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Immunological Analysis of Allergenic Tree Nut Proteins

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IMMUNOLOGICAL ANALYSIS OF ALLERGENIC TREE NUT PROTEINS

By

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ABSTRACT

Allergies are a growing problem in industrialized countries, with food allergies affecting 6% of children and 3-4% of adults. Tree nuts are a common cause of food-induced allergy and include walnut, cashew, almond, hazelnut, pistachio, pecan, chestnut, and Brazil nut. Tree nut allergy, in particular, affects 0.5% of the US population. Unlike other food allergies, tree nut allergy persists throughout life and is known to cause severe allergic reactions. Therefore, it is critical to identify allergenic proteins in tree nuts that are directly involved in the allergic response and study these proteins on both the biochemical and immunological level.

To identify allergenic proteins in pistachio and almond, polymerase chain reaction (PCR) was preformed using degenerate or gene specific primers, designed to amplify likely tree nut allergens in complementary cDNA libraries. The coding genes for the 7S vicilin from pistachio and lipid transfer protein (LTP) from almond were identified and were ligated into an expression vector to be expressed as fusion proteins. The generated fusion proteins were purified and used to screen almond- or pistachio-allergic individuals for IgE reactivity using immunoblot, ELISA, and dot blot assays.

IgE reactivity to the pistachio 7S was found in 7 of 19 (37%) patients tested, and the allergen was designated Pis v 3. Inhibition immunoblots using the 7S fusion protein lead to the identification of the native protein in the crude nut extract. It is not uncommon for food-allergic individuals to react to more than one allergen, and tree nut allergic individuals are often sensitized to multiple nuts. In particular, patients allergic to cashew frequently report allergy to pistachio as well, which is likely a result of cross-reactivity between the two closely related tree nuts. Analysis of the 7S vicilin pistachio allergen revealed that it was highly homologous to the 7S vicilin allergen, Ana o 1, from cashew. Cross-reactivity between these two allergens was investigated by inhibition dot- and immunoblot assays using serum IgE from pistachio and/or cashew-allergic individuals. The seven patients with IgE that recognized Pis v 3 also recognized Ana o 1. Similar results were obtained using a panel of murine anti-rAna o 1 monoclonal antibodies (MAbs), as six of nine (67%) MAbs tested showed reactivity to Pis v 3 on dot-blots. The data does not identify the primary sensitizing agent but suggests that IgE reactivity to Pis v 3 and Ana o 1 is focused on the most conserved regions of the proteins. The results demonstrate that Pis v 3 is a likely contributor to the observed co-sensitivity to pistachio and cashew in some patients.

Plant LTPs have been identified as allergens in a variety of fruits, vegetables, and nuts, and several studies have demonstrated cross-reactivity among LTPs. Immunoblotting using a rabbit polyclonal antibody (pAb) raised against peach LTP identified native LTP in the crude nut extract. IgE reactivity to almond LTP was found in 5 of 25 (20%) patients tested and designated Pru du 3. LTP is found in low abundance in the seed, therefore, an enriched almond extract was used in immunoblotting assays with LTP-reactive patients. Of the five LTP-reactive patients, only two recognized native LTP in the enriched nut extract. The lack of IgE reactivity by immunoblot suggests that some patients recognize primarily conformational epitopes on LTP that are destroyed under the denaturing conditions of immunoblotting. Subsequent dot blot assays confirm this hypothesis as IgE reactivity was lost when LTP was treated with common reducing reagents used in immunoblotting. Overall, the results demonstrate that LTP is an

allergenic protein in almond and IgE antibodies in allergic individuals are directed against both conformational and linear epitopes.

CHAPTER # 1

FOOD HYPERSENSITIVTY AND TREE NUT ALLERGY

The immune system is the elaborate system of defense used to protect the body from pathogens and is composed of a variety of tissues, cells, and molecules. Immunity can be broken down into two types, innate and acquired immunity. Innate immunity is an evolutionarily older defense system and forms the first line of defense against infection [1;2]. An important component of innate immunity is the epithelial surfaces, which provide a large physical barrier that prevent infection by microorganisms and colonization through the production of microbicidal substances [1]. If a microorganism evades the epithelial barriers it is immediately recognized by phagocytic cells such as macrophages, neutrophiles and dendritic cells which are localized in the tissues and blood. These cells recognize pathogens via cell surface receptors; once a pathogen is bound it is taken in by phagocytosis and degraded in the phagolysosome [1;3]. Activated macrophages are important in initiating the inflammatory response through the release of chemokines, cytokines, and mediators of inflammation. These molecules are critical for recruiting additional neutrophiles and macrophages to the site of infection to effectively remove the microorganism [1;4]. If a microorganism cannot be successfully cleared from the body by the innate immune response these activated cells initiate the acquired immune response through the interaction with lymphocytes [4].

The acquired immune response is the result of antigen specific lymphocytes, B lymphocytes (B-cells) and T lymphocytes (T-cells), which are both highly specific and lead to the generation of immunological memory. Both T-cells and B-cells are derived from a hematopoietic stem cell; but mature in different locations of the body, T-cells mature in the thymus, and B-cells mature in the bone marrow [5]. T-cells contain a membrane bound receptor for antigen and recognize antigen displayed on the cell surface of antigen presenting cells (APC) - macrophages, dendritic cells and B-cells. These APCs internalize pathogens and present peptide fragments on the surface by the major histocompatability complexes (MHC). Upon interaction with a particular antigen:MHC complex, the T-cell will become activated and differentiate into a specific effector T-cell [1]. The antigen recognition molecules of B-cells are immunoglobulins which can be membrane bound, or secreted [1;5]. The interaction between the antigen:MHC class II complex presented on B-cells with the $CD4^+$ helper T-cell, is critical for differentiation of the B-cell into immunoglobulin secreting plasma cells or memory B-cells. [5;6]. In humans there are five isotypes of immunogloblins; IgG, IgA, IgM, IgE, and IgD. The production of a particular immunoglobulin isotype is dependent on which cytokines are secreted by the helper T-cell upon interaction with the antigen:MHC complex presented on the B-cell surface. The memory B-cells generated in the acquired immune response continue to circulate through the body long after the infection is cleared. They provide a rapid immune response, termed immunological memory, when the antigen is encountered again by rapidly differentiating into antibody secreting plasma cells. The production of these long living memory cells that provide immunological memory is an important attribute of acquired immunity [6].

Food hypersensitivity

An allergic reaction is an immune system response known as a hypersensitivity reaction. Hypersensitivity reactions are harmful immune responses that often damage the body's tissue. Allergies have become a growing problem in industrialized countries and it is estimated that food allergies affect

6% of young children and 4% of adults in the United States [1;7]. Food allergy is an adverse immunological (hypersensitivity) reaction, to a normally harmless agent in food and occur rapidly after ingestion or inhalation of the offending food [8;9]. It is a type I hypersensitivity reaction that is IgE mediated and to date is the best studied. Adverse food reactions result when food-specific IgE antibodies that are bound to mast cells and basophils by the FcεRI receptor, recognize and bind circulating food allergens, cross-linking between two IgE antibodies subsequently causes effector cell degranulation [1;7;10]. Degranulation releases histamines, which increases blood flow, and enzymes such as tryptase and chymase, which break down the tissue matrix proteins fibronection and non-helical collegens, causing tissue destruction [11]. Also upon stimulation the mast cell synthesizes and releases cytokines, chemokines, and leukotrienes, which are important mediators of inflammation and perpetuate the allergic response [1;7]. The release of the chemical mediators listed above are responsible for causing the allergic symptoms experienced by the individual, which can include allergic rhinitis, allergic conjunctivitis, allergic asthma, urticaria, angioedema, and systemic anaphylaxis depending on the route of allergen entry [1].

Tree nut allergy

The average human diet contains a variety of different foods; however, only a few foods are responsible for the majority of food allergies [7]. The most common food allergens include milk, egg, wheat, soy, fish, peanuts, tree nuts, and shellfish. Often allergies to wheat, egg, milk, and soy are outgrown but peanuts, shellfish, and tree nut allergies are known to cause severe allergic reactions and persist throughout life [8]. Allergic reactions to tree nuts are common and it is estimated that 0.5% of the US population is allergic to one or more tree nuts. Tree nuts are typically eaten as a snack or incorporated into foods, and include walnuts, almond, cashew, pistachio, pecan, hazelnut, Brazil nut, macadamia nut, and chestnut. In 2003, a random digit dialed phone survey conducted in the US revealed that, of 82 tree nut-allergic individuals, 62% reported allergy to walnut, 44% to cashew, 39% to almond, 35% to pecan, 29% to hazelnut, 30% to brazil nut, and 22% to pistachio [12].

Plant food allergens

Specific proteins in food have been found to be directly responsible for food allergies, some of which have been extensively studied at the molecular level for laboratory diagnosis, and to clarify our understanding of type I hypersensitivity. Plant food allergens can be grouped based on their sequence, function, and structural similarities [13;14]. One group of proteins identified in plant foods includes seed storage proteins, which are abundant proteins in the plant. They make up the majority of tree nut and peanut allergens and include 7S vicilins, 11S legumins, and 2S albumins [13;15]. These proteins have been identified as allergens in a variety of plant foods such as cashew (Ana o 1, Ana o 2, Ana o 3), peanut (Ara h 1), soybean, sesame (Ses i 1, Ses i 2, Ses i 3), walnut (Jug r 1, Jug r 2), lentils (Len c 1), mustard, and hazelnut (Cor a 9, Cor a 11) [13;16]. Another group of plant food allergens include profilins, which are small proteins that bind actin in eukaryotic cells and regulate the polymerization of actin filaments. Profilins are highly conserved among plants with 70-85% sequence identity [13;14]. Finally, pathogenesis-related proteins (PR-proteins) are proteins that are produced in the plant upon attack by bacteria/fungi, or when abiotically stressed [17]. Members of the PR-proteins include chitinases, thaumatin-like proteins, peroxidases, endoproteinases, Bet v 1 homologues, and lipid transfer proteins.

Lipid transfer proteins

Plant lipid transfer proteins (LTP) are small proteins with a typical molecular weight of approximately 9 kDa and contain 91-95 amino acid residues but lack tryptophan residues. They are typically basic polypeptides with a pI of ~9 and contain eight conserved cysteine residues that form four disulfide bridges that are responsible for the compact folding found in LTP [18]. Analysis of 3-D models has revealed that the compact structure of plant LTPs is a result of 4 α-helixes, 3 interchain loops, and a long unstructured C-terminal coil [18-21]. In the plant, LTP is typically localized in the seeds, flowers, or fruits with little to no expression in the roots. Several studies have noted their abundance in the peel of the fruit [22;23]. Fruits from the *Rosaceae* family commonly trigger IgE mediated food allergy and the importance/role of LTP in IgE mediated food allergy was first discovered in peach. The peach allergen was subsequently named Pru p 3 [24]. Since then, LTP has been identified as an allergen in other fruits of the *Rosaceae* family such as apple, apricot, plum, cherry, and strawberry, as well non *Rosaceae* fruits and vegetables including; grape, orange, lemon, tomato, lettuce, asparagus, and maize [13;25]. LTP has also been identified in tree nuts including walnut, chestnut, and hazelnut. In several studies, screening with allergic patient sera has demonstrated that LTP's are, in fact, potent allergens (hazelnut, Cor a 8; chestnut, Cas s 8; walnut, Jug n 3) [26-28]. Their highly conserved structural and sequence similarity, is believed to be responsible for the cross-reactivity found between members of different species. Consequently, LTPs have been classified as pan-allergens [27;29].

The 7S globulins (vicilins)

The 7S globulins or vicilin like proteins are members of the cupin superfamily which are composed of a 6 stranded β–barrel conformation [14]. They are trimeric proteins that are 150 to 190 kDa in weight and composed of ~50 kDa subunits. In the plant 7S globulins function as seed storage proteins, where they serve as amino acid reserves for the developing seed and act as a sink for surplus nitrogen [30;31]. Seed storage proteins are abundant in the plant and it is estimated that they can account for ~50% of the total protein [16]. To date, several 7S globulins have been identified as important allergens in both peanuts and tree nuts, including peanut (Ara h 1), cashew (Ana o 1), walnut (Jug r 2), and hazelnut (Cor a 11) [32-35] [36].

Cross-reactivity among tree nuts

Several studies have observed that individuals, who are allergic to one tree nut, often show IgE reactivity to others [12;37;38]. One particular study done by Rance *et al* found that of 42 cashew allergic individuals, 29 had positive skin pricks tests to other nuts; with 67% showing reactivity to pistachio, 23.8% to almond, 14.3% to hazelnut, 9.5% to walnut, and 4.8% to pecan [39]. This reactivity could be a result of multiple sensitization events leading to the generation of specific IgE to each nut, or to IgE cross-reactivity between nuts [15;40]. Cross-reactivity between two tree nuts is a result of immunological recognition where the antibody, IgE, is unable to differentiate between the two allergens [40]. The majority of plant food allergens that have been identified can be grouped into a few protein families. The seed storage proteins; 11S legumins, 7S vicilins, 2S albumins, represent a major component of the total nut protein, whereas profilins, and the pathogenesis-related proteins are found in lower quantities. The highly conserved structural and sequence similarity between allergens from the same protein family is believed to contribute to the molecular basis of cross-reactivity between plant foods [41;42].

