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Cloning, Expression, and Characterization of Ara h 3, a Major Peanut Allergen

A Thesis

Submitted to the Graduate Faculty of the
University of New Orleans
in partial fulfillment of the
requirements for the degree of

Master of Science
in
Biological Sciences

by

Cathryn E. Garvey

B.S. University of New Orleans, 2009

December, 2012

Dedication

This thesis is dedicated to two of my family members Christopher H. Aldrich and Patrick G. Garvey, who both have severe peanut allergy.

Acknowledgement

I would like to give special thanks to Dr. Soheila Maleki for giving me the opportunity to work and do my research in her laboratory where I learned more about peanuts, molecular biology and protein biochemistry than I could have ever imagined. She was always there to guide me through the process of the demanding and laborious work that research presents and how it can give a person the feeling of defeat. Dr. Maleki was not only a mentor in science and the laboratory but in life experiences. She always presented me with mental challenges and problem solving skills that would prepare me for life not only as a scientist but as a person. I thank her for everything she has given me.

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me in my coursework and any other chance he got. He is a not only a great professor, but is also very passionate in all aspects of science. He amazes me with his vast knowledge and how every time I speak with him, I learn something new. I anticipate the day when I will be able to speak with that kind of knowledge and passion about all areas of science. Dr. Liu is one of UNO's best professors and assets.

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Abstract

There are eight foods that contribute to food allergies in the western world and peanut is the most common. Currently, there are no medical treatments that can cure an individual of food allergy, so avoidance of the allergic food is the only option. In the United States, there are three immunodominant allergic proteins accountable for patient sensitization to peanut, *Arachis hypogea* 1, 2, and 3 (Ara h 1, Ara h 2, Ara h 3). Therefore, research into why peanuts are more allergic than other foods that have homologous proteins is critical and may be obtained by studying the structural and allergenic properties of individual allergens and the changes that occur due to food processing. In this study, the basic and acidic subunits of Ara h 3 were cloned, expressed, and purified, and compared with each other and with the native Ara h 3 purified from peanut for differences in binding to IgE from peanut allergic individuals. Also, an in vitro Maillard reaction was performed on purified native raw Ara h 3 and patient serum IgE western blots were performed. This study concluded that an in vitro Maillard reaction enhanced IgE binding to Ara h 3, IgE binding to native Ara h 3 was in most cases higher than to the recombinant Ara h 3 subunits, and recognition of the acidic subunit was much higher than the and basic subunits in both the recombinant and native forms of the protein were investigated.

Keywords: Peanut Allergy, Ara h 3, Allergic Reaction, Immunoglobulin E (IgE), Antigen, Sensitization, FcεRI Receptor, Epitope, Mast Cell, Maillard Reaction, Simulated Roasted Model (SRM), Simulated Roasted (SR)

Introduction

Prevalence of Peanut Allergy

Food allergy has become recognized worldwide and appears to be increasing in developing countries.[1] There are eight major allergenic foods that contribute to immunoglobulin-E[i] (IgE)-mediated food allergy both in the United States and internationally. These foods, peanuts, tree nuts, cow's milk, soy, wheat, hen's eggs, fish, and crustaceans, are responsible for approximately 90 % of food allergies in the United States.[2] According to a survey in 2008, approximately 1.2 – 1.6 % of the American population has peanut allergy.[3] In 1997, a nationwide, random telephone survey for self-reported or parent-reported peanut and tree nut allergy was conducted in order to determine on average how many children under the age of 18 in the United States were affected by peanut allergy. It reported a prevalence of peanut allergy in children under the age of 18 to be 0.4 %.[4] In 2002, a follow up study to the one in 1997, concluded that peanut allergy affected approximately 0.8 % of children and 0.6 % of adults in the United States.[5] In the most recent study done by Sicherer et.al., in 2008, there was a reported a prevalence of peanut allergy in children to be 1.4 % and peanut and tree nut allergy increased to 2.1 % from 1.2 % in 2002, and 0.6 % in 1997.[3] These data indicate that peanut allergies have doubled in American children twice in a ten-year period.[3, 5]

Hypersensitivity Disorders and Allergen Characteristics

In 2004, the European Academy of Allergy and Clinical Immunology revised the nomenclature for allergy.[6] Food allergies were defined as hypersensitive reactions initiated by specific immune mechanisms that occur after an allergen is encountered.[6] A food has been defined as any substance intended for human consumption whether it is processed, semi-processed, or raw, however, this does not include drugs, cosmetics, or tobacco products.[7] Allergen-specific IgE-mediated food allergy requires both the presence of clinical symptoms and the ability to sensitize an individual on exposure to a specific food and upon subsequent exposures to the food, allergic reactions ensue.[7] There are foods that can elicit reproducible negative clinical reactions but do not have established immunological mechchanisms or likely immunological mechanisms. These foods are usually not considered food allergens.[7] These non-immunological reactions are termed food intolerances. For example, an individual can have IgE-mediated food allergy to the protein, casein, in milk versus the non-immunological reaction termed lactose intolerace, which is where the individual is unable to digest the lactose sugar in milk.[7]

Most patients with clinical food allergy symptoms that occur from an allergen encountered at mucosal membranes in the airways or gastrointestinal tract are said to have IgE-mediated food allergy.[6] Food hypersensitivity disorders are categorized into two categories, IgE-mediated and non-IgE-mediated

[i] IgE- class of antibodies important in allergy that elicits an immune response when a food allergen is encountered

responses.[6] For the purpose of this study, only IgE-mediated reactions and hypersensitivity disorders are going to be covered. IgE-mediated reactions typically occur rapidly after the allergen is encountered.[1] There are four hypersensitivity disorders that can occur depending on the severity of the patient's allergy and are listed in Table 1-1.[1]

Table 1-1 IgE-Mediated Food Hypersensitivity Disorders and Symptoms

IgE-mediated	
Gastrointestinal	• Oral allergy syndrome (OAS) consisting of mild pruritus, tingling, and/or angioedema of lips, palate, tongue, or oropharynx, tightness of the throat • Gastrointestinal anaphylaxis including nausea, abdominal pain, cramps, vomiting, diarrhea, and usually occurs with allergic manifestations in other organs
Cutaneous	• Acute urticaria and angioedema includes morbilliform rashes and flushing (pruritus, hives, and/or swelling) • Chronic urticaria and angioedema includes pruritus, hives, and/or swelling longer that 6 weeks • Atopic dermatitis: marked pruritus and eczematous rash in a classical distribution
Respiratory	• Acute rhinoconjunctivitis, bronchospasm (wheezing), periocular pruritus, tearing, and conjunctival erythema, nasal congestion, rhinorrhea, sneezing
Generalized	• Anaphylaxis • Presence of cutaneous, respiratory, and gastrointestinal symptoms, and possible cardiovascular symptoms including hypotension, vascular collapse, and cardiac dysrhythmias

Table 1-1[1,8]

There are some crucial characteristics of food allergens and their ability to provoke hypersensitivity disorders in allergic individuals. Food allergens have been identified as proteins with low molecular weights ranging in size from approximately 10 kDa to 70 kDa with acidic isoelectric points and commonly have sugar moieties.[9] It has been proposed that the carbohydrate moieties on allergens can play a key role in recognition by the immune system.[10]

The structure of a protein can contribute to the characteristics of whether or not it will be allergic or not. The amino acid sequence is the primary structure of a protein and defines the overall structure. Linear epitope sequences can be found within the amino acid sequence. The primary structure determines the secondary structure (α helices, loops, and β sheets). Interactions of the secondary structures within a three dimensional space is known as the tertiary structure of the protein. Binding of the individual protein to itself or other proteins is known as the quaternary structure. This is seen when a protein is made up of multiple subunits. Each of these elements can contribute to the allergic nature of the protein and define the characteristics of the allergen including: digestibility and absorption upon ingestion, recognition by

the immune system, and stability to heat and other processing methods possibly due to their sugar moieties and formation of higher order structures.[9]

IgE-Mediated Immune Response to Allergy

The mucosal immune system in the gut encounters a high number of both harmless and "hazardous" antigens on a daily basis.[1] The small intestine uses physiochemical and cellular factors to maintain its physical barrier. Both the innate and adaptive immune responses give extra support to the physical barrier of the small intestine by providing an active barrier to foreign antigens.[1] In a non-allergic individual, food fragments regularly resist different pH levels between the mouth, stomach, and small intestine and the different enzymes that are present to degrade the fragments rendering them non-immunogenic.[1] By contrast, in an allergic individual, once allergenic fragments have been absorbed into the bloodstream, they are encountered by the immune system cells that are capable of provoking allergic responses.[11, 12] Being resistant to digestive enzymes is thought to be characteristic of allergens and an example of this was shown by a study on immunodominant peanut allergen, Ara h 1. Digestion resistant fragments of Ara h 1 contained multiple IgE binding sites capable of initiating immune responses. This study concluded that the quaternary structure of Ara h 1 may protect the IgE-binding sites from enzymatic digestions. Therefore, resistance to human digestive enzymes contributes to allergenic properties by allowing prolonged access of these intact proteins or peptides to the gastrointestinal system which may enhance absorption of allergic proteins through the gut mucosa.[12, 13]

In the case of peanut allergy, allergic reactions are usually severe and most often hypersensitivity continues throughout the patients' lives.[2] When a food is ingested, it travels through the gut where it is subjected to many enzymes from α-amylase in the mouth, pepsin in the stomach, to trypsin and chymotrypsin in the small intestines.[14] Food proteins are broken down into peptides and amino acids in the small intestine and pass from the lumen into the bloodstream. After absorption into the bloodstream, food proteins are engulfed and processed by enzymes within antigen-presenting cells (APCs). The APCs, in turn, present the processed protein fragments to specific immune cells such as, T-helper 2 (T_H2) cells and B cells.[15]

Allergen-specific T_H2 cells proliferate, produce and secrete immune response factors known as cytokines. Cytokines known as interleukin-4 (IL-4) and interleukin-13 (IL-13) are highly specific in stimulating and promoting IgE antibody production by B cells against specific allergens.[12, 15] Specific IgE antibodies then bind the high affinity IgE receptor, FcεRI, on surfaces of mast cells and basophils.[16] When allergen-specific IgE antibodies bind FcεRI receptors, a patient is now considered sensitized to that protein.[12, 15]

