the total intensity of stained anacardein in a single NDND PAGE lane, as
estimated using densitometric scanning and (2) Per cent immunoreactivity of each of the
three bands with mAb 4C3 relative to whole anacardein, as estimated using a sandwich
ELISA. (Please refer to Materials and Methods section for details).

The results obtained in NDND PAGE of amandin and anacardein are in agreement with
those obtained by Sathe et al. (2002) and Sathe et al. (1997) respectively.
NDND PAGE separates proteins based on charge differences reflected in electrophoretic mobilities of the isoforms. A more common protocol of 2D proteomic separation of seed proteins involves denaturing IEF as the first dimension of separation (Maruyama et al., 2003, Chua et al., 2007). A limitation of this technique is its inability to characterize differences in electrophoretic mobilities of proteins not subjected to denaturing conditions.

To examine whether these bands were isoforms of each other, or distinct proteins, the amandin and anacardein bands separated in the NDND PAGE gel were then subjected to SDS PAGE in presence of a reducing agent (2% v/v βME) to compare polypeptide composition.

For the second dimension of PAGE, strips were cut from the NDND PAGE gels and then treated for SDS PAGE separation. Results of 2D SDS PAGE in presence of βME are shown in Figure 14.

In the second dimension (SDS-PAGE), protein preparations used for isoform separation were heat denatured in the SDS-PAGE sample buffer containing 2% (v/v) βME were loaded next to the NDND PAGE strips labeled ‘R’ for ‘reference protein’.

In amandin, two major clusters of polypeptides: one for acidic (36,000-42,000-Mr) and the other for basic polypeptides (20,000-24,000-Mr) were observed, consistent with previous report (Sathe et al., 2002).

In anacardein, the acidic polypeptide cluster was estimated to have molecular weights of 30,000-35,000-Mr, while the basic polypeptide cluster could be located at 18,000-24,000-Mr. In addition, a distinct band could be observed at ~51,000-Mr. These results are in agreement with those obtained by Sathe et al., 1997.

As seen in figures 14 and 15A, polypeptide composition of A1 and A2 was qualitatively identical. The main difference between A1 and A2 appeared to be the relative intensity of the constituent polypeptides. A3, however, had a distinctly different polypeptide
composition with the peptide at ~46,000-Mr being the most abundant. The peptide is a glycoprotein (figure 15B, square bracket).

Figure 14: Two-dimensional PAGE for amandin and anacardein (150 µg, each), stained with CBB. S: Fisher molecular weight ladder, R: whole protein (amandin or anacardein) used as a reference. Direction of migration from left to right (NDND PAGE) and top to bottom (SDS-PAGE).

Western blots probed with 4C10 (Figure 15C) and 4F10 (Figure 15D), revealed the differences in immunoreactivity of constituent polypeptides of the isoforms. A band ~59,000-Mr (indicated with an arrow) was detected by both 4C10 and 4F10 in A2, but not in A1 and A3. Both 4C10 and 4F10 failed to detect the major band, 46,000-Mr, in A3. However, 4C10 detected a band in A3 at 30,000-Mr (figure 15C, filled circle). Thus, A3 seems to consist predominantly of a 46,000-Mr and a 30,000-Mr peptide. Further, A3 appears to migrate in NDND PAGE as a single band (figure 13A).

These two observations indicate two possibilities: (a) the 46,000-Mr and the 30,000-Mr are two distinct peptides with similar electrophoretic mobilities in NDND PAGE or (b) the two peptides (46,000-Mr and 30,000-Mr) are associated with each other through chemical bonds.
As in case of A1 and A2, polypeptide composition of C1 and C2 was almost identical (figure 16A). Similarly, just as in case of A3, C3 had a distinctly different polypeptide composition when compared with the polypeptide composition of isoforms C1 and C2.

![Figure 15](image)

**Figure 15:** Isolated amandin isoforms A1, A2 and A3 were subjected to SDS PAGE under reducing conditions and stained with CBB (30 µg, each) (A), subjected to glycoprotein staining (90 µg, each) (B), or subjected to Western immunoblotting (30 µg, each) and probed with amandin-specific mouse monoclonal antibodies 4C10 (C) and 4F10 (D). S: Fisher molecular weight ladder, AMP: whole amandin for reference.

Based on band width and intensity a ~50,000-Mr polypeptide appeared to be the dominant component of isomer C3. The peptide stained positive when subjected to glycoprotein staining (Figure 16B). When 30 µg of each isoforms was subjected to Western immunoblotting and then probed with anacardein-specific monoclonal antibodies 4C3 (Figure 16C) and 1F5 (Figure 16D), C1 and C2 failed to show differences in immunoreactivity of constituent polypeptides. Both 4C3 and 1F5 failed to detect the major band, 50,000-Mr, in C3. However, both the monoclonal antibodies detected a band in C3 at ~37,000-Mr. A similar observation was made earlier in case of amandin isoform A3. Once again, these observations reveal complexity of tree nut 11S globulins as several distinct polypeptides make up the mature, native protein molecules. Piersma et al., (2005) have similarly noted existence of several distinct polypeptides in purified peanut glycinin. It is likely that these polypeptides are products of post-translational proteolytic processing of 11S globulins, and are associated with the core 11S globulin structure through weak chemical interactions (Koppelman et al., 2003, Natarajan et al., 2005).
Three observations: 46,000-50,000-Mr molecular weight in reducing SDS PAGE; positive glycoprotein staining and a native molecular weight of 140,000-160,000-Mr point to A3 and C3 being vicilin-like protein. Vicilins are typically trimeric glycoproteins composed monomers of 40,000-60,000-Mr (Dunwell, 1998). Vicilin-like proteins have been identified as human allergens in both almond (Poltronieri et al., 2002) and cashew (Wang et al., 2002). Soybean SRP, a 7S basic globulin has been reported to co-purify with soybean glycinin (Sathe et al., 1991).

Figure 16: Isolated anacardein isoforms C1, C2 and C3 were subjected to SDS PAGE under reducing conditions and stained with CBB (90 μg, each) (A), subjected to glycoprotein staining (90 μg, each) (B), or subjected to Western immunoblotting (30 μg, each) and probed with anacardein-specific mouse monoclonal antibodies 4C3 (C) and 1F5 (D). S: Fisher molecular weight ladder, C: whole anacardein for reference.
CHAPTER 5

CONCLUSIONS

1. Amandin and anacardein secondary structures are characterized by β-strands (37.7% in amandin and 28.7% in anacardein), turns (18.7% in amandin and 20.3% in anacardein) and α-helix (14.3% in amandin and 23.7% in anacardein).

2. Among the chemical denaturants assessed, urea and SDS caused most unfolding of amandin and anacardein, with concurrent loss of immunoreactivity in both proteins at ≥ 1.5 mM SDS.

3. β-strands and turns in amandin and anacardein were largely retained even after chemical denaturation.

4. Amandin and anacardein were antigenically stable.

5. Amandin and anacardein exhibited isoforms.

6. Vicilin-like minor impurity was noted in amandin and anacardein.

Future research directions

1. Purification protocols for pistachio, pecan, walnut and brazil nut native 11S globulins need to be developed.

2. The β-barrel portion of tree nut 11S globulin should be targeted for development of possible immunotherapies.

3. Immunodominant isoforms should be identified and fully characterized with respect to molecular properties including immunochemical, biochemical or biophysical properties.
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