Aims of the thesis work

In this study we aim to identify allergenic proteins from both pistachio (*Pistacia vera*) and almond (*Prunus dulcis*) that are directly involved in IgE mediated hypersensitivity, as well as perform

biochemical and immunological studies to analyze cross-reactivity between homologous allergens identified in pistachio and cashew nut. Food allergies affect many individuals in industrialized countries, with tree nut allergies causing severe and even life threatening reactions. The use of tree nuts in the food industry is an important concern for tree nut allergic individuals, as contamination between food products can be fatal. Therefore, it is critical to not only identify tree nut allergens that are directly involved in the allergic response but also to study these proteins on both the biochemical and immunological level. These findings may directly contribute to advancements in diagnosis and the future treatments of tree nut allergies.

CHAPTER # 2

PISTACHIO VICILIN, PIS V 3, IS IGE-REACTIVE AND CROSS-REACTS WITH THE HOMOLOGOUS CASHEW ALLERGEN, ANA O 1

Introduction

Allergic reactions to tree nuts are common and it is estimated that 0.5% of the US population is allergic to one or more tree nuts [12]. Recently, a case matched comparison of cashew- and peanut-allergic children found that cashew nut caused more severe reactions than peanut in a UK population [43]. The consumption of tree nuts is steadily increasing due to the general perception of their health benefits [15]. According to the USDA (http://www.ers.usda.gov/publications/FTS/2006/Yearbook/FTS2006.pdf) in 2005, the per capita tree nut consumption was 2.7 pounds, with cashew nut being the most commonly consumed imported nut [44]. The consumption of pistachio has been steadily rising over the last few years, which can be attributed to their incorporation into baked goods, ice cream, candies, and other food dishes. In 2003, a random digit dialed phone survey revealed that, of 82 tree nut-allergic individuals, 44% reported allergy to cashew and 22% to pistachio [12].

To date, three major cashew allergens, Ana o 1 (7S vicillin), Ana o 2 (11S globulin), and Ana o 3 (2S albumin), have been identified, all of which are characterized as seed storage proteins [33;45;46]. Ana o 1, a 7S vicilin, is a homotrimer of 45 kDa subunits, recognized by 10 of the 20 cashew-allergic patients' sera and identified as a major allergen (i.e., ≥50% reactive) [33]. Epitope mapping performed using synthetic overlapping peptides on this allergen identified eleven epitopes that bind IgE from cashew allergic individuals [33].

Vicilins are typically homotrimeric proteins with a molecular mass of 150 to 190 kDa, composed of protomers of 40 to 80 kDa [13]. Vicilins have previously been identified as allergens in tree nuts including cashew (Ana o 1), walnut (Jug r 2), and hazelnut (Cor a 11), and certain aspects of their cross-reactivity have been investigated [33-35;47;48]. An epitope map comparison revealed that Ana o 1 does not share common linear epitopes with the peanut vicilin, Ara h 1 [33;49]. Recombinant (r) walnut Jug r 2 was recognized by 9 of 15 walnut allergic patients, and was thus classified as a major walnut allergen. This allergen did not show any cross-reactivity to the homologous pea or peanut vicilins in IgE binding inhibition experiments [34]. The hazelnut 7S vicilin-like protein (rCor a 11) was recognized by 43% of tested hazelnut allergic patients. An analysis of the allergen demonstrated that IgE reactivity was not influenced by the presence or absence of glycans [35]. The authors noted a 67% and 44% amino acid (aa) sequence similarity, between two IgE binding epitopes identified on Ara h 1 and Cor a 11 respectively, suggesting the possibility of cross-reactivity, however, direct evidence of cross-reactivity was not presented [35]. In a comparative homology modeling study, Barre *et al*. identified structural features associated with the epitopes on the vicilin allergens in peanut, lentil, and pea [50]. Comparison of the epitopes identified on Ara h 1 with the corresponding amino acid sequence of pea (Pis s 1) and lentil (Len c 1) vicilins, revealed a high degree of sequence similarity and three-dimensional conformation, which could account for the observed cross-reactivity between legumes for some patients [50].

There have been several reports of cross-reactivity between pistachio and cashew nut proteins, which is not surprising since both are members of the *Anacardiaceae* family [39;51-54]. In one study, two pistachio-allergic individuals who had never eaten cashews exhibited IgE specific to both cashew and pistachio nuts using skin prick tests, immunoblotting, and radioallergosorbent tests (RAST) [51].

Western blot assays demonstrated IgE binding to pistachio proteins ranging from 14 to 70 kDa and cashew proteins from 20 to 67 kDa. In another study, ImmunoCAP-inhibition assays were used to demonstrate cross-reactivity between pistachio and cashew using three patients; one allergic to only pistachio and two allergic to pistachio but had never eaten cashew [52]. The results showed that pre-incubation of patients' sera with cashew extract could significantly inhibit IgE binding to pistachio nut on the solid phase [52]. IgE binding to 34, 41, 52, and 60 kDa bands in pistachio nut extract were detected by immunoblotting and, in agreement with Fernandez *et al*. [51], the 34 kDa band exhibited the strongest IgE binding signal [52]. Also, Goetz *et al*. [53] demonstrated cross-reactivity between cashew and pistachio proteins using rabbit anti-pistachio antisera in a double immunodiffusion assay.

In none of the studies described above were the specific proteins recognized by patient IgE identified or characterized beyond estimates of their molecular masses [51-53]. In this study we (1) report the identification and immunological characterization of a cloned pistachio allergen, a vicilin designated Pis v 3, and (2) show that the cashew and pistachio vicilin homologues are highly cross-reactive when assayed with serum IgE from allergic individuals and with mouse anti-cashew vicilin monoclonal antibodies (MAbs). These studies have been published in Clin Exper Allergy 2008 (Willison 2008) [55].

Methods

Human sera

Blood samples were drawn after informed consent from patients to cashew and pistachio nut. The study was approved by the human subjects review committee of the University of California at Davis (Davis, CA). Sera were frozen at -70°C until use. The presence of pistachio- and cashew-reactive IgE was confirmed by means of Pharmacia ImmunoCAP assay or Western immunoblotting, as described below. Clinical characteristics of the subjects are shown in Table 2.1. Control sera were obtained from patients with histories of pollinosis to weeds, trees, and/or grasses but who were not food-allergic.

Table 2.1. Clinical characteristics of pistachio and/or cashew-allergic subjects [55].

No	Sex/Age	Age on onset of pistachio/cashew allergy	Pistachio allergic	Cashew allergic	Other Atopy history	Food Allergy	[2]ImmunoCap, RAST, or positive IgE Immunoblot	Positive Dot-blot to Pis v 3/Ana o 1
1	M/25	3	Yes	Yes	Asthma	walnut, pecan, hazel	Pistachio 5.65 Cashew 6.95	No/No
3	F/26	2	Yes	Yes	AD, AR, Asthma	peanut, walnut	Pistachio = Class 5 Cashew 9.51	**Yes/Yes***
5	F/54	10	Yes	Yes	AR, Asthma	walnut, pecans, hazel	Pistachio 7.24 Cashew 1.62	No/No
7	F/30	10	NE[1]	Yes	AD, AR	Peanut, walnut, hazel	Pistachio 2.80 Cashew 4.04	No/No
9	F/35	2	NE	Yes	AD, AR, Asthma	Walnut, pecans, almond	Pistachio = Class 5 Cashew 35.1	**Yes/Yes**
11	M/50	1	Yes	Yes	AD, AR, Asthma	Multiple tree nuts	Pistachio 4.60 Cashew 5.19	No/No
12	F/26	3	NE	Yes	AR, Asthma	Multiple tree nuts	Pistachio 2.22 Cashew 2.41	No/No
13	F/39	1	Yes	NE	AD, AR, Asthma	Peanut, walnut, hazel, pine nut, brazil	Pistachio 12.5 Cashew 9.53	**Yes/Yes**

Table 2.1. Continued

No	Sex/Age	Age on onset of pistachio/cashew allergy	Pistachio allergic	Cashew allergic	Other Atopy history	Food Allergy	[2]ImmunoCap, RAST, or positive IgE Immunoblot	Positive Dot-blot to Pis v 3/Ana o 1
14	F/39	5	Yes	Yes	Asthma	Tree nuts	Pistachio 57.4 Cashew 94.7	**Yes/Yes**
20	F/48	1	NE	Yes	AD, AR, Asthma	Peanut, walnut, hazel	Pistachio 0.56 Cashew +blot	No/No
29	F/49	3	Yes	Yes	AD, AR, Asthma	Peanut, sesame, tree nuts	Pistachio 0.38 Cashew 0.52	No/No
30	F/53	15	Yes	Yes	AD, AR, Asthma	Tree nuts except almond	Cashew <0.35 +blot Pistachio +blot	No/No
32	M/38	1	Yes	Yes	AD, Asthma	Walnut, pecan, hazel	Pistachio 1.17 Cashew 1.64	No/No
33	F/63	53	NE	Yes	AD, AR, Asthma	Peanut, almond, fish,eggs	Pistachio 66.9 Cashew 81.3	**Yes/Yes**
35	F/54	2	Yes	Yes	AD, AR Asthma	Walnut, hazel, pecan, brazil	Pistachio = Class 2 Cashew 0.85	**Yes/Yes**
46	M/39	18	Yes	No	AR, Asthma	Sunflower seed, mango, fruit	Pistachio 2.29 Cashew <0.35	No/No
47	F/65	child	Yes	Yes	AR, Asthma	Banana, avocado, mango, melon	Pistachio 38.2 Cashew 52.9	No/**Yes**
48	M/59	4	Yes	Yes	AD, Asthma	Peanut, walnut, almond, pecan, hazel, brazil, pine	Pistachio 3.53 Cashew 7.82	**Yes/Yes**
49	M/35	6	Yes	Yes	AR, Asthma	Walnut, pecan, hazel, brazil	Cashew +blot Pistachio +blot	No/No

[1] NE= never eaten, AD = Atopic dermatitis; AR = allergic rhinitis
[2]ImmunoCAP results are shown as kU/1, RAST as class.
*Positive results indicated in bold

Cashew and pistachio protein extract

Cashew and pistachio protein extracts were obtained from defatted cashew or pistachio flour by extraction with buffered saline borate (BSB) pH 8.2 (0.1 M boric acid, 0.025 M sodium borate, 0.075 M sodium chloride) at room temperature (RT) for 1 h and stored at -20°C for later analysis as previously described [56]. Protein concentrations were measured using the Bradford protein assay (BioRad Laboratories, Inc, Hercules, CA).

cDNA production, PCR amplification, and DNA sequencing

Mature pistachio nuts were frozen in liquid nitrogen and ground with a mortar and pestle. Total RNA was extracted as described earlier [57] using TRIzol (Gibco BRL, New York, NY). mRNA was isolated using a PolyAtract mRNA Isolation Kit (Promega, Madison, WI) as described by the manufacturer. Both 5'- and 3'-RACE were used to generate pistachio cDNA as described in the SMART RACE cDNA Amplification Kit user manual (BD Biosciences Clonetech, Palo Alto, CA).

Degenerate primers were designed based upon conserved homologous sequences found in 7S globulins from cashew, hazelnut, sesame, soybean, and fava bean. The degenerate primer, 5'-

IGIKATYTTYGTTGCMIKCGAGTTGTA-3', and a universal primer based on the 5' linker sequence on the 5'-RACE cDNA were used. Sequencing of the PCR products lead to the identification of the pistachio 7S globulin. Gene specific primers (forward: 5'-TGCTCTAGAAAGACAGACCCAGAGC TGAAAC-3', reverse: 5'-AAACTGCAGTCATTCATCAGCACGCCCTTG-3') were then designed and used to amplify full length pistachio 7S globulin cDNA which was then TA cloned (TOPO TA Cloning Kit, Invitrogen, Carlsbad, CA) and sequenced on an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, Calif.).

Cloning, expression and purification of cDNA-encoded proteins

As described in detail (for cashew nut [33]), the pistachio cDNA coding sequences were ligated into a modified version of the maltose-binding protein (MBP) fusion expression vector pMAL-c2 (New England BioLabs Inc, Beverly, Mass). The modified vector, pMAL-c2-His, contained a 8-residue histidine tag downstream of the *mal*E gene and *Sac*I restriction site and the factor Xa cleavage site along with the corresponding *Xmn*I and *Eco*RI sites were replaced with a thrombin cleavage site. The use of modified pMal-c2-His ensured additional purification of the fusion protein (rPis v 3-MBP) by a nickel affinity column if needed. The cloning, expression, and purification of rAna o 1- and rPis v 3-MBP fusion proteins were carried out as previously described for rAna o 1[47]. Briefly, cDNA/pMAL-c2-His plasmids encoding rAna o 1 or rPis v 3 were used to transform competent *E. coli* BL21 (DE3) cells (Novagen Inc, Madison, WI). Bacterial colonies were grown at 37°C with shaking to an OD_{600nm} of 0.5, followed by incubation with 0.3 M isopropyl-D-thiogalactopyranoside (IPTG). The cells were harvested, resuspended in amylose resin buffer (20 mmol/L Tris-HCL, pH 7.4, 200 mmol/L β-mercaptoethanol, and 1 mmol/L EDTA), lysed with mild sonication, centrifuged at 10,000g, and the supernatant passed over amylose affinity column. The fusion protein (rAna o 1-MBP or rPis v 3-MBP) was eluted with column buffer containing 10 mmol/L maltose and stored at 4°C until use or for long term storage, frozen at -80°C.