An IgE-mediated allergic response occurs when re-exposure to the same allergen happens. However, the allergen fragment that is passed into the bloodstream must be large enough to contain at

least two IgE-binding sites[12, 16] in order to crosslink at least two IgE antibodies that bound FcεRI receptors on surfaces of mast cells and basophils.[16] Crosslinking of IgE antibodies by the allergen fragment promotes degranulation of immune cells and release of histamine and other pro-inflammatory chemical mediators into the bloodstream, eliciting an allergic reaction.[16]

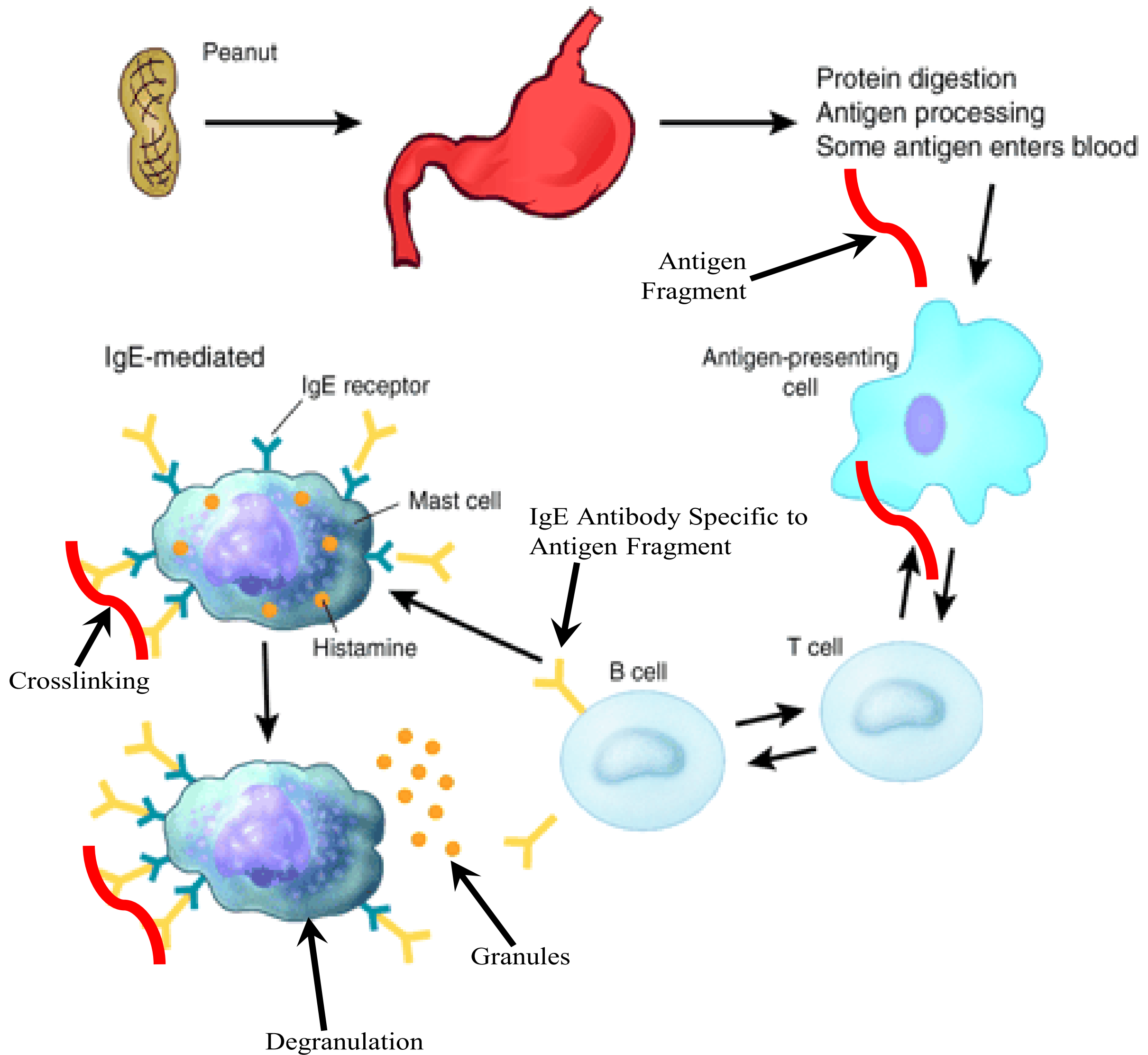


Figure 1-1 IgE Mediated Allergic Response. Red squiggle line: Antigen fragment containing two IgE binding epitopes. The antigen fragment crosslinks 2 or more IgE antibodies specific for the antigen fragment that are bound to FcεRI receptors on the surface of the sensitized mast cell. The crosslinking causes the mast cell to degranulate and release chemical mediators into the bloodstream (histamine etc.) inducing an allergic response.[12]

Anaphylaxis

Peanuts are widely used in a variety of foods in America because of their important nutritional properties.[3] This has led to peanut allergy being the most common cause of life-threatening allergic reactions and food related deaths[3] due to increased rates of accidental ingestion by peanut allergic patients.[1]

Anaphylaxis occurs when mast cell activation causes an increase in vascular permeability and there is a widespread constriction of smooth muscles. Anaphylaxis involves fluid leaving the blood which causes the blood pressure to drop significantly, connective tissues continue to swell, and organs of the body sustain damage and their functions are impaired. Death can occur by asphyxiation caused by constriction of the airways and swelling of the epiglottis. Anaphylaxis can be provoked by many allergens, but foods tend to cause it more often because these allergens are rapidly absorbed from the gut to the bloodstream.[17]

Since peanut allergies are rising[3, 5] and peanuts are used in so many foods in the United States, it is important that people with life threatening peanut allergy carry an epinephrine pen with them. Epinephrine stimulates reformation of tight junctions between endothelial cells, reducing permeability, preventing fluid loss from the blood, constricting connective tissues, and raises blood pressure during allergic reactions. Epinephrine can usually bring an anaphylactic reaction under control.[17]

Food Processing Contributions to Food Allergy

Thermal processing, including roasting, frying, and other cooking methods, causes a multitude of various non-enzymatic reactions to occur in foods[18] and all have a range of effects on the primary, secondary, tertiary, and quaternary structure of proteins and/or peptides that can alter their allergenic properties. It has been speculated that IgE epitopes identified in raw food sources might not be the same in processed foods due to the chemical reactions that occur.[19] During processing, existing conformational and linear IgE binding epitopes are likely to be altered and may cause a change in protein conformation or immunogenicity. Changes in protein conformation can cause new epitopes to form called neoallergens.[20] All of these characteristics brought about by food processing either enhance or reduce allergen recognition by IgE, potentially altering the allergenicity of the food.[21]

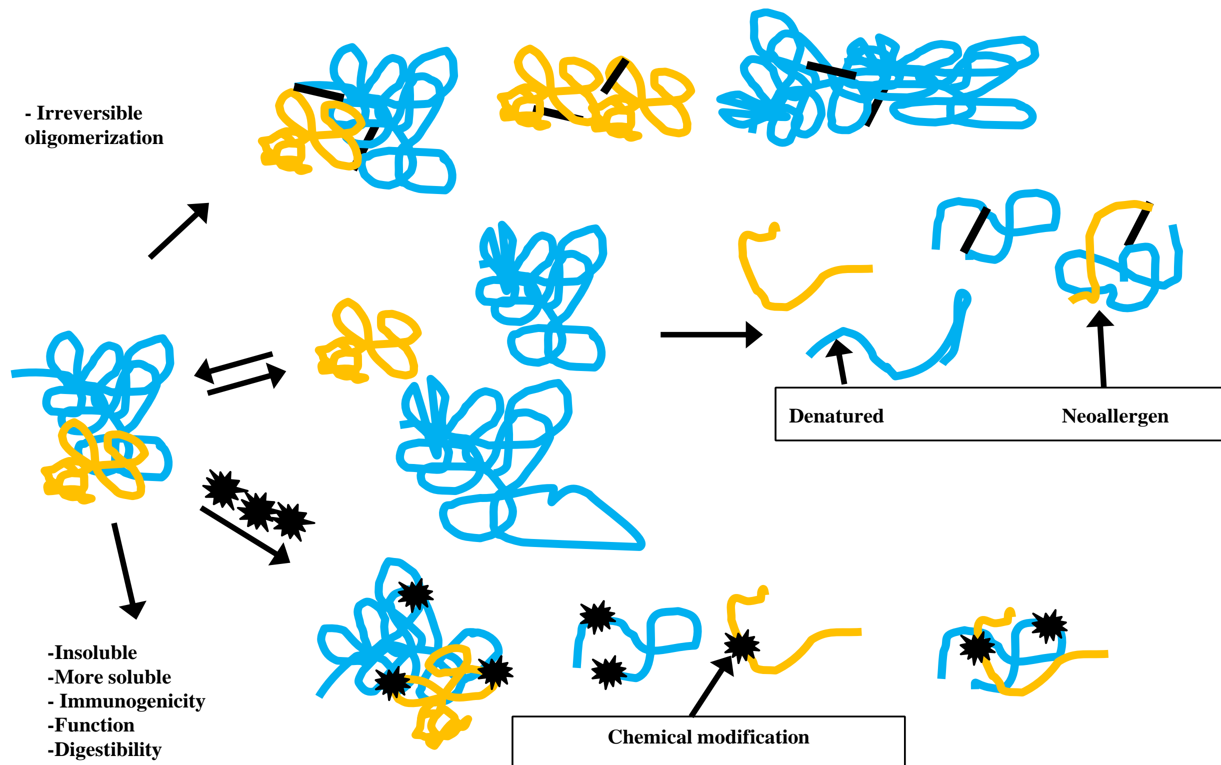


Figure 1-2 Different effects of food processing on a protein. Irreversible oligomerization: alteration in the quaternary structure. Reduction: alteration in the tertiary structure by breaking disulfide bonds. Denaturation and chemical modification: alteration that affect the secondary, tertiary, and quaternary structures of proteins. Neoallergn: new epitopes formed as a result of changes in protein conformations. Stars indicate chemical modifications; black bars indicate chemical crosslinks.