Polyacrylamide gel electrophoresis and protein transfer

Recombinant proteins (0.5 μg per 4mm well width) or aqueous total cashew/pistachio extracts (12 to 14 μg per 4mm well width) were subjected to SDS-PAGE (12%). Samples were boiled in reducing sample buffer containing β-mercaptoethanol then subjected to electrophoresis and either stained with Coomassie Brilliant Blue R (Sigma-Aldrich, St. Louis, MO) or transferred to nitrocellulose (NC) membranes as previously described [58].

Dot-blot analysis and inhibition

Recombinant Pis v 3 and rAna o 1 were applied to NC membranes using a 96-well Bio-Dot Microfiltration Apparatus (BioRad Laboratories) as previously described [59]. Briefly, recombinant proteins (0.5 μg per 2mm dot) were applied to NC and strips containing dotted rPis v 3 and rAna o 1 were excised and probed as described below. For inhibition dot-blots, rPis v 3 and rAna o 1 were used as inhibitors at 100 μg/mL (100 to 200 μl total volume) and pre-incubated with patients' sera at 1:50 dilution (8 μl in 400 μl total volume) overnight (o/n) at 4°C prior to incubation with the dotted protein.

IgE immunoblotting and inhibition

NC strips (4 mm wide) from gel transfers containing 12 to 14 μg of nut protein extract or 0.5 μg of recombinant/native Pis v 3 protein per strip were blocked o/n at 4°C using phosphate buffered saline - Tween® 20 (PBS-T)/5% (v/w) nonfat dry milk. Dotted protein strips were similarly blocked and NC strips/dots were incubated with sera diluted 1:5 v/v or 1:50 v/v (for highly reactive sera) o/n at 4°C. The

probed strips/dots were then washed for 90 min in (PBS-T) at RT with the PBS-T, changed 3X, before being incubated o/n at 4°C with ^{125}I-labeled anti-human IgE (Specific IgE Tracer, Hycor Biomedical Inc, Garden Grove, CA) diluted 1:10 in nonfat milk buffer. Membranes were washed again as above and exposed to X-ray film (Kodak X-OMAT, Kodak Molecular Imaging, New Haven, CT).

For inhibition immunoblots and dot-blots, human sera at 1:5 or 1:50 dilution (80 or 8 µl in 400 µl total volume) were pre-incubated with 100 µg/mL (100 to 200 µl total volume) of rAna o 1/rPis v 3 (both with associated MBP) or 7 µg (1.2 µl total volume) of MBP inhibitor o/n at 4°C or at 37°C for 1 h and used as described above. Controls included strips/dots exposed to IgE without inhibitor and strips/dots exposed to serum from an atopic individual without a history of tree nut allergies.

Monoclonal anti-cashew antibodies

MAbs against rAna o 1 were raised in the Hybridoma Facility at Florida State University (FSU) using standard techniques [60]. The guidelines for animal care and welfare described in the 'Guide for the Care and Use of Laboratory Animals' prepared by the Institute of Laboratory Animals Resources (National Research Council, National Academy Press, revised 1996) were followed. Briefly, mice were immunized with 40 µg of rAna o 1 in RIBI adjuvant (RIBI ImmunoChem Research, Inc., Hamilton, MT), boosted with 20 µg of rAna o 1 in RIBI adjuvant at 3-week intervals, and were given a final injection of 25 µg of rAna o 1 in saline equally split between the intravenous and subcutaneous routes. The resulting hybridomas were screened and assayed for relative strength and specificity by direct-binding ELISA [61].

MAb immuno-dot-blotting

Dot-containing NC strips were prepared as described above and probed with the rAna o 1-specific MAbs at 1:400 or 1:500 dilutions in TBS-T at RT for 1 h. Dots were washed 3X with Tris buffered saline -Tween® 20 (TBS-T) for 20 min each wash and were then incubated with horse radish peroxidase (HRP) labeled goat anti-mouse reagent (Jackson Immunoresearch Laboratories Inc, West Grove, PA) at a 1:3000 v/v dilution in TBS-T for 1 h at RT, and washed as above. Amersham ECL (GE Healthcare, Piscataway, NJ) was used to detect reactivity upon exposure of dot strips to Kodak XAR film (Kodak Molecular Imaging, New Haven, CT, USA).

Results

Gene characterization

The 7S globulin gene was amplified from the pistachio 5` RACE cDNA by means of PCR with a degenerate forward and universal lock dock reverse primer. Subsequently, gene specific primers were used to clone the full length gene. The resulting 1560-bp PCR product (GenBank ID EF116865) encodes a 519-amino acid (aa) protein designated Pis v 3 according to the guidelines of the IUIS Allergen Nomenclature Subcommittee (Fig 2.1). The SignalP program (www.expasy.org, Swiss Institute of Bioinformatics, 4056 Basel, Switzerland) was used to identify a 26 aa presumptive signal sequence (in red, Fig 2.1).

(A)

```
   1  atgggttcgc gaacaaagtt ttgtttaact ctttttctcg tttctgtttt gattctgtgt
  61  gccggtttag ctttggctaa gacagaccca gagctgaaac aatgcaagca ccagtgcaaa
 121  gtccagaggc agtatgacga ggaacagaag gagcagtgtg cgaaaggatg tgaaaagtac
 181  tacaaagaga agaaaggacg cgagcaagag gaagaggaag aagaggaatg gggaagcggt
 241  cgcggtcggg gtgatgaatt cagcacgcat gaacccggtg aaaagcgttt gagccagtgc
 301  atgaagcagt gcgagagaca agacggaggg cagcagaagc agctgtgccg cttcaggtgt
 361  caggagaagt ataagaaaga gagaagagaa catagttaca gtagagacga agaagaggaa
 421  gaggaaggcg atgaggaaca agaggaagaa gatgagaatc cttacgtatt tgaagacgaa
 481  catttcacca ccagagtcaa gaccgaacaa ggaaaagttg ttgttcttcc caagttcact
 541  aaacgatcaa agcttctccg tggcctggag aaataccgtc tggcctttct tgtcgctaat
 601  cctcaagctt ttgtagttcc aaaccacatg gatgctgaca gtattttctt tgtttcctgg
 661  ggacgaggaa caatcaccaa gattcgtgag aataagagag agagcatgaa cgtcaaacag
 721  ggagatataa ttaggattcg tgctggtact ccttttttata tcgtcaatac cgatgaaaat
 781  gagaagcttt acattgtcaa actccttcaa cccgtcaatc ttcctggcca ttacgaagta
 841  tttcatggac caggaggtga aaacccagag tcgttctaca gagctttcag cagggaagta
 901  ctcgaagccg ctctgaagac tccaagggac aaactggaga aattgttcga gaaacaggac
 961  gagggagcca tcgtaaaagc ctccaaagaa caaattcggg ctatgagccg gaggggtgaa
1021 ggtcctagca tttggccatt tacagggaaa tcaacgggta cattcaatct cttcaaaaag
1081 gatccctctc aatccaataa ctatggccaa ctctttgaaa gcgaattcaa agattatccg
1141 ccactccaag agctcgacat tatggtctct tatgtcaaca tcaccaaggg aggaatgtca
1201 ggtccattct acaactcaag ggcaacgaag atagccattg ttgtttcagg agagggacgc
1261 cttgaaatag cctgccctca cctctcctct tccaaaaact caggccagga aaaaagtggc
1321 ccgagttaca agaaattaag ctcgagtatc agaaccgatt cagtgttcgt tgtcccggcg
1381 ggtcaccctt ttgtcaccgt tgcttctgga aaccaaaact tggaaatcct ctgttttgaa
1441 gttaatgcag aaggaaatat caggtatact cttgctggga agaagaacat tatagaggtg
1501 atggagaagg aagcgaaaga attggcattt aaaacgaaag gagaggaggt ggacaaagtg
1561 tttggaaaac aagatgaaga gttcttcttc caggggccga aatggcgaca acatcaacaa
1621 gggcgtgctg atgaatga
```

(B)

```
   1  MGSRTKFCLTLFLVSVLILCAGLALAKTDPELKQCKHQCKVQRQYDEEQKEQCAKGCEKY
  61  YKEKKGREQEEEEEEEWGSGRGRGDEFSTHEPGEKRLSQCMKQCERQDGGQQKQLCRFRC
 121  QEKYKKERREHSYSRDEEEEEEGDEEQEEEDENPYVFEDEHFTTRVKTEQGKVVVLPKFT
 181  KRSKLLRGLEKYRLAFLVANPQAFVVPNHMDADSIFFVSWGRGTITKIRENKRESMNVKQ
 241  GDIIRIRAGTPFYIVNTDENEKLYIVKLLQPVNLPGHYEVFHGPGGENPESFYRAFSREV
 301  LEAALKTPRDKLEKLFEKQDEGAIVKASKEQIRAMSRRGEGPSIWPFTGKSTGTFNLFKK
 361  DPSQSNNYGQLFESEFKDYPPLQELDIMVSYVNITKGGMSGPFYNSRATKIAIVVSGEGR
 421  LEIACPHLSSSKNSGQEKSGPSYKKLSSSIRTDSVFVVPAGHPFVTVASGNQNLEILCFE
 481  VNAEGNIRYTLAGKKNIIEVMEKEAKELAFKTKGEEVDKVFGKQDEEFFFQGPKWRQHQQ
 541  GRADE
```

Figure 2.1. Nucleotide and derived amino acid sequence of Pis v 3 cDNA [55]. (A) Nucleotide sequence (GenBank accession no. EF116865) and (B) amino acid sequence of the Pis v 3 coding region. The predicted signal peptide is indicated in red.

Protein sequence homology characterization

Comparison of the aa sequence with the NCBI database using BLAST analysis identified homology with other members of the 7S globulin family of seed storage proteins, several of which are known food allergens (Table 2.2). In line with the familial relationship between pistachio and cashew, their respective vicilins are 90% aa sequence similar and 80% identical. In contrast, the aa sequence comparisons with the 10 nut and seed proteins listed in Table 2.2 revealed only 51-72% similarity and 31-55% identity to pistachio vicilin.

Table 2.2. Sequences demonstrating the greatest homology to Pis v 3 [55].

Protein Description	Organism	Accession No.	% Identity	% Similarity	Ref.	Allergen Designation
Vicilin-like protein	*Anacardium occidentale* (cashew)	AAM73730	80	90	[33]	Ana o 1
48-kDa glycoprotein precursor	*Corylus avellana* (hazelnut)	AAL86739	55	72	[35]	Cor a 11
7S globulin	*Sesamum indicum* (sesame seed)	AAK15089	47	65	[62]	Ses i 3
Sucrose binding protein homolog S-64	*Glycine max* (soy bean)	AAF05723	46	65	[63]	
Sucrose-binding protein 2	*Glycine max* (soy bean)	AAO48716	46	65	[64]	
7S globulin	*Elaeis guineensis* (African oil palm)	AAK28402	41	60	[65]	
Vicilin-like protein precursor	*Juglans regia* (English walnut)	AAF18269	41	65	[34]	Jug r 2
Vicilin seed storage protein	*Juglans nigra* (black walnut)	AAM54366	40	63	[66]unpublished	Jug n 2
Vicilin precursor	*Macadamia integrifolia* (smooth shelled macadamia)	AAD54244	39	60	[67]	
Vicilin	*Pisum sativum* (pea)	CAF25232	35	52	[68]	Pis s 1
Convivilin	*Pisum sativum* (pea)	CAB82855	35	52	[68]	Pis s 2
Allergen Len c 1.0102	*Lens culinaris* (lentil)	CAD87731	34	51	[69]	Len c 1
allergen Len c 1.0101	*Lens culinaris* (lentil)	CAD87730	33	51	[69]	Len c 1
Vicilin-like protein	*Lupinus albus* (white lupine)	CAI84850	32	53	[70]	
7S seed storage protein (vicilin)	*Arachis hypogaea* (peanut)	AAL27476	31	51	[41]	Ara h 1

Protein sequence characterization

The entire Pis v 3 cDNA, beginning at K27 following the presumptive signal peptide was cloned. The DNA segments were ligated into an expression vector designed to yield a MBP fusion protein. The resulting ~102 kDa Pis v 3-MBP fusion protein was affinity purified with the aid of an amylose affinity column, as previously described [33].

Comparison of the Pis v 3 protein sequence to that of Ana o 1 and its IgE-reactive peptides.

A sequence alignment of the pistachio vicilin, Pis v 3, and the cashew homologue, Ana o 1, was used to evaluate their structural similarity and compare the aa sequence of the 10 known cashew IgE binding peptides [33] with the corresponding aa sequence on pistachio (Fig 2.2). As described above, the comparison reveals 80% overall aa identity and 90% similarity. Of the two peptide segments previously shown to contain immunodominant epitopes in rAna o 1, peptide #3 had 13 of 15 identical residues and one similar residue, and peptide #10 had 8 of 15 identical residues and 4 similar residues. All of the variant amino acids were clustered at the C-terminal end of peptide #10 leaving the N-terminal end, which is 100% identical, as a potential source of cross-reactivity. Comparisons of the sequences for the other epitope sites show similar degrees of homology with the exception of peptide #9 where minimal homology is evident. The high degree of sequence homology between the two allergens suggests the likelihood of considerable cross-reactivity and prompted additional studies.