When a protein is processed, covalent crosslinking may occur and produce large irreversible oligomeric complexes.[21] A protein can react with sugars, phospholipids, and chemicals in the environment and can cause chemical modifications to occur. These chemical modifications alter its secondary structure thereby altering the entire conformation of the protein. The Maillard reaction, a major reaction during roasting of peanuts, can cause a loss, disruption, or rearrangement of sulfhydryl groups leading to denaturation of the protein.[12] Disruption of the disulfide bonds can alter conformational epitopes influencing an allergen's biological activity most likely by affecting solubility, IgE binding, and digestibility.[12, 13, 21]

It is possible that proteins that are allergenic in the same food have become modified during processing and the presence of other modified allergenic proteins induces a protective effect on other proteins.[22] This has been shown in Ara h 1 and Ara h 2 where roasted Ara h 2 was more resistant to trypsin digestion than raw Ara h 2. Roasted Ara h 2 protected Ara h 1 from trypsin digestion leading to the conclusion that processing caused enhancement of allergenic properties of different proteins in the same food.[22]

Maillard Reaction

A major biochemical reaction that occurs during thermal processing such as cooking, curing, and roasting is the Maillard reaction.[9, 23, 24] The Maillard reaction is a non-enzymatic browning reaction important in the development of flavor and color in peanuts. The Maillard reaction occurs between a protein's free amino groups and reducing sugars which causes the protein to become glycosylated.[9, 23, 24]

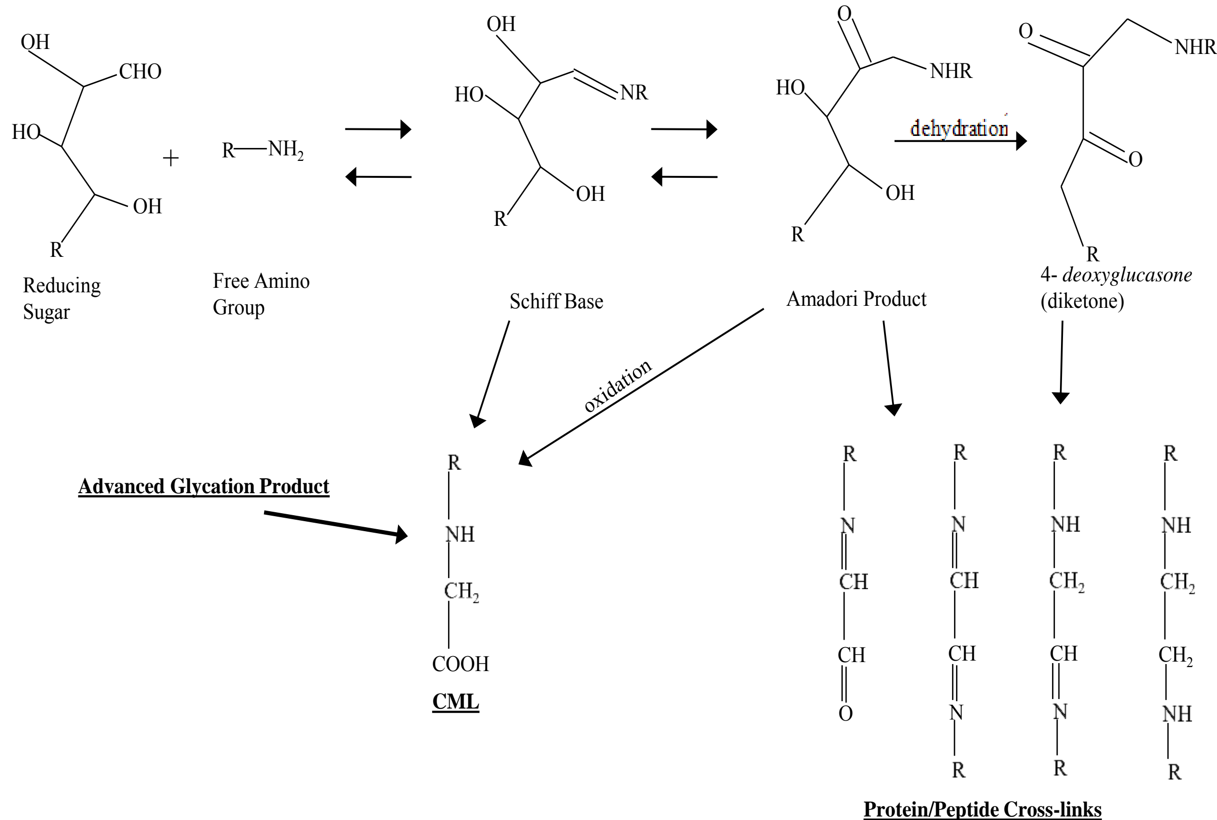


Figure 1-3 Depiction of the Maillard reaction and the formation of advanced glycation end products. The early stages of protein glycation leads to the formation of Amadori products from the Schiff base adduct. The Amadori product undergoes a dehydration reaction to form a glucasone (diketone), proximate intermediate in the production of AGEs and protein cross-linking. The AGE (CML) is one of the predominant modifications found on proteins after the Maillard reaction.[9]

Figure 1-3 shows the general reaction mechanism of the Maillard reaction.[9] The reaction of reducing sugars and free amino groups on amino acid residues in a protein or peptide leads to the formation of Schiff bases with the release of water molecules. Schiff bases undergo rearrangements to form Amadori products. Amadori products are more reactive than their respective parent sugars and are readily able to react with amino groups of proteins to form crosslinked, stable end products called Maillard reaction products (MRPs), or advanced glycation end products (AGEs).[24]

Amadori products undergo a dehydration reaction to form dicarbonyls which then react to form protein and peptide crosslinks. Amadori products can undergo an oxidation reaction that leads to the loss

or modification of amino acids, such as the modification of amino acid lysine to carboxymethyllysine (CML), malanoidin formation, and other non-crosslinking modifications to proteins that all have detrimental effects on nutrition, disease states, and allergy.[24]

Using a competitive inhibition ELISA, Maleki et.al in 2000 showed that roasted peanut extracts bound serum IgE from peanut allergic patients at approximately 90-fold higher levels than raw peanuts from the same cultivars as shown in Figure 1-4.[9] In the same study, purified native major peanut allergens, Ara h 1 and Ara h 2 underwent an in vitro Maillard reaction, also known as the Simulated Roasting Model (SRM) in the presence of different reducing sugars to decipher if the Maillard reaction alone could enhance the allergenic properties of Ara h 1 and Ara h 2.[9] Ara h 1 and Ara 2 subjected to an in vitro Maillard reaction in the presence of different sugars bound higher serum IgE levels versus their raw counterparts and the binding curves mimicked the ones shown in Figure 1-4.[9] When simulated roasted (SR) Ara h 1 and Ara h 2 were analyzed by SDS-PAGE, the SR proteins were more resistant to heat and degradation. Upon incubation in the presence of gastric secretions to look at impact of the Maillard reaction on digestibility of peanut allergens, both SR Ara h 1 and Ara h 2 proteins exhibited more resistance to gastrointestinal enzymes after they had undergone the SRM.[9]

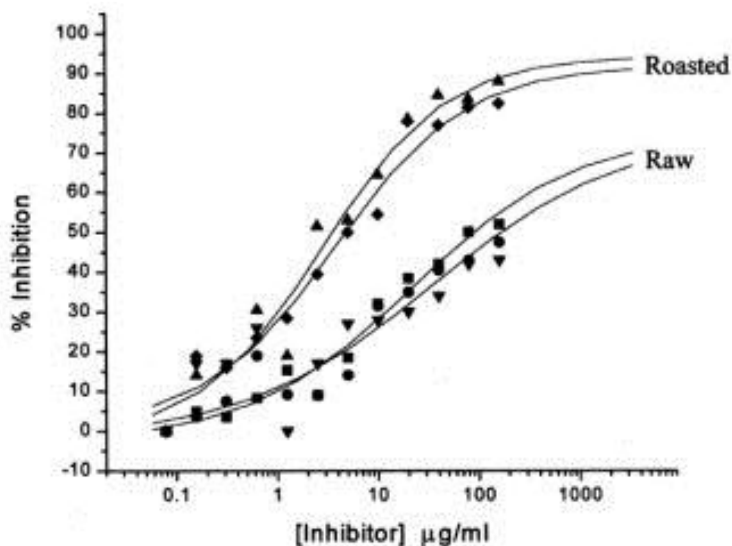


Figure 1-4 IgE-binding properties of roasted vs. raw peanuts. Increasing concentration of raw peanut extracts from brand 1 (raw, *circles*; roasted, *triangles*) and brand 2 (raw, *inverted triangles*; roasted, *diamonds*) were used to compete for IgE binding with the raw Flowrunner whole peanut extracts (WPEs) that coats the wells. Increasing concentrations of the raw Flowrunner extract (*squares*) were used as free antigen to compete with itself for IgE binding as a standard for comparison.[9]

The Maillard reaction causes covalent modifications of proteins during roasting.[9, 23, 24] Since IgE-binding by patient serum IgE was higher in roasted versus raw peanuts,[9] there is speculation that IgE-recognition sites in roasted peanuts are different than in raw due to the fact that heat treatment exposes possible sites that were previously hidden.[25] The covalent modifications that occur via the

Maillard reaction can influence other allergenic properties such as resistance to heat and digestion by gastrointestinal enzymes possibly by chemically modifying the protein or altering the protein structure and enabling large protein fragments to enter the bloodstream.[9, 25]

Peanut Allergens

The scientific name for peanut is *Arachis hypogea.* The peanut allergens are named by the first three letters of the genus followed by the first letter of the species and are numbered in the order they have been identified. Currently, there have been 12 peanut allergens identified that are responsible for sensitization and IgE-binding-reactivity in allergic patients.[26] Three of these 12 peanut allergens are considered major immunodominant seed storage proteins. They are members of vicilin (Ara h 1), conglutin (Ara h 2), and glycinin (Ara h 3/4) protein families. These three proteins families are considered immunodominant because greater than 50 % of the United States peanut allergic individuals recognize these allergens.[27]