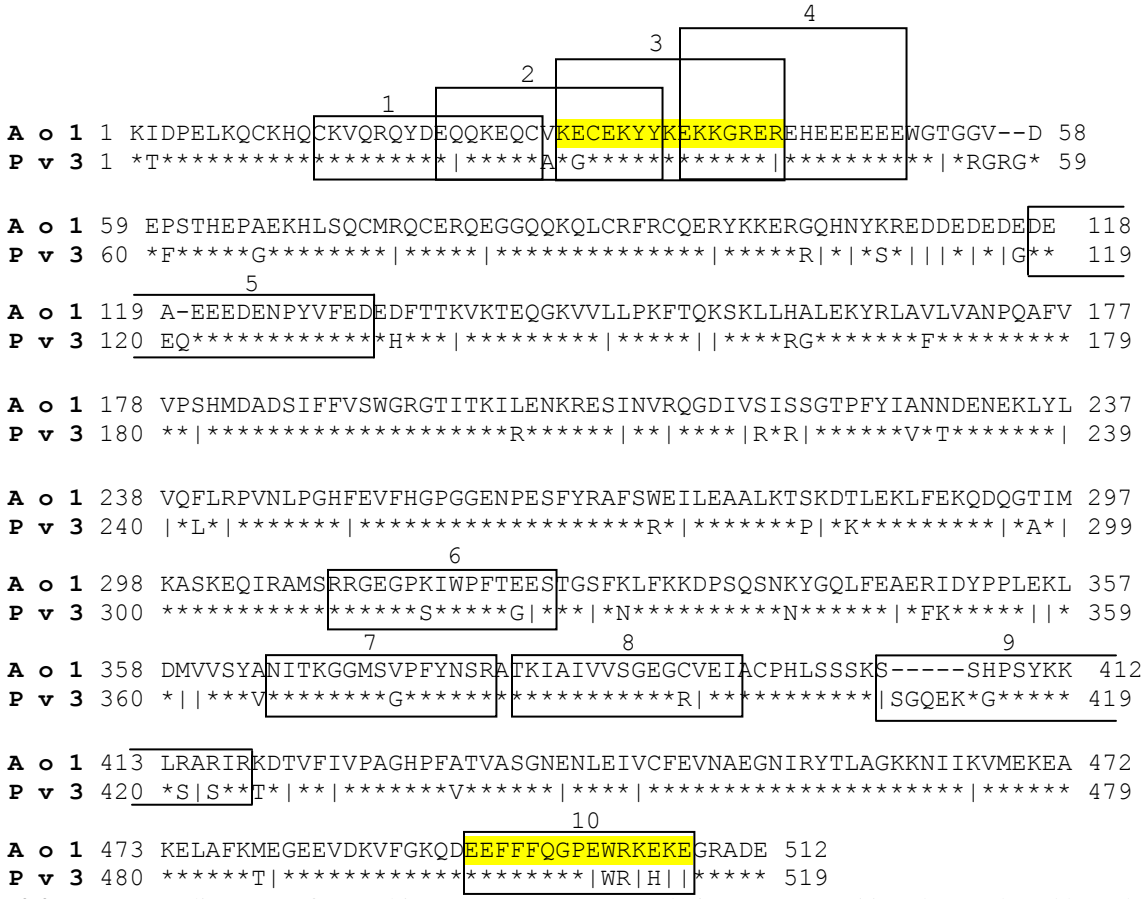

Figure 2.2. Sequence alignment of recombinant Ana o 1 (A o 1) and Pis v 3 (P v 3) [55]. The numbered boxed sequences indicate linear epitope-bearing peptides previously identified on Ana o 1 [33]. Peptides #3 and #10 (highlighted in yellow) contain Ana o 1 immunodominant epitopes. "*" = identical amino acid, "|" = similar amino acid.

Reactivity of recombinant proteins with human IgE

Reactivity to rPis v 3 and rAna o 1 was screened using 19 patients' sera: 12 cashew- and pistachio-allergic, five cashew-allergic but who had never eaten pistachios, one pistachio-allergic but who had never eaten cashew, and one only pistachio-allergic. Of the 14 pistachio-allergic patients (#'s 1, 3, 5, 11, 13, 14, 29, 30, 32, 35, 46, 47, 48, 49), five (36%) showed IgE reactivity to rPis v 3 by dot-blot (Fig. 3A). Interestingly, of the five cashew-allergic patients (#'s 7, 9, 12, 20, 33) who report that they had never eaten pistachio, two (40%) showed IgE reactivity to rPis v 3 by dot-blot. Each of the seven rPis v 3-reactive sera (patient #'s 3, 9, 13, 14, 33, 35, 48) also recognized rAna o 1. Only one patient, number 47, was reactive to the cashew vicilin alone and not to the pistachio vicilin (Fig 2.3A). Pre-incubation of patient sera with MBP did not inhibit IgE binding to rPis v 3 demonstrating that no MBP specific antibodies were present in the patients' sera (Fig 2.3B). One patient, # 33, was not tested for MBP inhibition due to unavailability of serum. Similar results were obtained with MBP inhibition of Ana o 1 reactivity.

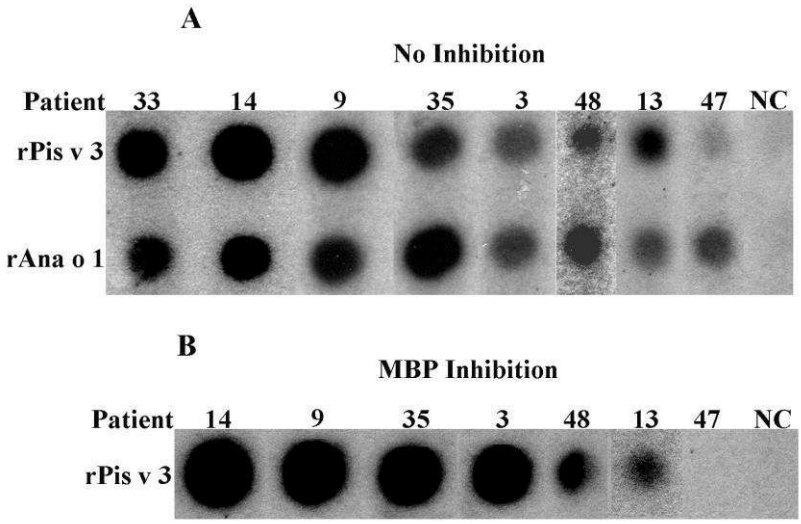

Figure 2.3. Dot blot and inhibition dot blot (A) Dot blot with cashew-allergic patients' sera showed IgE reactivity to both rAna o 1 and rPis v 3 (sera showing no signals not shown). (B) Inhibition dot blot of rPis v 3 probed with rPis v 3/rAna o 1 reactive sera pre-incubated with MBP [55]. NC = negative control.

The similarity in signal intensity between the two probed allergens for any given serum sample suggested the likelihood of considerable cross-reactivity. To investigate this potential cross-reactive relationship, SDS-PAGE of cashew and pistachio extract followed by Coomassie Brilliant Blue staining indicated the presence of similar molecular mass proteins in both nut extracts (Fig. 4A) as previously reported by Fernandez et al.[51]. This pattern is consistent with the close family relationship between the two nut trees. In order to identify the vicilin-like protein band(s) in pistachio nut extract, inhibition immunoblotting (Western Blotting), in which rPis v 3 or rAna o 1 served to inhibit the reaction between a pool of patient sera (# 3, 9, 14) and pistachio extract was preformed. Pre-incubation of sera with either inhibitor shows inhibition of IgE binding to a 45 kDa band in the nut extract indicating that this band represents the native vicilin like protein, Pis v 3 (Fig 2.4B). Additionally, a 15 kDa band shows considerable inhibition and could potentially be a vicilin fragment (Fig 2.4B). This interpretation is

supported by the identification of IgE-reactive vicilin fragments in both pea (16 and 13 kDa) and lentil (26 and 16 kDa) by N-terminal sequencing [67;68].

It is possible that the signal intensity of IgE binding to other proteins present in whole nut extract (for example a legumin like protein) may overshadow IgE inhibition to any other vicilin like fragments present in the whole extract. To investigate this possibility, an inhibition immunoblot was preformed using rPis v 3 or rAna o 1 to inhibit the reaction between patients' sera and pistachio extract. rAna o 2 (cashew legumin) was used as a negative control. Patient #3 was chosen for inhibition immunoblotting because previous studies demonstrated strong reactivity to two protein bands, 47 and 38 kDa in pistachio nut extract by this serum. The results show complete inhibition of IgE binding by both inhibitors (Fig 2.4C). Inhibition of IgE binding to the 47 kDa band in the nut extract indicates that this band represents the native vicilin like protein, Pis v 3 (Fig 2.4C). Inhibition of IgE binding to the 38kDa band in the nut extract suggests that this band is potentially a vicilin like fragment similar to the 36 and 32 kDa vicilin like fragments previously identified in pea [67]. Similar results were obtained using patient #35, 9, and 14 in inhibition dot-blots (Fig 2.4D).

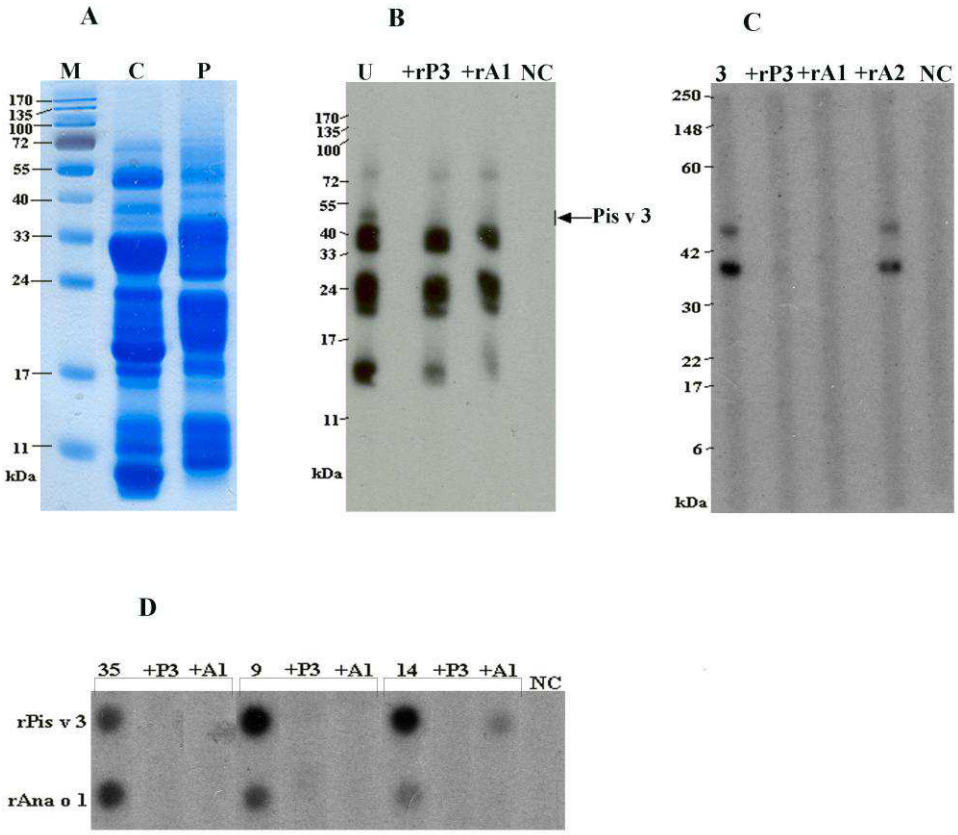

Figure 2.4. Coomassie Blue Stain and inhibition blots [55] (A) SDS-PAGE and Coomassie Blue stain of cashew (C) and pistachio (P) protein extract. M= molecular weight marker. (B) Inhibition immunoblot of pistachio protein extract probed with a pool of patient sera (# 3, 9, 14) either unabsorbed (U), pre-absorbed with rPis v 3 (+rP3), or pre-absorbed with rAna o 1 (+rA1). NC = negative control. Putative Pis v 3 band indicated by arrow. (C) Inhibition immunoblot of pistachio extract probed with serum from patient #3 either unabsorbed (3) or pre-incubated with recombinant allergen inhibitors, rPis 3 (+rP3), rAna o 1 (+rA1) [33], or rAna o 2 (+rA2) [45], NC = atopic serum negative control. (D) Inhibition dot-blot with recombinant allergens in which patients' sera #35 (35), #9 (9), and #14 (14) were pre-incubated with the indicated recombinant allergens [55].

Reactivity of cashew MAbs with recombinant proteins

The above described sequence alignment and specific IgE binding data revealed a high degree of homology between the cashew and pistachio vicilin. To further assess the nature of the cross-reactivity between cashew and pistachio vicilin, a panel of murine IgG MAbs, previously generated against cashew rAna o 1, was assayed. Of the nine MAbs tested, six (67%) also recognized rPis v 3 to varying degrees on dot-blots (Fig 2.5) indicating considerable epitope homology between rPis v 3 and rAna o 1.

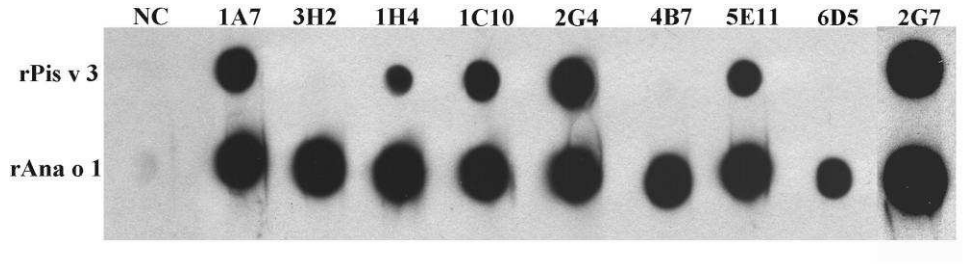

Figure 2.5. Dot-blot containing rPis v 3 and rAna o 1 probed with cashew MAbs raised against rAna o 1 [55]. The negative control (NC) contained no primary antibody.

Discussion

In recent years a number of allergens have been identified in a variety of foods and plants. Not only has this information defined the proteins that are directly responsible for food allergies but it has also revealed structural relationships between allergens including, in some cases, the structural basis for allergen cross-reactivity [13;16;40-42;71].

It is not uncommon for food-allergic individuals to react to more than one allergen, a pattern which can be a result of several independent sensitization events. Another potential factor contributing to complex allergen sensitivity patterns is allergen cross-reactivity [40]. Cross-reactivity between allergens occurs when IgE originally raised against one allergen recognizes and binds to a structurally similar protein from a different source even in the absence of prior exposure to the cross-reacting agent [40;41;72;73]. Such situations may or may not be apparent through examination of clinical histories. The term "co-recognition", which includes cross-reactivity, has been used to define an alternative situation wherein possible co-exposure to two or more agents that contain homologous (and likely cross-reactive) molecules masks the identity of the primary sensitizing agent [42]. From an immunological perspective, the degree of epitope sharing may be sufficiently greater in co-recognition such that the cross-reactivity may be described as symmetric. In such cases the epitope reactivity profiles induced by sensitization to any one of the two or more cross-reactive allergens are essentially equivalent [72]. This situation contrasts with the more typical asymmetric cross-reactivity which, when assayed *in vitro*, shows that one allergen inhibits IgE binding to a second allergen better than the second allergen inhibits binding to the first [72].

Patients allergic to cashew often report allergy to pistachio as well, which is likely a result of cross-reactivity between the two closely related tree nuts [39;51-54]. In this study the pistachio 7S vicilin-like protein was identified as an allergen (37% of patients' serum were reactive) and designated Pis v 3. A sequence alignment of Pis v 3 with the vicilin-like allergen, Ana o 1, from the closely related

cashew revealed a high degree of homology (80% identity and 90% similarity) between the two proteins. This finding, coupled with considerable similarity between the two nut aa sequences in the regions corresponding to the previously identified linear epitopes of cashew vicilin [33], is a strong predictor of cross-reactivity. The results obtained from the IgE binding studies provide further support for this supposition since all but one of the tested pistachio and/or cashew allergic patients' sera that recognized the vicilin from one nut, also reacted with the other in an IgE dot-blot assay. Included in this population are patients that report previous exposure to only one of the two nuts. Inhibition dot- and Western immunoblots wherein IgE binding to either allergen could be completely prevented by pre-incubation of sera with either allergen demonstrates not only cross-reactivity but that the cross-reactivity is symmetric in these patients, at least with respect to this IgE-binding protein. Cross-reactivity is not limited to patient IgE as six of nine randomly selected murine IgG anti-rAna o 1 MAbs also bound to Pis v 3. Together, these data suggest that antibody recognition of these proteins, whether by patient IgE in a natural allergenic situation or in an artificial murine immunization/hybridoma situation, is focused on the most conserved regions of the proteins.

If allergy to pistachio follows a pattern similar to that for cashew, the recognition of several different allergenic proteins by patient IgE can be expected. To date three major cashew allergens, a 7S vicilin (Ana o 1), an 11S legumin (Ana o 2), and 2S albumin (Ana o 3), have been identified which are characterized as seed storage proteins [33;45;46]. For each, their recombinant molecules have been cloned, sequenced, expressed and their linear epitopes mapped. Several studies have demonstrated that multiple proteins in pistachio extract exhibit IgE binding [51;52]. On the basis of these previously published immunoblots and comparison to similar blots for cashew extract [51;52], it could be surmised that the 45 kDa pistachio vicilin-like protein, identified as Pis v 3, and a 33 kDa legumin-like protein, are important allergens and that other allergenic pistachio proteins remain to be characterized.

CHAPTER # 3

IDENTIFICATION AND IMMUNLOGICAL ANALYSIS OF ALMOND (*PRUNUS DULCIS*) LIPID TRANSFER PROTEIN

Introduction

Tree nuts are known to cause food allergic reactions. Unlike other food allergies, tree nut allergy persists throughout life and cause allergic reactions that range from mild urticaria to life-threatening anaphylaxis [74]. Almond (*Prunus dulcis*) is a commonly consumed tree nut that is important both commercially and economically in the US. According to the USDA in 2007/08, the United States produced 65% of the commercial almond crop worldwide, with the state of California being the largest tree nut producer [75]. To date several allergens have been identified in almond including; profilin- Pru du 4, and the seed storage protein- almond major protein (AMP) [59;76].

Almond is a member of the *Rosaceae* family, which includes apple, peach, cherry, strawberry, and apricot. Fruits from *Rosaceae* commonly trigger IgE mediated food allergy, and one particular protein, lipid transfer protein, has been identified as an important allergen in these fruits [24;25;77]. Plant lipid transfer proteins (LTP) are small proteins with a typical molecular mass of approximately 9 kDa. They contain eight conserved cysteine residues that form four disulfide bridges that are responsible for the compact folding found in LTP [18]. The generation of 3-D models has determined that the compact structure of plant LTPs is is comprised of 4 α-helixes, 3 interchain loops, and a long unstructured C-terminal coil [18-21]. In the plant, LTP is typically localized in the seeds, flowers, and/or fruits and several studies have noted their abundance in the peel [22;23].

The role of LTP in IgE mediated food allergy was first discovered in peach. Pasterollo *et al.* determined that Mediterranean patients with and without pollinosis displayed different patterns of IgE reactivity to proteins in peach extract using Western blotting assays [77]. The results showed that patients without associated pollinosis predominantly recognized a single low molecular weight (LMW) protein in peach extract (> 13 kDa), whereas those with pollinosis showed specific IgE to 13, 17 and 20 kDa bands [77]. In further studies, the native protein from peach extract was purified and trypsin digestion followed by amino acid sequencing was used to identify the LMW protein as LTP. Subsequently the allergen was named Pru p 3 [24]. Since then, LTP has been identified as an major allergen in other fruits of the *Rosaceae* family such as apple, apricot, plum, and cherry, as well non *Rosaceae* fruits and vegetables including, grape, orange, lemon, tomato, lettuce, asparagus, and maize [13;25;78].

Recently, LTP has also been identified in tree nuts including walnut, chestnut, and hazelnut [26-28]. To evaluate the role of LTP in hazelnut allergy, Schocker *et al.* recruited 26 Spanish patients allergic to hazelnut without associated birch pollen allergy. Western blotting revealed that 62% recognized the native protein in hazelnut extract and 77% of tested patients showed specific IgE to recombinant protein [79]. Similarly, Sanchez-Monge *et al.* identified chestnut LTP as an important allergen in chestnut allergic individuals without associated latex allergy, through ELISA and skin prick testing [80]. Walnut LTP was identified as a major allergen in an Italian patient population and inhibition Western blotting assays demonstrated cross-reactivity between walnut and peach LTP [28]. Overall these studies demonstrated that LTPs are, in fact, potent allergens in tree nuts (hazelnut, Cor a 8; chestnut, Cas s 8; walnut, Jug n 3) [26-28].

In none of the studies described above has the role of LTP in almond allergy been evaluated. To date, LTP has been identified as major allergens in fruits and several tree nuts; therefore, it is likely that LTP is present and allergenic in almond. In this study we aim to report the identification and immunological characterization of LTP as an allergen in almond.

Methods

Human sera

Blood samples were drawn after informed consent from patients allergic to almond by our collaborator Dr Suzanne Teuber at University of California, Davis, in the Department of Internal Medicine, School of Medicine or purchased from PlasmaLab International (Everett, WA, USA). The study was approved by the human subjects review committee of Florida State University and the University of California at Davis (Davis, CA). Sera were frozen at -70°C until use. The presence of almond-reactive IgE was confirmed by means of Pharmacia ImmunoCAP assay (Pharmacia Diagnostics, Uppsala, Sweden) or by Western immunoblotting as described below. Clinical characteristics of the subjects are shown in Table 3.1. Control sera were obtained from patients with histories of pollinosis to weeds, trees, and/or grasses but who were not food-allergic.

Table 3.1. Clinical characteristics of almond-allergic subjects.

Serum No	Sex/Age	Age of onset	Other Atopy	Other food allergy	[1]ImmunoCap, RAST, or positive IgE Immunoblot	Positive dot blot to Pru du 3
7	F/25	child	AD, AR	Peanuts, walnut, other tree nuts	Class 2	No
9	F/34	1	AD,AR, As	Walnut, cashew, pecan	Class 3	No
11	M/50	1	AD, AR, As	Walnut, other tree nuts, mustard	0.64	No
12	F/26	3	AR, As	Pistachio, cashew, hazelnut	<0.35	No
13	F39	1	AD as child, AR, As	Peas, walnut, pistachio, peanut, Brazil nut	<0.35	No
14	F/38	5	As a child	Cashew, pistachio, brazil nut, walnut	1.9	No
18	F/62	1	AR	Peanut, walnut, cashew	1.15	No
20	F/48	4	AD, AR, As	Peanut, walnut, pecan, cashew, hazelnut	<0.35	No
34	M/33	child	AR	Multiple fresh fruits, cooked are ok	<0.35	No
38	F/50	2	AD, AR, As	walnut	0.5	No
39	F/32	1	AD, As	Peanut, tree nuts, raw carrots, avacodo, and bananas	0.49	**YES**

Table 3.1. Continued

Serum No	Sex/Age	Age of onset	Other Atopy	Other food allergy	[1]ImmunoCap, RAST, or positive IgE Immunoblot	Positive dot blot to Pru du 3
43	F/48	30	AR	Raw walnuts, pecans, pistachios, cherries, peaches, apricot, avocado	<0.35	No
44	M/30	child	AD as child, AR, As	Never eaten other nuts	2.88	No
48	M/59	child	AD, As	Peanut, walnut, pecan, hazelnut, brazil nut	+blot	**YES**
50	M/43	2	AD, AR, As	Walnut	+blot	**YES**
51 *	M/33		OAS, D	Chestnut, hazelnut, peach, mustard	0.92	**YES**
52 *	M/32		U, AE	Walnut, chestnut, peach	+blot	**YES**
53	F/40	teens	U	None	<0.35	No
54	F/22	child	U	Walnuts, pecan, pine nut, cocnut, sunflower and pumpkin seed, raw carrots, cucumber, bananas, avocados, cantaloupe, watermelon, apple	+blot	No
55	F/7	3	AE, U	walnut	<0.35	No
56	M/25	child	U	Chestnut, Pecan, peanut, soybean, Brazil nut,	16.4	No
57	F/44	child	AD, U, AR, As	Egg, soybean, crab, shrimp, tomato, beef, pork, carrot, potato, coconut, apple, milk, peach	15.8	No
58	F/33	22	U	Peanut, hazelnut, Brazil nut, pecan, cashew, pistachio, walnut,	7.15	No
59	M/39	10	U, AE	Meat, egg, peanut, soybean, hazelnut, brazilnut, fish, shellfish, carrot, orange apple, corn, potato, coconut, rice	9.05	No
60	?/?	unknown	?	Peanut, pistachio, walnut	10.8	No

AD = atopic dermatitis; AR = allergic rhinitis, AE= angioedema, As= asthma, U= urticaria, D= dyspnea, OAS= oral allergy syndrome

[1]ImmunoCAP results are shown as kU/1, RAST as class.

* Indicates Spanish almond-allergic patients

Construction of almond cDNA library

Almond cDNA library construction was previously preformed by Dr. Fang Wang in the lab using mRNA derived from immature almond kernels as previously described in detail for cashew library generation [33;45]. Briefly, developing nuts were chopped, frozen in liquid nitrogen, and ground with a mortar and pestle. mRNA was isolated with a PolyATtract kit (Promega, Madison, WI). The construction of the cDNA library was performed with the Uni-Zap XR Gigapack Cloning Kit (Stratagene Inc, Ceder Creek, TX). The double-stranded cDNAs with *Eco*RI (using a 5′ end adapter) and *Xho*I (using a 3′ end PCR primer) cohesive ends were cloned into the lambda Uni-ZAP XR expression vector. The cDNA was ligated into *Escherichia coli* strain XL1-Blue and amplified to generate the library.