Food Allergy Varies by Geographical Location

There are theories that different culinary preparations, eating habits and different environmental pollen exposures that allow for cross-reactivity between allergens can possibly account for differences in food allergy and the recognition of different peanut allergens globally.[28, 29, 30, 31] One example of global eating habits and correlation with different geographical distribution of food allergy was shown by a study done in Israel.[28] Peanut allergy in Israel is less common than in the United States and Europe and sesame-seed allergy dominates this country only behind cow's milk.[28]

Not only do food allergies differ by geographical location, but recognition of individual peanut proteins also varies by global location. A study using patients with known histories of peanut allergy from three different countries was conducted to compare their serum IgE recognition to the different peanut proteins.[29] In the three countries compared, peanuts are typically eaten after being dry roasted, which has been shown to increase allergenicity in peanuts.[9] It was concluded that patients from the three different countries reacted to different peanut proteins and the age of onset of allergy differed.[29] In Spain and Sweden, the age patients became allergic was approximately two years old as compared to American children starting at around age one.[29] Swedish patients were found to be sensitized to the birch allergen, Bet v 1 which is homologous to Ara h 8. Ara h 8 was the second most common allergen recognized in Swedish patients, but major allergens Ara h 1, Ara h 2, or Ara h 3 were also recognized.[29] A separate study showed that Swedish patients also recognized Ara h 5.[30] A majority of Spanish patients were found to be sensitized to only Ara h 9, a lipid transfer protein important in peanut allergy in the Mediterranean area.[29] In the United States, the major allergens recognized were Ara h 1, Ara h 2, and Ara h 3.[29] Peanut allergy is not usually considered a major allergy in Italy, but Restani et.al. identified the basic subunit of

major peanut allergen Ara h 3 as being more allergenic than the acidic subunit in a group of children.[31] This was in contradiction to previous conclusions in the United States population stating that the acidic subunit was more clinically relevant.[32]

Genomic Organization of Immunodominant Ara h 3

Ara h 3 and Ara h 4 have been identified as two variants of the same gene.[33] Nucleotide analysis of the open reading frame (ORF) of the Ara h 3 gene revealed a full length genomic sequence spanning 3,828 base pairs containing the plant consensus sequence for initiation of transcription and four exons.[27] The inferred amino acid sequence of 538 amino acids with a molecular weight of to 61.7 kDa is comparable to the molecular masses of 57 kDa and 61 kDa of Ara h 3 and Ara h 4, respectively. When the N-terminal amino acid sequence was analyzed further, it revealed that an N-terminal transit peptide consisting of 20 amino acids (MGKLLALSVCFCFLVLGASS) rich in hydrophobic amino acid residues was not removed post-transcriptionally in the Ara h 4 protein.[27] This N-terminal transit peptide is the leader sequence important for translocation to the vacuole of the cell and most likely accounts for the size differences in Ara h 3 and Ara h 4.[27]

11S Globulin Structure and Ara h 3

Ara h 3 belongs to the family of 11S globulin seed storage proteins.[33] Analysis of the primary amino acid sequence of Ara h 3 shows it is a member of the cupin superfamily and composed of two subunits.[33] The cupin superfamily consists of 7S (vicilins, such as Ara h 1 and the walnut homolog Jug r 2) and 11S (legumins, such as Ara h 3; the cashew homolog Ana o 2; the walnut homolog Jug r 4; and the soybean homolog glycinin) seed storage proteins.[34] Members of the cupin (Latin for small cask or barrel) superfamily assume a "double stranded β-helix or jelly-roll barrel" structure which is connected to a domain consisting of all-α-helices.[34] 7S and 11S globulins are considered bicupins, which have a repeat of a double stranded β-helix domain and an α-helical domain.[34, 35, 36, 37]

11S globulins are synthesized as single polypeptide precursor, preproprotein, and the signal sequence is removed post-translationally.[35] After synthesis and removal of the signal peptide of Ara h 3 is stored in protein storage vacuoles (PSVs).[33, 35, 36] In the PSVs, the proprotein undergoes extensive proteolytic processing and is cleaved at a conserved aspartic acid-glycine bond by a specific aspartic acid endoprotease to yield an acidic and basic subunit that remain covalently linked by an intermolecular disulfide bond.[38] The acidic subunit undergoes further processing including proteolytic truncation and yields multiple bands ranging from 14 kDa to 45 kDa seen on SDS-PAGE.[33] The expected whole acidic subunit has molecular weights ranging from 42 kDa to 45 kDa and the entire basic subunit, which doesn't undergo any proteolytic truncation is observed at approximately 22 kDa.[33] The truncated acidic subunit polypeptide products are required for the assembly of the subunits into trimers of 7S in the PSVs.[34, 39]

Two 7S trimers fold back to back and associate into a very heat stable 11S hexmer[35, 36] with a molecular weight of approximately 300 kDa – 380 kDa.[38] The proteolytic cleavage by the aspartic acid endoprotease producing the acidic and basic subunits is essential for the hexamer formation.[33, 35, 36, 37, 39]

IgE Epitopes within Ara h 3 Protein

Originally, Ara h 3 was said to have four IgE epitopes identified within only the acidic subunit.[32] The identification of the IgE epitopes in Ara h 3 was performed by Spots technique using synthetic 15-mer peptides that overlapped by eight residues.[32] In 2005, researchers identified the basic subunit as being more allergic than the acidic subunit,[31] which raised the possibility that there are unidentified epitopes in the basic subunit as well. In 2009, a group of scientists performed epitope mapping on the entire Ara h 3 protein by Spots technique using synthetic 15-mer peptides that overlapped by 12 residues in order to confirm the absence or presence of IgE binding epitopes in the basic subunit.[37] Rougé et.al. identified eight epitopes in both the acidic and basic subunits and two of the eight identified epitopes partially overlapped with two of the previously four epitopes.[37]

The modeled Ara h 3 monomer shown in Figure 1-5 indicates a central core made up of two bundles of antiparallel β-sheets associated at both ends into loop regions that consist of three short α-helical stretches.[37] This is consistent with members of the cupin superfamily previously described.[34] Two *N*-glycosylations occur at sites Asn267-Glu-Ser and Asn351-Arg-Ser along the amino acid sequence of the Ara h 3 monomer as shown in Figure 1-6 and Figure 1-7.[37]

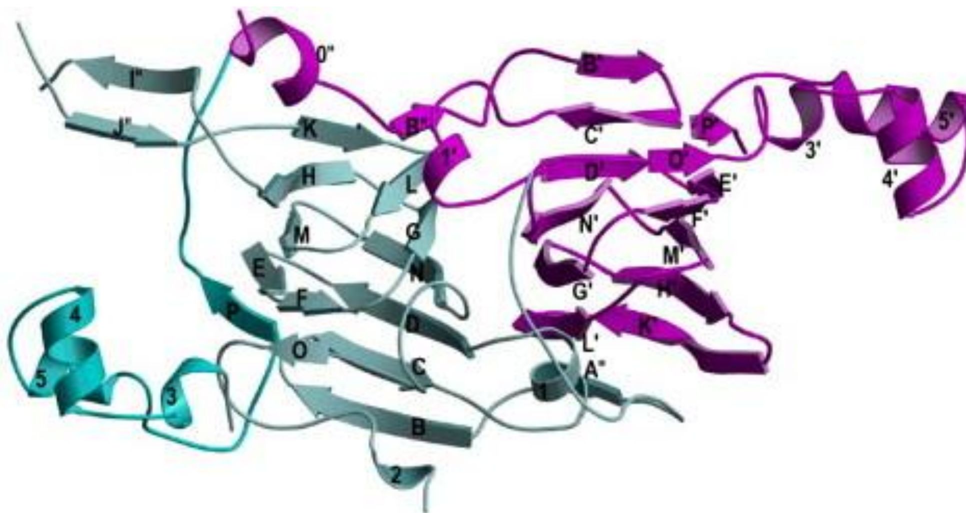


Figure 1-5 Ribbon diagram of the Ara h 3 monomer. N-terminal domain is shown in cyan with the region homologous to peanut trypsin inhibitor lightened and the C-terminal domain shown in magenta. The β-strands are labeled alphabetically and α-helices are numbered. Element labels in the C-terminal domain are primed. First strand in the N-terminal peptide is part of a β-sheet of the C-terminal domain and double primed and so is the C-terminal peptide. Strands I and J in the N-terminal domain are double primed because they become part of a β-sheet in another monomer in the native hexameric state.[36]

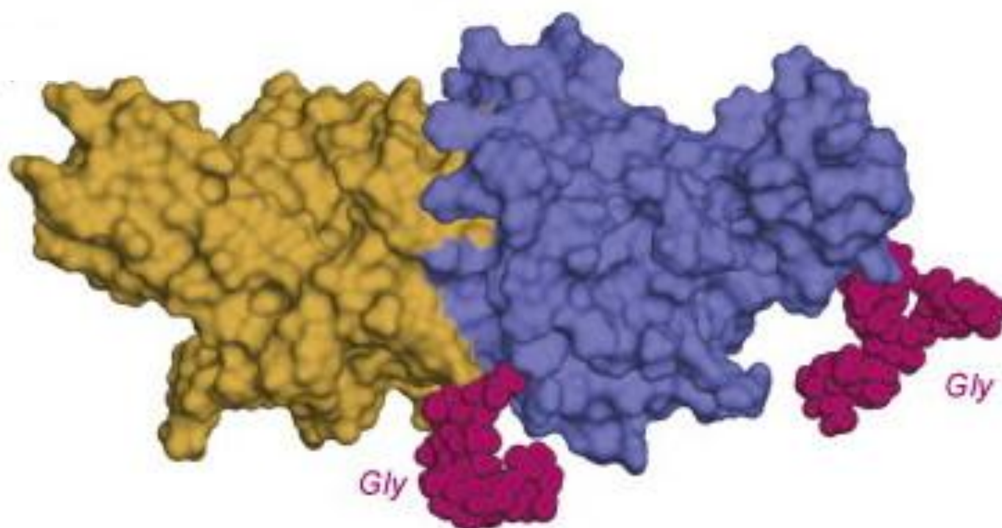


Figure 1-6 Modeled Ara h 3 monomer showing the two *N*-glycosylation sites. Two cupin motifs are colored orange and violet and the *N*-glycan chain is colored pink.[37]