PCR amplification and DNA sequencing

The sequence for the lipid transfer protein from almond (*Prunus dulcis*) was available on the NCBI database, accession number CAA65477. From this sequence, gene specific primers (forward: 5'-AAAGGATCCGTTGGTGGTCCCAAGGC-3' and reverse: 5'-TGCCTGCAGTCACTTGATCGTTTTGCAGTC-3') were designed and used to amplify full length almond LTP cDNA which was then TA cloned (TOPO TA Cloning Kit, Invitrogen, Carlsbad, CA) and sequenced on an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Cloning, expression and purification of cDNA-encoded proteins

The almond cDNA coding sequences were ligated into the fusion expression vector pMAL-c4X that contains a factor Xa cleavage site (New England BioLabs Inc, Beverly, MA). The cloning, expression, and purification of almond LTP was carried out as described [33], cDNA/pMAL-c4X plasmids encoding rLTP were used to transform competent *E. coli* Rosetta gamiB(DE3)pLysS cells (Novagen Inc, Madison, WI). Bacterial colonies were grown at 37^{o}C with shaking to an OD_{600nm} of 0.5, followed by incubation with 0.3 M isopropyl-D-thiogalactopyranoside (IPTG). The cells were harvested, resuspended in amylose resin buffer (20 mmol/L Tris-HCL, pH 7.4, 200 mmol/L β-mercaptoethanol, and 1 mmol/L EDTA), lysed with mild sonication, centrifuged at 10,000g, and the supernatant passed over amylose affinity column. The fusion protein was eluted with column buffer containing 10 mmol/L maltose and subsequently cleaved with factor Xa at a 1% (w/w) ratio to fusion protein MBP-rLTP. The liberated recombinant protein was purified by means of size exclusion HPLC using a Superdex 75 column (Amersham Pharmacia), concentrated, and stored at 4^{o}C until use or frozen at –20^{o}C.

Almond and peach protein extract

Almond protein extracts were obtained from defatted almond flour by extraction with buffered saline borate (BSB) pH 8.2 (0.1 M boric acid, 0.025 M sodium borate, 0.075 M sodium chloride) at room temperature (RT) for 1 h and stored at -20°C for later analysis as previously described [56].

Ripe peaches were purchased from a local grocer. Peach extract was prepared using the previously described protocol [24]. Briefly, peach peel was diluted 5:1 (w/v) in 10 mmol/L PBS (pH 7), 2 mol/L EDTA, 10 mmol/L sodium diethyldithiocarbamate, 3 mmol/L sodium azide and 2% solid polyvinylpolypyrrolidone and homogenized. The slurry was centrifuged at 16,000 rpm for 30 minutes at 4°C and dialyzed against 10 mmol/L PBS with 3 mmol/L sodium azide for 48 hours using Spectrapor 6-8,000 MWCO dialysis tubing (Spectrum Laboratories Inc, Rancho Dominguez, CA). The extract was stored at 4^{o}C until use or frozen at –20^{o}C. Protein concentrations were measured using the Bradford protein assay (BioRad Laboratories, Inc, Hercules, CA).

Production of enriched almond extract by gel filtration

Almond extract was loaded onto a Sephacryl S200 HR column (2.6 × 72 cm) equilibrated with 0.02 M Tris-HCl (pH 8.1) containing 0.1 M NaCl. The column flow rate was maintained at 24 mL/h, and fractions were collected every 15 min. Protein elution was monitored by UV absorbance at 280 nm and by gel electrophoresis. Fractions containing LMW proteins were concentrated using Amicon Ultra 3,000 MWCO (Millipore Corp., Bedford, MA) and used in subsequent immunoblotting and ELISA assays.

Polyacrylamide gel electrophoresis and protein transfer

Recombinant protein (5 μg per 4mm well width), aqueous total almond extract or peach extract (12 to 14 μg per 4mm well width) was subjected to SDS-PAGE (4% stacking, 15% resolving). Samples were boiled in reducing sample buffer containing β-mercaptoethanol then subjected to electrophoresis and either stained with Coomassie Brilliant Blue R (Sigma-Aldrich, St. Louis, MO) or transferred to nitrocellulose (NC) membranes as previously described [58].

Dot-blot analysis

Recombinant LTP or MBP was applied to NC membranes using a 96-well Bio-Dot Microfiltration Apparatus (BioRad Laboratories). The recombinant protein (3 μg per 2mm dot) was applied to 0.2 μm NC and strips containing dotted LTP were excised and probed as described below.

IgE immuno-dot blotting

NC strips (4mm wide) containing 3 μg of recombinant LTP protein per dot were blocked o/n at 4°C using phosphate buffered saline -Tween® 20 (PBS-T)/5% (v/w) nonfat dry milk. Dots were incubated with sera diluted 1:3 (v/v) o/n at 4°C. The probed dots were then washed for 90 min in (PBS-T) at RT with the PBS-T, changed 3X, before being incubated o/n at 4°C with ^{125}I-labeled anti-human IgE (Specific IgE Tracer, Hycor Biomedical Inc, Garden Grove, CA) diluted 1:10 in nonfat milk buffer. Membranes were washed again as above and exposed to X-ray film (Kodak X-OMAT, Kodak Molecular Imaging, New Haven, CT).

IgE immunoblotting

NC strips (4 mm wide) from gel transfers containing 12 to 14 μg of almond nut protein extract were blocked o/n at 4°C using phosphate buffered saline -Tween® 20 (PBS-T)/5% (v/w) nonfat dry milk. Strips were incubated with almond-allergic sera diluted 1:3 or 1:5 (v/v) o/n at 4°C. The probed strips were then washed for 90 min in (PBS-T) at RT with the PBS-T, changed 3X, before being incubated o/n at 4°C with ^{125}I-labeled anti-human IgE (Specific IgE Tracer, Hycor Biomedical Inc) diluted 1:10 in nonfat milk buffer. Membranes were washed again as above and exposed to X-ray film (Kodak X-OMAT, Kodak Molecular Imaging).

Enzyme linked immunosorbant assay (ELISA) and inhibition

ELISAs were preformed as previously described [59]. Briefly, 96 well microtiter plates (Seracluster "U" Vinyl, no 2797, Costar, Cambridge, MA) were coated with 50 μl/well of protein solution (almond rLTP) at 40 μg/ml in coating buffer (0.1 M carbonate–bicarbonate buffer, pH 9.6). Sera from almond-allergic patients (diluted 1:3, 50 μL/well) was added and incubated for 3 h at 37°C. After washing, bound IgE was reacted with HRP-conjugated mouse anti-human IgE (Zymed Laboratories, Inc.) at a dilution of 1:1000 and incubated for 1 h at 37°C. IgE reactivity was detected by colorimetric reaction using o-phenylenediamine (OPD, Zymed Laboratories Inc.) and H_2O_2 as substrate.

Optical density (OD) was measured in a KC4 v2.5 ELISA reader (Bio-Tek Instruments Inc, Winooski, VT) at 495 nm.

For inhibition ELISA, human sera at 1:3 (v/v) dilution was pre-incubated with enriched almond extract as the inhibitor o/n at 4°C or at 37°C for 1 h and used as described above. Controls include wells exposed to IgE without inhibitor and wells exposed to serum from an atopic individual without a history of tree nut allergies.

Polyclonal rabbit anti-peach LTP immunoblotting and inhibition

NC strips (4 mm wide) from gel transfers containing 12 to 14 μg of peach protein extract were prepared as described above and probed with the LTP-specific polyclonal antibodies (generously provided by ALK-Abello, Madrid, Spain) at 1:3000 dilution in PBS-T at RT for 1 h. Strips were washed 3X with PBS-T for 20 min each wash and were then incubated with horse radish peroxidase (HRP) labeled goat anti-rabbit reagent (Promega, Madison, WI) at a 1:50000 v/v dilution in PBS-T for 1 h at RT, and washed as above. Amersham ECL Plus (GE Healthcare, Piscataway, NJ) was used to detect reactivity upon exposure of strips to Kodak XAR film (Kodak Molecular Imaging, New Haven, CT, USA).

For inhibition immunoblots, polyclonal antibody at 1:3,000 dilution (v/v) was pre-incubated with rLTP inhibitor at 37°C for 1 h and used as described above. Controls include strips exposed to polyclonal without inhibitor.

Results

Gene characterization

The almond LTP gene was amplified from the almond cDNA library by means of PCR using a gene specific forward and reverse primer. The resulting 306-bp PCR product encodes a 123-amino acid (aa) protein (Fig 3.1) and was submitted to GenBank (accession number FJ652103). The SignalP program (www.expasy.org, Swiss Institute of Bioinformatics, 4056 Basel, Switzerland) was used to identify a 21 amino acid (aa) presumptive signal sequence (in red, Fig 3.1).

(A)

```
  1 ATGGCTAGCT CTGGACAGCT CCTCAAGCTC GTTTGCCTTG TGGCGGTGAT GTGCTGCATG
 61 GCGGTTGGTG GTCCCAAGGC CATGGCAGCT GTGTCATGCG GCCAGGTGGT GAACAATCTG
121 ACCCCATGCA TAAACTACGT GGCAAACGGT GGGGCTTTGA ACCCTAGTTG CTGCACTGGG
181 GTCAGGTCTC TCTACAGCTT GGCTCAGACC ACAGCTGACC GCCAGAGCAT CTGCAACTGC
241 TTGAAGCAAG CCGTCAATGG CATCCCTTAC ACCAATGCAA ATGCTGGGCT TGCGGCTGGC
301 CTTCCTGGCA AGTGTGGGGT CAATATTCCT TACAAGATCT CTCCTTCTAC TGACTGCAAA
361 ACGATCAAGT GA
```

(B)

```
  1 MASSGQLLKLVCLVAVMCCMAVGGPKAMAAVSCGQVVNNLTPCINYVANGGALNPSCCTG
 61 VRSLYSLAQTTADRQSICNCLKQAVNGIPYTNANAGLAAGLPGKCGVNIPYKISPSTDCK
121 TIK
```

Figure 3.1. Nucleotide and derived amino acid sequence of almond LTP cDNA. (A) Nucleotide sequence (GenBank accession no. FJ652103) and (B) amino acid sequence of the LTP coding region. The predicted signal peptide is indicated in red.

Protein sequence homology characterization

Comparison of the derived aa sequence with the NCBI database using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi, National Center for Biotechnology, Bethesda, MD) analysis revealed that the sequence is that of almond lipid transfer protein. A single amino acid difference, serine to a tyrosine residue at position 121, suggests that we have identified an LTP isoform (underlined aa in Fig 3.1). BLAST analysis also showed sequence homology with other lipid transfer proteins, several of which are known food allergens (Table 3.2).

Table 3.2. Sequences demonstrating the greatest homology to almond LTP.

Protein Description	**Organism**	**Accession No.**	**% Identity**	**% Similarity**	**Ref.**	**Allergen Designation**
Non-specific lipid-transfer protein precursor	*Prunus avium* (cherry)	AAF26449	62	78	[81]	Pru av 3
Lipid transfer protein precursor	*Corylus avellana* (hazelnut)	AAK28533	60	72	[26]	Cor a 8
Non-specific lipid-transfer protein precursor (LTP)	*Malus x domestica* (apple)	Q9M5X7	59	75	[82]	Mal d 3
Lipid transfer protein isoform 1.1 precursor	*Lactuca sativa* (lettuce)	ABK96813	58	76	[83]	Lac s 1
Non-specific lipid-transfer protein 1	*Prunus persica* (peach)	P81402	58	74	[24]	Pru p 3
Non-specific lipid-transfer protein precursor	*Pyrus communis* (pear)	Q9M5X6	52	68	Unpublished	Pyr c 3

Production and purification of recombinant LTP

The entire almond LTP cDNA sequence, beginning at V22 following the presumptive signal peptide, was cloned. Cloned DNA segments were ligated into an expression vector designed to yield a MBP fusion protein. The resulting ~53 kDa MBP-LTP fusion protein was affinity purified with the aid of an amylose affinity column, cleaved from the carrier protein using factor Xa, and purified by size exclusion chromatography (Fig 3.2).

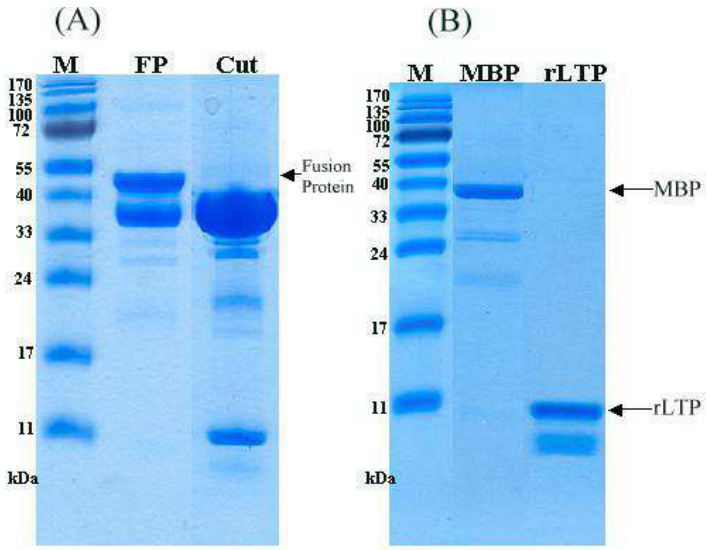

Figure 3.2. Coomassie blue stain of cleaved and purified almond rLTP. (A) FP- purified fusion protein: MBP-rLTP, by amylose affinity chromatography. Cut- factor Xa digested fusion protein generated free MBP and rLTP. (B) Purified MBP and rLTP (indicated by arrows) by size exclusion chromatography. M= molecular weight marker.