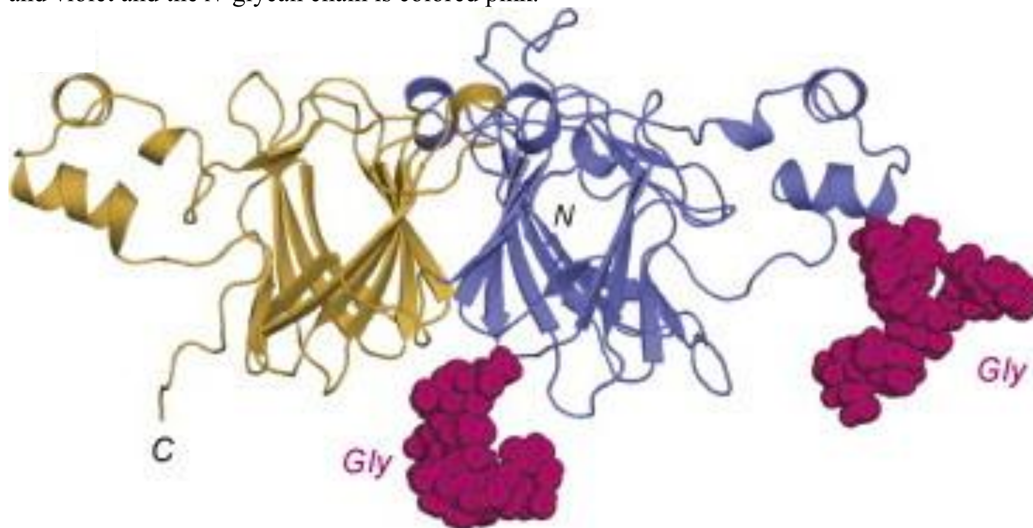


Figure 1-7 Ribbon Diagram of the Ara h 3 monomer. The two cupin motifs are colored in orange and violet and the *N*-glycan chains attached to the monomer are shown in pink. N and C refer to the N- and C- termini of the polypeptide.[37]

Spots experiment using Ara h 3 specific sera from peanut allergic patients as the primary antibody allowed Rougé et.al. to identify eight IgE-binding epitopes spanning the entire amino acid sequence of Ara h 3.[37] Figure 1-8 (A) represents the Ara h 3 monomer as a ribbon diagram[36] depicting three of the four original IgE epitopes shown in the N-terminal domain (acidic subunit) only.[32, 36] Figure 1-8 (B) is a representation of Ara h 3 as a ribbon diagram in its native hexameric form.[36] It is illustrated in the gray monomer in Figure 1-8 (B), the locations of the original IgE epitopes.[32, 36] Figure 1-9 illustrates Ara h 3 in a ribbon diagram showing the locations of the newly identified epitopes and *N*-glycan chains.[37] See Table 1-3 for a list of the IgE-binding epitopes identified by Rabjohn et.al.[32] and Table 1-4 for a list of the IgE-binding epitopes identified by Rougé et.al.[37]

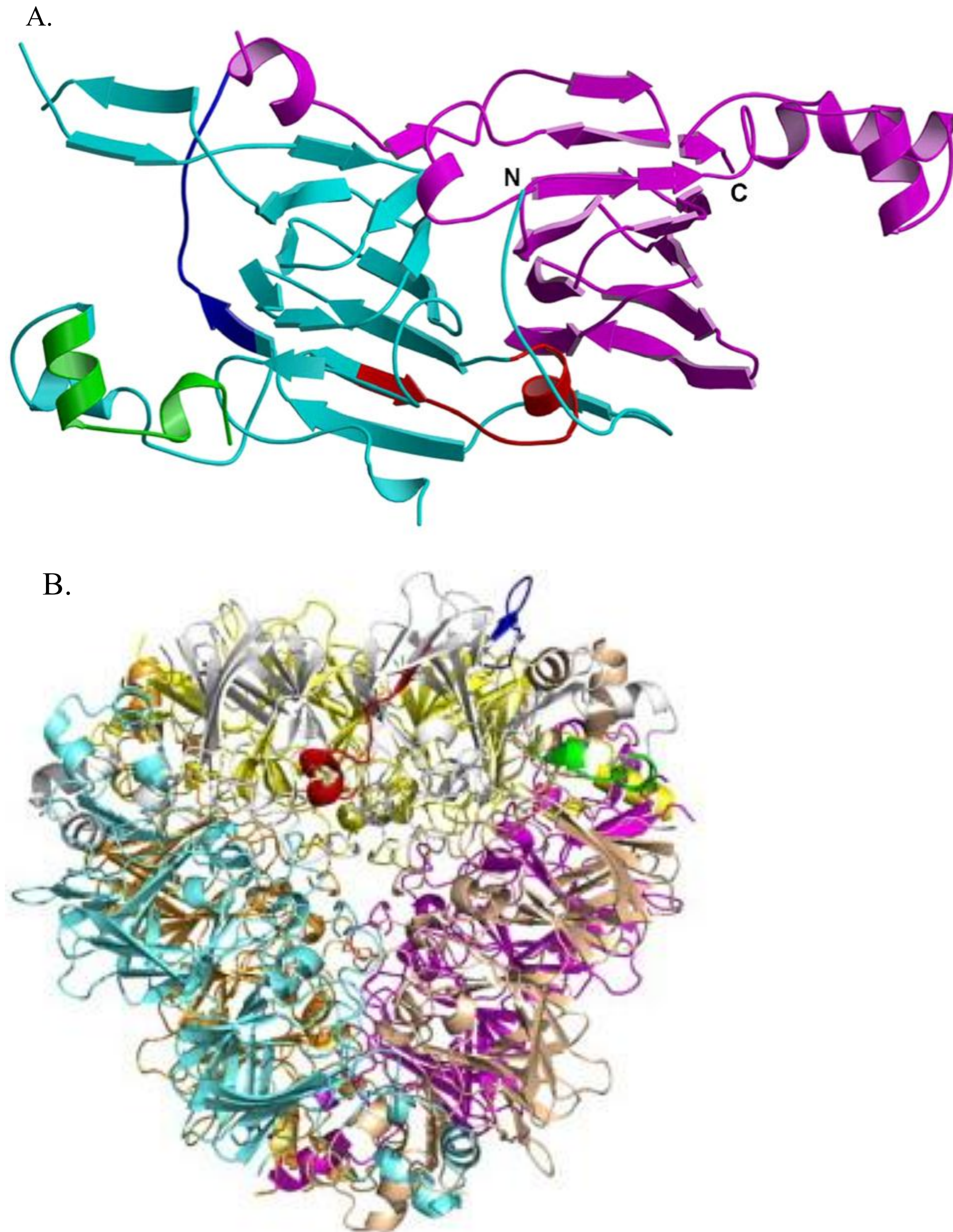


Figure 1-8 (Figure A) Ribbon diagram of the Ara h 3 monomer identifying the original IgE epitopes as described by Rabjohn et.al. N-terminal domain is in cyan with the identification of epitope 1 in red, epitope 2 in green, and epitope 3 in blue. Epitope 4 was disordered and could not be depicted in the crystal structure. C-terminal domain is in magenta. (Figure B) Hexameric Ara h 3 with each individual monomer individually colored. In the gray monomer, epitopes 1, 2, and 3 are shown in red, green, and blue respectively.[36]

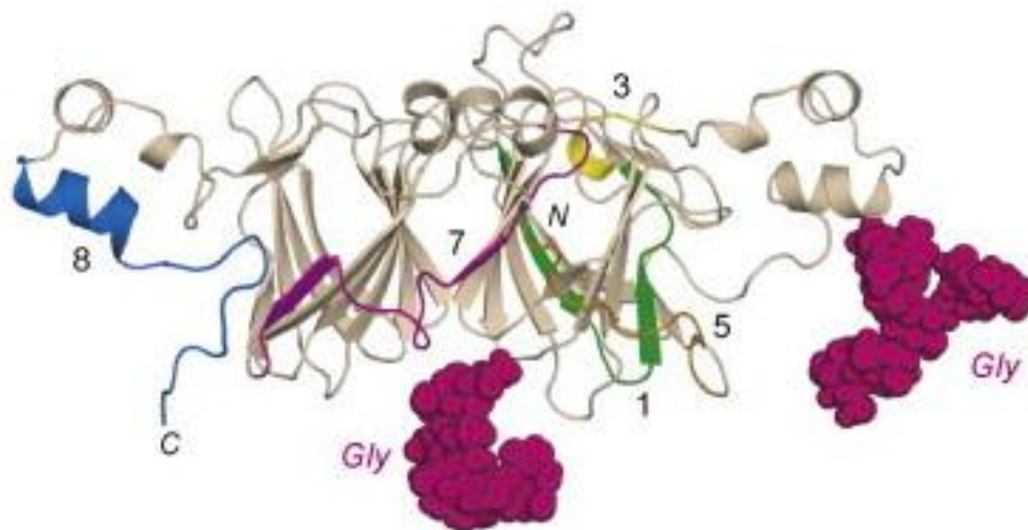


Figure 1-9 Ribbon diagram of the Ara h 3 monomer depicting the 8 newly identified IgE-binding epitopes and the *N*-glycan chains attached to the monomer. Epitopes are numbered and colored (#1: green, #3: yellow, #5: orange, #7: magenta, #8: blue) Regions containing epitopes #2, #4, and #6 are unable to be shown. The *N*-glycan chains are shown in pink and labeled Gly.[37]

Table 1-2 Original Four IgE-Binding Epitopes Identified

IgE Binding Epitope Identified	Ara h 3 Amino Acid Stretch
#1 (located in acidic subunit)	33 IETWN**PN**NQEFECAG 47
#2 (located in the acidic subunit)	240 GNI**F**SG**F**TPE**FL**EQA 254
#3 (located in the acidic subunit)	279 VTVRGG**L**PR**IL**S**P**DRK 293
#4 (located in the acidic subunit	303 DEDEY**EYDE**E**DR**G 317