SDS-PAGE analysis of enriched almond extract

The native LTP from almond extract was enriched by means of gel filtration. Because there are several proteins of various molecular weights in almond extract, the degree of LMW enrichment was assessed by SDS-PAGE. The analysis reveals that a majority of the higher molecular weight proteins found in almond extract were removed by gel filtration and successful enrichment of LMW proteins occurred (Fig 3.3). This enriched almond extract was used in subsequent immunoblots.

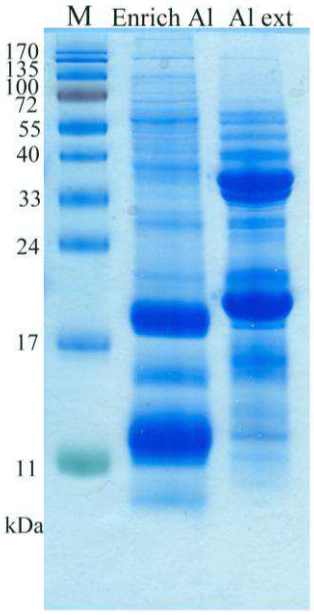

Figure 3.3. SDS-PAGE and Coomassie Blue stain of almond (Al Ext) and enriched almond (Enrich Al) protein extract. M= molecular weight marker.

Reactivity of peach LTP rabbit antisera with recombinant LTP

Several studies have demonstrated a high degree of cross-reactivity among plant LTPs [80;83;84], therefore we attempted to identify almond LTP using rabbit polyclonal antibody (Ab) to peach LTP. Western blotting analysis revealed Ab reactivity to both the uncleaved and cleaved recombinant protein, and to a LMW protein (~9 kDa) in almond extract (Fig 3.4A). Due to the low abundance of native LTP found in fruits and nuts, an enriched almond extract was prepared. This enriched extract showed stronger polyclonal reactivity as compared to unenriched almond extract, suggesting the successful enrichment of native LTP (Fig 3.4A). An inhibition immunoblot was preformed to identify LTP in almond extract and to assess reactivity between native and recombinant LTP (Fig 3.4B). Polyclonal antisera was pre-absorbed with either rLTP or almond extract, and then probed against the alternate sample (almond extract or rLTP) blotted onto NC. The inhibition of polyclonal antiserum with rLTP resulted in a loss of binding to the LMW (~9 kDa) band in almond extract as expected. Similarly, polyclonal reactivity to rLTP was inhibited after pre-absorption with almond extract (Fig 3.4B).

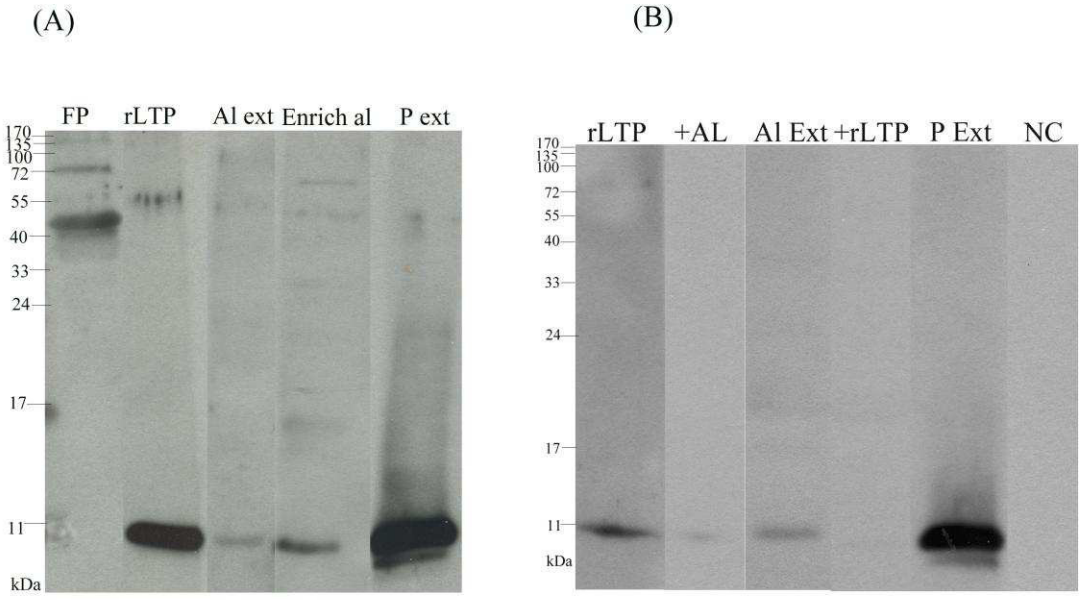

Figure 3.4. Immunoblot and inhibition immunoblot of rabbit anti-peach LTP polyclonal antibody. (A) Immunoblot with FP-fusion protein (MBP-rLTP), cleaved and purified rLTP, Al ext: almond extract, Enriched al: enriched almond extract, and P ext: peach extract. (B) Inhibition immunoblot of rLTP pre-absorbed with almond extract (+AL), and almond extract pre-absorbed with rLTP (+rLTP). Peach extract served as a positive control. The negative control (NC) contained no primary antibody.

Reactivity of recombinant LTP with human IgE

Reactivity to recombinant LTP was screened using 25 almond-allergic patients' sera: 23 almond-allergic North American patients and two almond-allergic Spanish patients. Of the patients tested, only five (20%) showed IgE reactivity to rLTP by dot-blot (Fig 3.5). Following the guidelines of the IUIS Allergen Nomenclature Subcommittee, the allergen was designated Pru du 3. Interestingly, of the two Spanish patients tested (#51 and 52), both (100%) showed reactivity to recombinant LTP by dot blot.

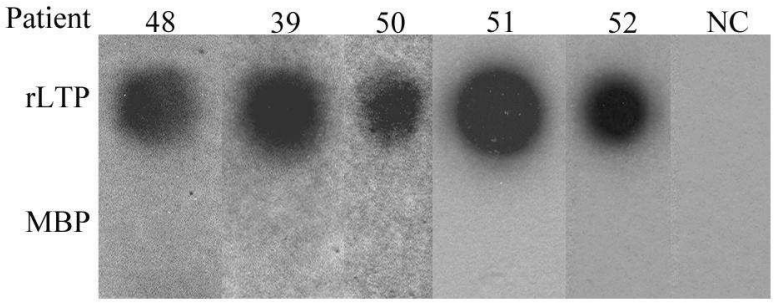

Figure 3.5. Dot blot with almond-allergic patients' sera showed IgE reactivity to rLTP (the 20 sera showing no signals are not shown). NC = negative control, non atopic control serum.

To identify the corresponding IgE reactive native LTP, an immunoblot was preformed using electrophoresed and blotted enriched almond extract, probed with recombinant LTP reactive patients (#48, 39, 50, 51, and 52). Of the patients tested, only two patients (#39 and 51) showed reactivity to LMW proteins, potentially native LTP (Fig 3.6A). This observation is similar to that observed by Schocker *et al.* for hazelnut LTP, where several patients did not show IgE reactivity to native LTP in hazelnut extract, but demonstrated specific IgE to the recombinant protein [79]. The above described immunoblot using rabbit Ab to peach LTP demonstrated the presence of native LTP in the enriched almond extract. To determine if native LTP in our enriched almond extract could inhibit IgE reactivity to the recombinant protein an inhibition ELISA was preformed. Preadsorption of patient sera with enriched almond extract abolished IgE reactivity to rLTP (Fig 3.6B).

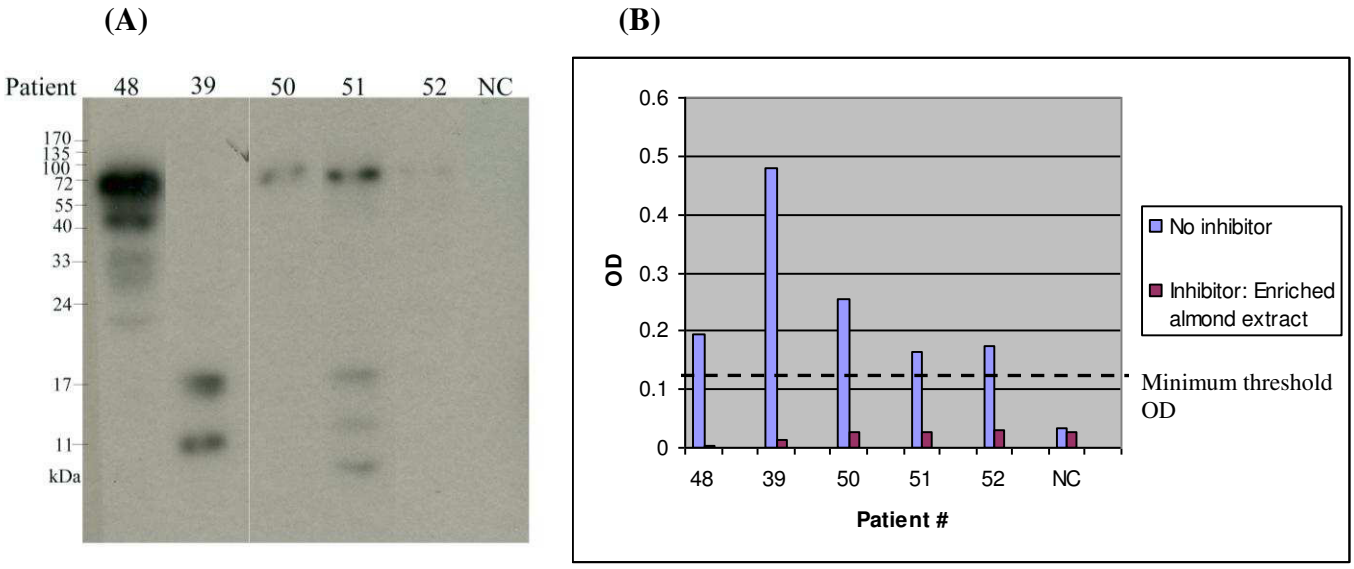

Figure 3.6. Immunoblot and inhibition ELISA. (A) Immunoblot using rLTP-reactive patient's sera. LMW-enriched almond extract was electrophoresed and blotted onto nitrocellulose membrane. (B) Inhibition ELISA using rLTP coated wells and reactive sera pre-incubated with enriched almond extract. NC= negative control.

Our initial screening of IgE reactivity to recombinant LTP was done by dot blot assay where the antigen is spotted onto NC under nondenaturing and nonreducing conditions. This is designed to allow the antigen to retain its native conformation and can aid in detecting reactivity toward conformational epitopes. In contrast immunoblotting uses SDS, β-mercaptoethanol, and boiling to denature the antigen before it is electrophoresed and transferred to NC. The lack of IgE reactivity to almond LTP by immunoblot suggests that patients #48, 50, and 52 are recognizing conformational epitopes on LTP that are lost under the denaturing conditions of immunoblotting. To further investigate this possibility, IgE reactivity to the untreated recombinant protein and the immunoblot-treated recombinant protein which was subjected to SDS/β-mercaptoethanol/boiling, was assayed by dot blot (Fig 3.7). A loss in IgE reactivity to the immunoblot-treated protein was observed, thus suggesting the presence of conformational epitopes on LTP.

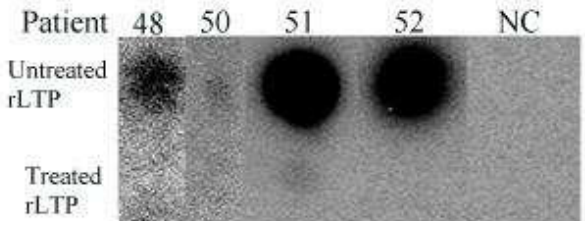

Figure 3.7. Dot blot using rLTP reactive patients sera probed against untreated rLTP and immunoblot treated rLTP which was subjected to SDS/β-mercaptoethanol/boiling. NC = negative control.

Discussion

Tree nuts are commonly consumed in the US, and tree nut allergy affects 0.5% of the US population [12]. Of tree nuts, almond allergy ranks third behind walnut and cashew nut [12]. In addition to almond, the *Rosaceae* family includes a variety of fruits; peach, apple, strawberry, pear, cherry, and plum. Initially it was believed that allergy to these fruits was primarily associated with birch pollinosis, a phenomenon known as birch-fruit syndrome. Birch-fruit syndrome is the result of cross-reactivity between the major birch pollen allergens, Bet v 1 (a pathogenesis related protein) and Bet v 2 (profilin), with similar homologues found in fruits [28;85;86]. Pastorello *et al.* was the first to determine that patients with and without associated birch pollinosis displayed different patterns of reactivity to proteins found in these fruits [77]. Subsequently, lipid transfer proteins were identified as important allergens in individuals suffering from food allergies without associated birch pollinosis [24]. Inhibition immunoblotting done by both Scheurer *et al.* and Pastorello *et al.* demonstrated that preincubation of IgE with birch pollen had no effect on IgE reactivity to cherry or peach LTP [77;81]. This lack of cross-reactivity between LTP and pollen indicates that LTP sensitization occurs primarily through the gastrointestinal tract and not through reactivity with inhalant allergens [87].

Sensitization to LTP has been primarily studied on European patients and a geographical difference in sensitization has been observed. In Northern and Central Europe, where birch pollen is high, sensitization to foods follows the birch-fruit syndrome [86]. However, in Southern Europe (predominantly in the Mediterranean), where there are lower quantities of birch pollen, individuals are

primarily directly sensitized to LTP in fruits and vegetables [13;25;82;88]. To date the basis for this difference in sensitization remains unclear.

The current study focuses on the identification and immunological analysis of almond LTP. In our patient population almond LTP was identified as a minor allergen (20% of patients' sera were reactive) and designated Pru du 3. A total of 25 patients were used in the study; 23 almond-allergic North American patients and two almond-allergic Spanish patients. Of the North American patients, only three (9%) demonstrated reactivity to the recombinant protein. Interestingly, both (100%) of the Spanish patients tested showed reactivity to almond LTP. This observation corresponds with the reported sensitization to LTP, as sensitization has been predominantly found in patients from the Mediterranean. A Western blot using LMW protein-enriched almond extract revealed that only two of the five LTP-reactive patients recognized native LTP, subsequently a dot blot confirmed that the denaturing conditions of immunoblotting eliminated IgE reactivity to the recombinant protein in three of the patients. These results indicated that certian patients recognize primarily conformational epitopes on almond LTP. Several studies have demonstrated that LTPs are resistant to both proteolytic digestion and heat treatments, which could be attributed to their compact folded structure [25;29;89;90] [91]. In a study done by Scheurer *et al.,* CD spectroscopy was used to analyze the effect of heat treatments on the structure of cherry LTP [90]. The results showed that LTP retained its conformation up to 70˚C but upon higher temperatures a heat induced loss of structure resulted [90]. Therefore, it is likely that LTP will retain its allergenic conformation during the sensitization process and may lead to the generation of IgE directed against conformational epitopes in addition to linear epitopes. Further studies involving epitope mapping must be done in order to identify the location and the amino acids involved in both the conformational and linear epitopes on LTP.

Our present study revealed that LTP is a minor (< 50% patient reactivity) but potentially important allergen in almond. This is not surprising as LTP is found in small amounts in the nut, and other allergenic proteins, such as almond major protein (AMP), have been found to constitute as much as 65% of the total aqueous extract [76]. According to the USDA Plants database (http://plants.usda.gov, Betula verrucosa), birch is prevalent in North American and distributed primarily throughout the Northeastern and Western states [92]. Unfortunately, the pollen sensitization profile for our patients is unknown; therefore we cannot make a direct correlation between the extent of pollinosis present in our patient population and LTP sensitization. It is possible that there may be geographical differences in LTP sensitization throughout the US, similar to that reported in Europe, especially if sensitization in some patient populations is linked to the presence or absence of birch pollen. Finally, it is also possible that LTP is a major allergen in the European population, as previous studies have demonstrated that LTPs are potent allergens in these patients. To further investigate these possibilities additional studies must be done using US patients with a confirmed presence or lack of birch pollinosis or similarly characterized European patient populations.

CONCLUSION

Allergies are a growing health concern and according to the National Institute of Health, allergies affect as many as 50 million Americans. Food allergies are classified as type 1 hypersensitive reactions that are mediated by IgE antibodies generated against proteins, allergens, in the offending food. Our studies were aimed at identifying and characterizing tree nut allergens from both pistachio and almond nut.

Extensive studies have lead to the identification of seed storage proteins as important allergens and the majority of tree nut allergens have been classified as 7S vicilins, 11S globulins, and 2S albumins. Pistachio is one of the five most commonly consumed tree nuts in the US along with walnut, cashew, almond, and pecan. However, allergy to pistachio has been largely ignored and was often only considered to be associated with cashew allergy, as the two nuts have a close phylogenetic relationship. In our first set of experiments we identified the 7S vicilin-like protein from pistachio and demonstrated that it was IgE-reactive using serum from allergic individuals. Several studies have demonstrated the presence of cross-reactive IgE in pistachio and cashew allergic individuals, which could be the result of cross-reactivity between the two nuts. This evidence, coupled with the fact that seed storage proteins are know to have both highly conserved sequence and structural similarities, prompted us to investigate cross-reactivity between the 7S vicilin from cashew and pistachio. By demonstrating that both human IgE, derived from naturally sensitized patients, and murine IgG, elicited during an artificial immunization/hybridoma regime, were unable to distinguish between the two vicilins, we provided evidence to support our claim of cross-reactivity between these two allergens. Overall, we are able to conclude that the considerable homology between these two allergens could serve as the molecular basis for their observed cross-reactivity.

Recently, lipid transfer proteins have been identified as important allergens in fruits and vegetables as well as tree nuts. In our second set of experiments we identified LTP in almond and demonstrated it is an IgE reactive allergen, designated Pru du 3. By demonstrating that treatment of LTP with reducing agents results in a loss of IgE reactivity, we provided evidence to support the presence of both linear and conformational epitopes on LTP.

Our data does not identify the location or amino acid sequence of the epitopes but suggests that both conformational and linear epitopes are involved in IgE recognition of LTP. At the present time, technology for identifying conformational epitopes is limited and less direct than the techniques used to identify linear epitopes. Additionally, there has been debate over the clinical importance of the two epitope forms, with some arguing that patients with more severe, lifelong allergy typically have IgE antibodies directed against linear, as opposed to conformational epitopes. However, recent data is challenging this concept and the true clinical importance of conformational/linear epitopes remains undetermined. In this regard, our efforts may be beneficial in the development of future assays to determine conformational epitope locations and sequences, as well help elucidate their clinical importance.

In conclusion, our experiments have identified allergenic proteins from both pistachio (*Pistacia vera*) and almond (*Prunus dulcis*) that are directly involved in IgE mediated hypersensitivity. The identification of tree nut allergens is crucial for both diagnosis and development of future treatments for tree nut allergy. Our findings provide the foundation for further work on these allergens to identify IgE-binding epitopes and generate modified allergens for use in immuno-therapeutic applications.

APPENDIX A

HUMAN SUBJECTS COMMITTEE APPROVAL MEMORANDUM

APPROVAL MEMORANDUM

Date: 5/19/2008

To: Shridhar Sathe

Address: 1493
Dept.: NUTRITION FOOD AND MOVEMENT SCIENCES

From: Thomas L. Jacobson, Chair

Re: Use of Human Subjects in Research
Identification and characterization of tree nut allergens

The application that you submitted to this office in regard to the use of human subjects in the research proposal referenced above has been reviewed by the Human Subjects Committee at its meeting on 05/14/2008. Your project was approved by the Committee.

The Human Subjects Committee has not evaluated your proposal for scientific merit, except to weigh the risk to the human participants and the aspects of the proposal related to potential risk and benefit. This approval does not replace any departmental or other approvals, which may be required.

If you submitted a proposed consent form with your application, the approved stamped consent form is attached to this approval notice. Only the stamped version of the consent form may be used in recruiting research subjects.

If the project has not been completed by 5/13/2009 you must request a renewal of approval for continuation of the project. As a courtesy, a renewal notice will be sent to you prior to your expiration date; however, it is your responsibility as the Principal Investigator to timely request renewal of your approval from the Committee.

You are advised that any change in protocol for this project must be reviewed and approved by the Committee prior to implementation of the proposed change in the protocol. A protocol change/amendment form is required to be submitted for approval by the Committee. In addition, federal regulations require that the Principal Investigator promptly report, in writing any unanticipated problems or adverse events involving risks to research subjects or others.

By copy of this memorandum, the Chair of your department and/or your major professor is reminded that he/she is responsible for being informed concerning research projects involving human subjects in the department, and should review protocols as often as needed to insure that the project is being conducted in compliance with our institution and with DHHS regulations.

This institution has an Assurance on file with the Office for Human Research Protection.
The Assurance Number is IRB00000446.

Cc: Bahram Arjmandi, Chair
HSC No. 2008.1343

HUMAN SUBJECTS COMMITTEE APPROVAL MEMORANDUM

Florida State
UNIVERSITY

Office of the Vice President For Research
Human Subjects Committee
Tallahassee, Florida 32306-2742
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APPROVAL MEMORANDUM

Date: 1/5/2007

To:
Shridhar Sathe
Mc 1493

Dept.: **NUTRITION FOOD AND MOVEMENT SCIENCES**

From: **Thomas L. Jacobson, Chair**

Re: **Use of Human Subjects in Research**
Identification and characterization of tree nut allergens

The forms that you submitted to this office in regard to the use of human subjects in the proposal referenced above have been reviewed by the Human Subjects Committee at its meeting on **12/13/2006**. Your project was approved by the Committee.

The Human Subjects Committee has not evaluated your proposal for scientific merit, except to weigh the risk to the human participants and the aspects of the proposal related to potential risk and benefit. This approval does not replace any departmental or other approvals which may be required.

If the project has not been completed by **12/12/2007** you must request renewed approval for continuation of the project.

You are advised that any change in protocol in this project must be approved by resubmission of the project to the Committee for approval. The principal investigator must promptly report, in writing, any unexpected problems causing risks to research subjects or others.

By copy of this memorandum, the chairman of your department and/or your major professor is reminded that he/she is responsible for being informed concerning research projects involving human subjects in the department, and should review protocols of such investigations as often as needed to insure that the project is being conducted in compliance with our institution and with DHHS regulations.

This institution has an Assurance on file with the Office for Protection from Research Risks. The Assurance Number is IRB00000446.

cc: Kenneth Roux
HSC No. 2006.1014

APPENDIX B

INFORMED CONSENT FORM

Informed Consent Form (Adult)

I, _____, freely and voluntarily and without element of force or coercion, consent to be a participant in the research project entitled **" Identification and Characterization of tree nut allergens"**

This research is being conducted by **Shridhar K. Sathe and Kenneth H. Roux** who are professors of Food Science and Immunology in the Department of Nutrition, Food and Exercise Sciences and Department of Biological Science, respectively, at The Florida State University. I understand that the purpose of their research project is to identify and characterize allergenic proteins in tree nuts. I understand that the blood collected will be used for identification and characterization of allergenic proteins in food and food ingredients. Information on my history of tree nut and other food allergies will also be obtained from my physician.

I understand that I will be asked to give up to 30 mL (approximately two table spoons) of blood for the tree nut allergen study.

I understand that the risks associated with venipuncture (blood sampling) include the possibility of fainting, bruising of the skin, local infection and hematoma (a mass of usually clotted blood that forms in a tissue, organ, or body space as a result of a broken blood vessel). I understand that these risks are minimal, as blood samples will be taken by a certified phlebotomist using correct sterile techniques, but that I may experience some pain as the needle pierces my skin. During the blood sampling an attempt to minimize the possibility of injury or risk through a detailed explanation of the procedures and a keen attention to my well being will be strictly adhered to. I fully understand that appropriate emergency protocols have been established and in case of emergency phlebotomists trained in First Aid and CPR will act rapidly and appropriately.

I understand that the information obtained in this investigation will be regarded as privileged and confidential to the full extent allowed by law. All my results and information will be kept confidential and identified only by a subject code. The link between my code and information will be properly stored in a locked cabinet during the investigation period and will be destroyed following the completion of this project. My name will not appear on any results.

I understand there is a possibility of a minimal level of risk involved if I agree to participate in this investigation. I am able to stop my participation at any time I wish to without prejudice, penalty or loss of benefits to which I am otherwise entitled. Questions regarding this investigation, if any, have been answered to my satisfaction.

I understand that I may contact Dr. Shridhar K. Sathe (NFES, 402 Sandels, Florida State University) at (850) 644-5837 or Dr. Kenneth H. Roux (Dept. of Biological Sciences, Florida State University) at (850) 644- 5037 for answers to any questions about this research or my rights. If I have questions regarding my rights as a human subject and participation in this study, I may call the Committee on Protection of Human Subjects at Florida State University at (850) 644-8633.

I have read and understand this informed consent form. The nature of this study, its possible risks, and the right to withdraw from participation in this study at any time without prejudice has been explained to me. I do hereby voluntarily consent to participate in this study.

_____
Subject Name (Printed)

_____ _____
Subject Signature Date (mm/dd/yy)

FSU Human Subjects Committee approved 5/14/2008. Void after 5/13/2009. HSC#2008.1343.

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BIOGRAPHICAL SKETCH

Personal

LeAnna N. Willison
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Education

December, 2009	M.S. in Biological Science Florida State University, Tallahassee, FL
August, 2005	B.S. in Biological Science Florida State University, Tallahassee, FL
June, 2000	Clay High School, Green Cove Springs, FL

Professional Experience

2006-2009	Graduate Teaching Assistant Department of Biological Science, Florida State University
2006-2009	Graduate Research Assistant Department of Biological Science, Florida State University

Publications

Willison LN, Tawde P, Robotham JM, Penny R, Teuber SS, Sathe SK, Roux KH. Pistachio vicilin, Pis v 1, is allergenic and cross-reactive with the homologous cashew allergen, Ana o 1. Clin. Exp. Allergy. 2008 Jul;38(7):1229-38.

Abstracts and Presentations

Cloning and expression of almond prunin 1 and prunin 2 and analysis of patient IgE reactivity. American College of Allergy, Asthma & Immunology XIII International Food Allergy Symposium, Miami Beach, FL, Nov 5, 2009.

Monoclonal antibody (mAb)-based enzyme linked immunoassay (ELISA) for sensitive, specific, and robust detection of trace quantities of almond (Prunus dulcis L.). Annual Meeting of the Institute of Food Technologists, Anaheim, CA, 2009.

Identification and classification of pistachio allergen that shows cross-reactivity with cashew (Anacardium occidental) allergen Ana o 1. Ferderation of Clinical Immunology Societies Conference in San Diego, CA, June 7-11 2007.