Table 1-3 Identified IgE-Binding Epitopes and Corresponding Amino Acid Sequence

IgE Binding Epitope Identified	Ara h 3 Amino Acid Stretch
#1 (located in acidic subunit)	49 ALS**R**LVL**RR**NAL**RRP** 63
#2 (located in acidic subunit)	94 EPAQQG**RR**HQSQ**R**PP 108
#3 (located in acidic subunit)	187 L**R**YQQQS**RRR**SLPYSPY 204
#4 (located in acidic subunit)	217 SPRGQHG**RR** 225
#5 (located in acidic subunit)	283 RILSPD**RKR** 291
#6 (located in acidic subunit)	319 D**RRR**G**R**GS**R** 327
#7 (located in basic subunit)	337 ICTASF**KK**NIG**R**N**R**SPDIYNP 357
#8 (located in basic subunit)	409 P**R**EQA**R**QL**K**NNNPF**K**FFVPPSEQS 513

Ara h 3 has been identified as a having homology with a known peanut trypsin inhibitor (TI).[40] Figure 1-5 depicts the Ara h 3 homologous peanut trypsin inhibitor located in the N-terminal domain (teal) lightened.[36, 40] Trypsin and chymotrypsin cleavage sites that are exposed on the surface of Ara h 3 illustrate that the central core structure remains preserved when attacked by gastrointestinal enzymes trypsin and chymotrypsin. Figure 1-10 shows the identified trypsin and chymotrypsin cleavage sites on the Ara h 3 monomer.

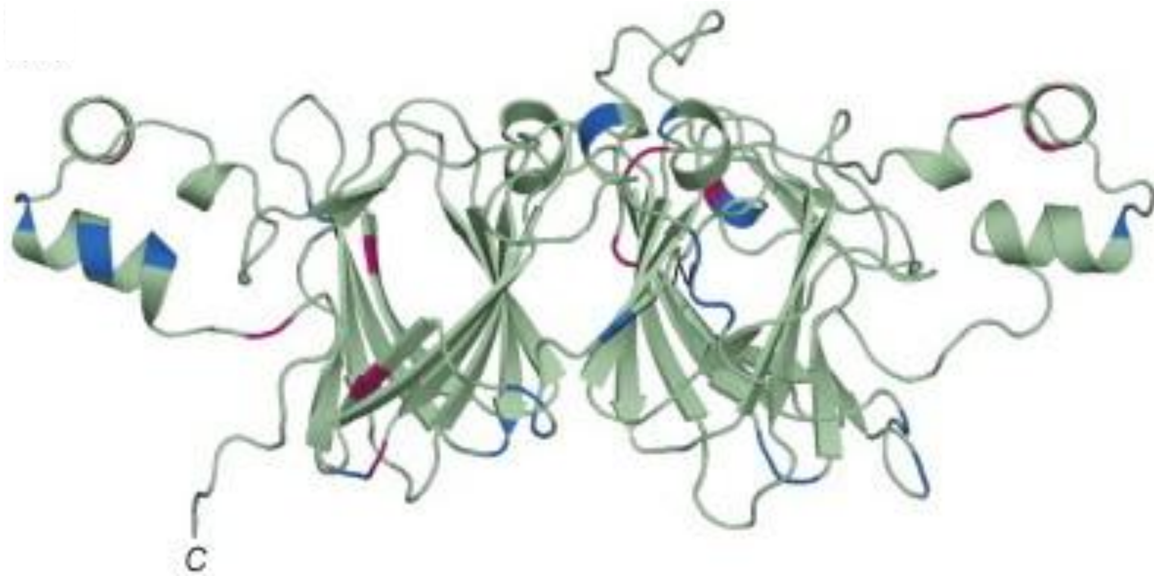


Figure 1-10 Ara h 3 monomer showing the exposed trypsin (pink) and chymotrypsin (blue) cleavage sites.[37]

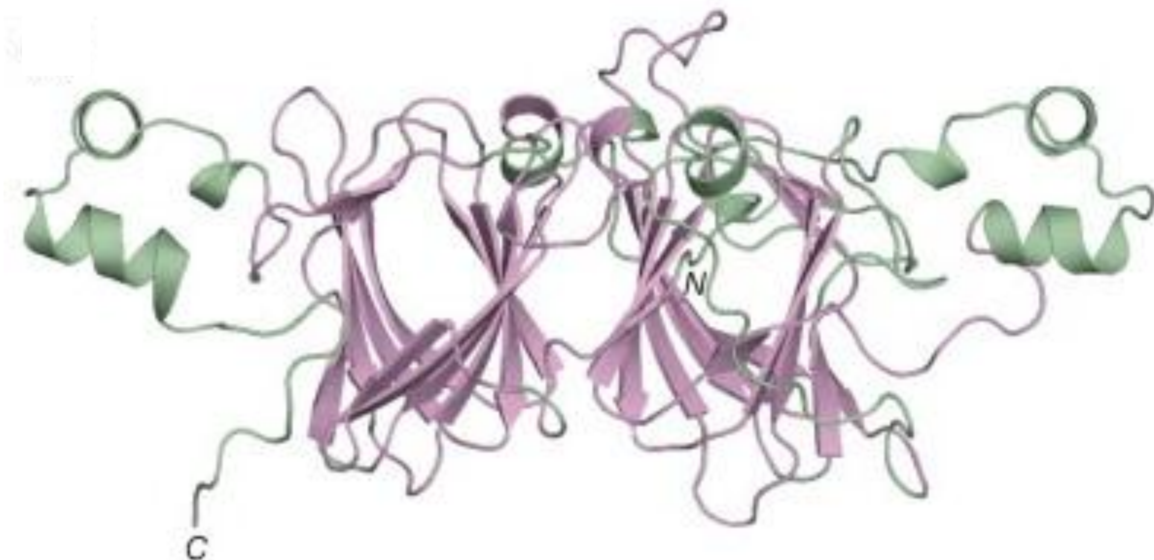


Figure 1-11 Ara h 3 monomer depicting the conserved core structure (pink) after proteolytic attack.[37]

Two of the IgE-binding epitopes originally identified by Rabjohn et.al. are present in the peanut TI sequence.[32, 40] As shown in Figure 1-11, the core structure of the Ara h 3 monomer contains epitopes numbers 1 and 7[37] that were identified by Rougé et.al. and remain protected against proteolytic degradation by digestive enzymes. All other epitopes appear to be destroyed upon proteolytic attack except epitopes 1 and 7.[37] These two epitopes are conserved in other homologous tree nut and legume allergens.[37, 41] It is important to note that the conserved and protected epitope 7, located in the basic subunit of Ara h 3, contains an *N*-glycosylation site at 351Asn-Arg-Ser and shown in Figure 1-12.[37]

The amino acid sequences used by Rabjohn et.al. and Rougé et.al. differed slightly and could have affected the results each group attained in regards to the identified IgE epitopes. A BLAST sequence analysis of the accession number AAC63045 used by Rabjohn et.al[32] against accession number AAM46958 used by Rougé et.al.[37] identified that the sequences have 89 % similarity. Despite the high similarity between the sequences, the slight dissimilarities could have affected the synthetic peptides each group made to use in their respective Spots experiments. Moreover, the serum IgE acquired from peanut allergic patients used in each of the studies was different. These small differences need to be considered when looking at the discrepancies between the two groups. Figure 1-12 depicts the sequence alignments used by both groups. The top sequence (ACC63045) and the bottom sequence (AAM46958) correspond to the amino acid sequences used by Rabjohn et.al.[32] and Rougé et.al.[37] respectively.

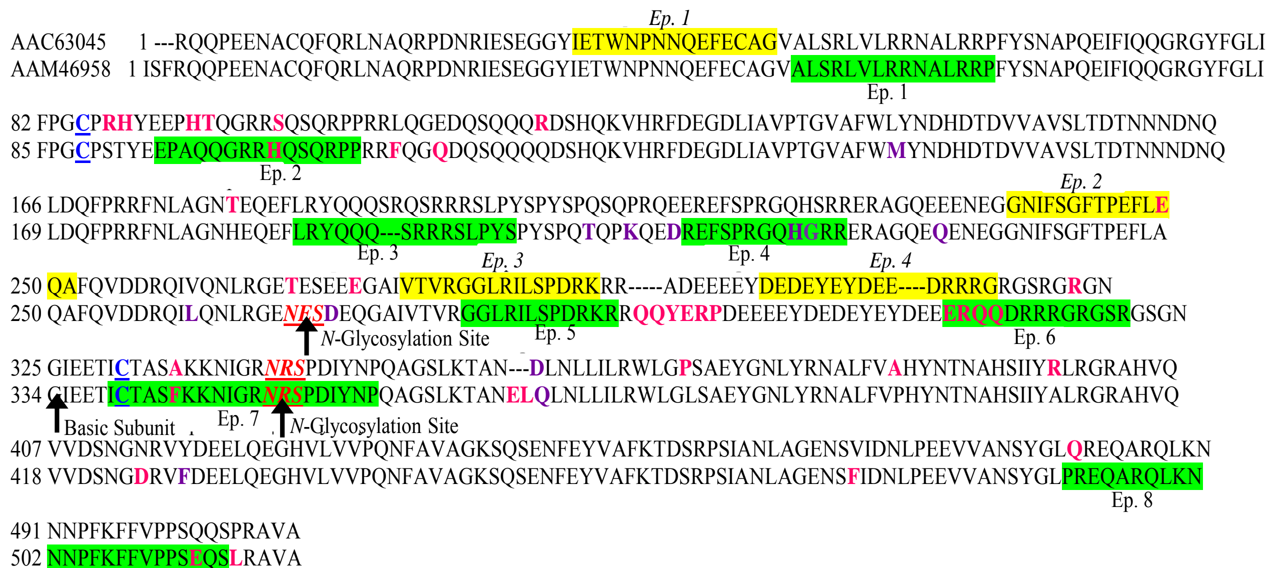


Figure 1-12 Sequence Alignments used by Rabjohn et.al (top sequence, accession number AAC63045) and Rougé et.al. (bottom sequence, accession number AAM46958). Epitopes (Ep.) are identified by Rabjohn et.al. labeled in italics above the sequence and highlighted in yellow. Epitopes identified by Rougé et.al. are labeled in non-italics under the sequence and highlighted in green. Conserved cysteine residue that is involved in the disulfide bond between the acidic and basic subunits is bolded, underlined, and in blue. The *N*-glycosylation sites are bolded, underlined, italicized, red, and labeled. The start of the basic subunit is indicated. Non-conserved residues are in magenta and similar residues are in purple

As seen in Figure 1-12, the cysteine residue involved in the disulfide bond between the acidic and basic subunits is the second residue located in in epitope #7. Also located in this epitope is the *N*-glycosylation site. Since epitope #7 is protected upon proteolytic attack, the *N*-glycan chain and the cysteine residue critical for the trimeric and hexmeric formation in the native structure are both preserved.[37] This could play a potential role in IgE recognition.

Materials and Methods

Materials

Restriction enzymes, enzyme buffers, BSA, T4 ligase, and 10x T4 DNA ligase reaction buffer were purchased from New England BioLabs (Ipswitch, MA, USA). The chemiluminscent detection reagents, ECL Plus was purchased from GE Healthcare (Piscataway, NJ, USA). Forward and reverse primers used in the amplification of Ara h 3 (1 kb) cDNA during polymerase chain reaction (PCR) were ordered from Integrated DNA Technologies (Coralville, IA, USA). PCR master mix, 1 kb DNA ladder, 6x DNA sample loading dye, and Wizard® *Plus* SV DNA mini-prep kits were purchased from Promega (Madison, WI, USA). Antibiotics, kanamycin and chloramphenicol, DNAse I, ethylenediaminetetraacetic acid (EDTA), and Millipore 0.45 µm polyvinylidene fluoride membrane (PVDF) were purchased from Merck KGaA (Darmstadt, Germany, EU). SeeBlue Plus 2 Prestained Protein Molecular Weight Marker, MagicMark™ XP Western Protein Standard, NuPage® 10x and 500 mM DTT protein loading dye, Novex® 4-20 % Tris-Glycine SDS-PAGE gels, pET-9a expression vector, *E. coli* DH5α competent cells, *E. coli* BL-21(DE3) and BL-21(DE3) pLysS protein expression cells were purchased from Invitrogen (Carlsbad, CA, USA). QIAEX II Gel Extraction kits were purchased from Qiagen (Valencia, CA, USA). The cDNA encoding Ara h 3 basic subunit was synthesized by Retrogen, Inc. (San Diego, CA, USA). Phenylmethylsulfonyl fluoride (PMSF) was purchased by Sigma-Aldrich (St. Louis, MO, USA). Primary chicken anti-Ara h 3 (40 kDa) antibody and secondary anti-chicken-HRP were produced by Sigma-Aldrich (St. Louis, MO, USA). Primary chicken anti-Ara h 3 basic subunit antibody was purchased from Pacific Immunology (Ramona, CA, USA). Secondary polyclonal mouse anti-Human IgE-HRP antibody was purchased from Southern Biotech (Birmingham, AL, USA). Ion exchange resins used for protein purification were Macro-Prep High Q and Macro-Prep High S, 4-20 % Tris-Glycine SDS-PAGE gels, and Precision Plus Protein™ WesternC™ Molecular Weight Marker (consisting of pre-stained marker and a StrepTactin-HRP conjugate) were purchased from Bio-Rad Laboratories (Hercules, CA, USA).

Polymerase Chain Reaction (PCR) and Ligation of Ara h 3 Acidic Subunit into pET-9a Expression Vector

Previously, analysis of codon usage by *E. coli* revealed that some codons were rarely used in *E. coli*. Therefore, using site-directed mutagenesis Arginine 91, Arginine 110, Leucine 112, and Threonine 83 were mutated to ones preferred by *E. coli* without altering the protein. In Table 2-1, the Nde I primer that incorporated an ATG start codon and the Bam HI primer which replaced the glycine codon at position 327 with a TAA translation stop site, were the primers used for mutagenesis and the altered codons are underlined.[42]

Table 2-1 Oligonucleotides Used for Mutagenesis and Cloning

Site 1 Mutation Top	cct ggt tgt cct cgt cac tat gaa gag cct cac aca caa ggt cgt cga
Site 1 Mutation Bottom	tcg acg acc ttg tgt gtg agg atc ttc ata gtg acg agg aca acc agg
Sites 2 and 3 Mutations Top	aa aga cca cca cgt cgt ctg caa gga gaa
Site 2 and 3 Mutations Bottom	ttc tcc ttg cag acg acg tgg tgg tct tt
Site 4 Mutation Top	ttg gct ggg aac acc gag caa gag ttg
Site 4 Mutation Bottom	caa ctc ttg ctc ggt gtt ccc agc caa
nde5prime	taa gaa gga gat ata cat atg gct agc ttc cgg ca
bam3prime	ttg gct ggg aac acc gag caa gag ttg

The Ara h 3 (1 kb) cDNA was amplified by PCR and introduced Nde I and Bam HI enzyme restriction sites for ligation into a kanamycin resistant low-copy number expression vector, pET-9a (Figure 2).

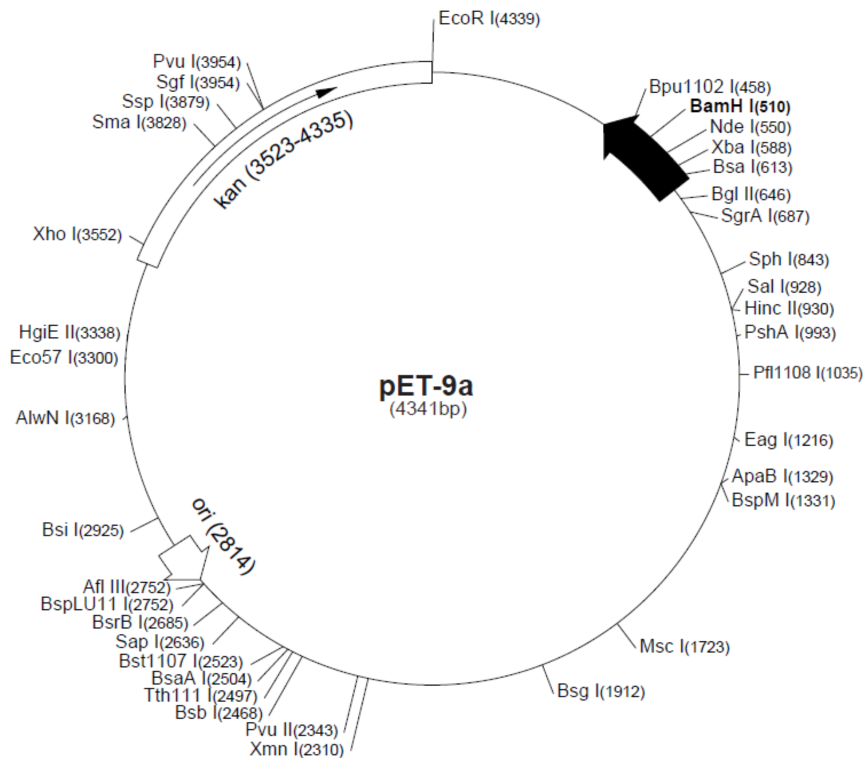


Figure 2 pET-9a Vector Map. It is 4341base pair vector that is kanamycin resistant. Expression of coding strand is transcribed by T7 RNA polymerase.

PCR primers are in the following Table 2-2 and the restriction sites are underlined and the ATG start site and TAG stop site are bolded.

Table 2-2 Primers Used to Amplify Ara h 3 Acidic Subunit cDNA

Primer
Nde I Forward Primer
5' ATTTCAT**ATG**CGCCAGCAACCGGAGG 3'
Bam HI Reverse Primer
5' ATGGATCC**CTA**ATTCCCCCTGCCTCTG 3'

The Ara h 3 (1 kb) cDNA used in the PCR reaction was the template that Hurlburt et al.[42] used and codons were altered already to ones preferred by *E. coli*. The concentration of the template was measured to be 89.5 ng/µl and was used at a final concentration of 190 ng/µl.

The resulting PCR fragment was digested with enzymes Nde I and Bam HI and subjected to agarose gel electrophoresis. The 1 kb PCR fragments were excised from the agarose gel and immediately gel-purified using QIAEX II Gel Extraction Kit following manufacturer's protocol. The expression vector, pET-9a, was digested with the same restriction enzymes Nde I and Bam HI and purified as described above.

Following gel purification of Ara h 3 (1 kb) insert and pET-9a expression vector, 20 µl ligation reactions were set up containing the following: pET-9a expression vector, Ara h 3 (1 kb) insert, 1 x T4 ligase buffer, T4 ligase enzyme, and nuclease-free water. The ligation reactions were set up as a vector to insert ratios, 1:5 (100 ng insert added), 1:12.5 (250 ng insert added), 1:25 (500 ng insert added), and a control reaction containing only the vector and no insert. pET-9a expression vector was added to each reaction at a constant concentration of 20 ng per reaction. Reactions were incubated in a water bath at 4 °C overnight. The ligation reactions are listed in Table 2-3:

Table 2-3 pET-9a Ara h 3 (1kb) Ligation Reactions

	Control Reaction	100 ng Reaction	250 ng Reaction	500 ng Reaction
pET-9a Vector	2.38 µl	2.38 µl	2.38 µl	2.38 µl
Ara h 3 (1kb) Insert	–	0.19 µl	0.48 µl	0.96 µl
10x T4 Ligase Buffer	2 µl	2 µl	2 µl	2 µl
T4 Ligase	1 µl	1 µl	1 µl	1 µl
Nuclease Free Water	14.62 µl	14.43 µl	14.14 µl	13.66 µl

Control Reaction: (-) no Ara h 3 (1 kb) insert added

The ligation reactions were transformed into DH5α competent cells following manufacturer's protocol and plated on LB kanamycin (50 µg /ml) agar. The next day, four colonies were picked to check for successful ligation. Overnight cultures containing 5 ml of LB broth, 5 µl of 1000 x kanamycin (50 µg/ ml), and 1 colony per 15 ml falcon tube were incubated at 37°C while shaking at 225 rpm. Overnight cultures were spun down for 10 minutes at 6,000 rpm, and 4 °C, and DNA was purified using Wizard® *Plus* SV DNA mini-prep following manufacturer's protocol.

Each DNA mini-prep was digested at 37 °C overnight with just Nde I enzyme and Bam HI enzyme was then added the following day for an additional incubation time of 30 minutes and subjected to agarose gel electrophoresis. The Ara h 3 (1 kb) coding region for the acidic subunit of Ara h 3 was released by digestion with Nde I and Bam HI. The correct sequences of the clones were confirmed by DNA sequencing (DNA Sequencing Laboratory, Little Rock, AR, USA) using the T7 promoter and terminator primers.

Ligation of Ara h 3 Basic Subunit into pET-9a Expression Vector

The DNA encoding for the basic subunit of Ara h 3 [Ara h 3 (0.5 kb)] was synthesized by Retrogen, Inc. to have codons preferred by *E. coli*. The cDNA received was transformed into DH5α competent cells according to manufacturer's instructions. DNA mini-preps were performed using Wizard® *Plus* SV mini-preps following manufacturer's protocol and confirmed by DNA sequencing

(DNA Sequencing Laboratory, Little Rock, AR, USA) using T7 promoter and M13 terminator primers. The Ara h 3 (0.5 kb) cDNA and pET-9a expression vector were digested as stated above with Nde I and Bam HI, electrophoresed on a 0.7 % agarose gel, and gel-purified as stated previously.

Following gel purification of Ara h 3 (0.5 kb) insert and pET-9a expression vector, 20 µl ligation reactions were set up as stated for recombinant Ara h 3 (1 kb) ligation into pET-9a. Reactions were incubated in a water bath at 4 °C overnight. The ligation reactions of Ara h 3 (0.5 kb) into pET-9a are listed in Table 2-4:

Table 2-4 pET-9a Ara h 3 (0.5kb) Ligation Reactions

	Control Reaction	100 ng Reaction	250 ng Reaction	500 ng Reaction
pET-9a Vector	1.36 µl	1.36 µl	1.36 µl	1.36 µl
Ara h 3 (0.5kb) Insert	–	1.7 µl	4.2 µl	8.6 µl
10x T4 Ligase Buffer	2 µl	2 µl	2 µl	2 µl
T4 Ligase	1µl	1µl	1µl	1µl
Nuclease Free Water	15.64 µl	13.94 µl	11.44 µl	7.04 µl

Control Reaction: no Ara h 3 (0.5 kb) insert added

The ligation reactions were transformed into DH5α competent cells following manufacturer's protocol and plated on LB kanamycin (50 µg/ml) agar. The next day, six colonies were picked to check for successful ligation. Overnight cultures containing 5 ml of LB broth, 5 µl of 1000 x kanamycin (50 µg/ml), and 1 colony per 15 ml falcon tube were incubated at 37 °C while shaking at 225 rpm. Overnight cultures were spun down for 10 minutes at 6,000 rpm at 4 °C and DNA was purified using Wizard® *Plus* SV DNA mini-prep following manufacturer's instructions.

Each DNA mini-prep was digested at 37 °C overnight with just Nde I enzyme and Bam HI enzyme was then added for an additional incubation time of 30 minutes the following day and subjected to agarose gel electrophoresis. Ara h 3 (0.5 kb) coding region for the basic subunit of Ara h 3 was released by digestion with Nde I and Bam HI. The clones were confirmed by DNA sequencing (DNA Sequencing Laboratory, Little Rock, AR, USA) using T7 promoter and terminator primers.

Transformation and Expression of Recombinant Ara h 3 Subunits

Recombinant Ara h 3 (1 kb) DNA was transformed into BL-21 (DE3) competent cells for expression following manufacturer's protocol and selection was made by plating the cells onto LB kanamycin (50 µg /ml) agar. One culture containing 5 ml of LB, 5 µl of kanamycin (50 µg/ml), and one colony from the overnight plate was started in a 15 ml falcon tube and incubated overnight at 37 °C, while shaking at 250 rpm. The 5 ml overnight culture was added to sterilized one liter LB in a baffled

flask with 1 ml of kanamycin (50 µg/ml) and incubated at 37 °C with shaking at 250 rpm. Samples were taken at time points 0 hr (prior to induction, uninduced), 1 hr, 2 hr, and 3 hr at 37 °C. Protein expression was induced using isopropyl β-D-thiogalactoside (IPTG) once the optical density (O.D.) reached 0.6 on the PharmaSpec UV-Vis spectrophotometer 1700 (Shimadzu, Columbia, MD, USA) at a wavelength of 600 nm, which indicated that the cells in the culture had reached exponential growth and were at an ideal point for induction of protein expression. SDS-PAGE analysis and chicken anti-Ara h 3 (40 kDa)-HRP (horse-radish peroxidase) western blot revealed that Ara h 3 (40 kDa) in BL-21 (DE3) competent cells had leaky protein expression occurring prior to addition of IPTG and post-induction expression levels were low.

To suppress leaky protein expression from occurring prior to induction with IPTG, recombinant Ara h 3 (40 kDa) was transformed into *E. coli* BL-21(DE3) pLysS competent cells for expression following standard manufacturer's protocol and selection was made by plating the cells onto LB kanamycin (50 µg/ml) and chloramphenicol (34 µg/ml) agar. One culture containing 5 ml of LB, 5 µl of kanamycin (50 µg/ml), 5 µl of chloramphenicol (34 µg/ml), and one colony from the overnight plate was started in a 15 ml falcon tube and incubated overnight at 37 °C, while shaking at 250 rpm. The 5 ml overnight culture was added to sterilized one liter LB in a baffled flask with 1 ml of kanamycin (50 µg/ml) and 1 ml of chloramphenicol (34 µg/ml).

The bacterial culture was induced with 200 mM IPTG to a final concentration of 1 mM once the O.D. of the culture reached 0.35 at a wavelength of 600 nm on the spectrophotometer at 37 °C while vigorously shaking. After induction, the culture was removed from the incubator and continued to shake at room temperature overnight. The culture was spun down at 6,000 rpm at 4°C, for 20 minutes the next day. The supernatant was discarded and the expressed cell pellet was stored at -20 °C until solubilization. Recombinant Ara h 3 (40 kDa) uninduced (0 hr) and induced (12 hr) fractions from BL-21 (DE3) cells and BL-21 (DE3) pLysS cells were analyzed by a 4 - 20 % Tris-Glycine SDS-PAGE.

Transformation and expression of the recombinant basic subunit proceeded using the same *E. coli* protein expression cell line BL-21 (DE3) pLysS and conditions of IPTG induction at 0.35 and expression overnight at 20 °C as the acidic subunit.

Anti-Ara h 3 (40 kDa) and (20 kDa) Western Blots

SDS-PAGE resolved the proteins of the bacterial lysates, following expression of recombinant Ara h 3 subunits and were transferred to a 0.45 µm pore size polyvinylidene fluoride membrane (PVDF) membrane electrophoretically using iBlot® Gel Transfer Device (Invitrogen, Carlsbad, CA, USA). The membranes were blocked in 50 ml phosphate-buffered saline containing 0.5 % Tween-20 plus 5 % non-fat dried milk (5 % Blotto) for 60 minutes. After blocking, 1:5,000 dilution of the primary chicken anti-Ara h 3 (40 kDa) antibody or 1:8,000 dilution of the primary chicken anti-Ara h 3 (20 kDa) antibody was

added to the 50 ml of 5 % Blotto and incubated while rocking gently for 60 minutes. After incubation with the primary antibody, the membranes were washed with phosphate-buffered saline containing 0.5 % Tween-20 (PBST) three times for five minutes. Detection of Ara h 3 was completed by using a polyclonal anti-chicken-HRP conjugated secondary antibody at a 1:100,000 dilution. The western blots were incubated in the presence of the secondary antibody for 30 minutes in 2 % Blotto, washed with PBST, and incubated with ECL Plus, a chemiluminscent substrate according to the manufacturer's instructions. Multiple images were captured at various exposure times using an incremental time setting on the FujiFilm LAS - 4000 (GE Healthcare, Piscataway, NJ, USA).

Solubilization of Recombinant Ara h 3 Acidic Subunit

The bacterial pellet containing the expressed recombinant Ara h 3 (40 kDa) was thawed on ice and then re-solubilized by pipetting up and down in 150 ml of buffer containing 0.5 M Tris pH 8.0, 300 mM NaCl, 5 mM EDTA at 4 °C and 3 ml of 50 mM PMSF. The bacterial lysate was sonicated on ice at one minute intervals with 30 seconds rest, three times or until viscosity was significantly reduced, then centrifuged at 10,000 rpm at 4 °C for 20 minutes. The insoluble pellet was stored at -20 °C and soluble recombinant Ara h 3 (40 kDa) was subjected to the preliminary step of protein purification.

Ammonium Sulfate Precipitation of Recombinant Ara h 3 Acidic Subunit

Recombinant Ara h 3 (40 kDa) was precipitated from the soluble bacterial lysate by the addition of ammonium sulfate while stirring at 4 °C to a final saturation of 60 %. The ammonium sulfate precipitated proteins were collected after 25 %, 50 %, and 60 % saturation by centrifugation at 10,000 rpm at 4 °C for 20 minutes and stored at -20 °C until use. Prior to ion exchange purification, 25 %, 50 %, and 60 % ammonium sulfate pellets were subjected to SDS-PAGE to determine which pellet contained recombinant Ara h 3 (40 kDa). The 50 % ammonium sulfate pellets were used and dissolved in 50 - 75 ml of 20 mM Tris, 1 mM EDTA - 0 (SJM - 0) salt buffer.

Ion Exchange Chromatography of Recombinant Ara h 3 Acidic Subunit

The 50 % ammonium sulfate pellet of recombinant Ara h 3 acidic subunit pellet was solubilized in 50 ml of SJM - 0 salt buffer at pH value of 9.0 and centrifuged for 15 minutes at 10,000 rpm at 4 °C to remove any insoluble particles. After centrifugation, the absorbance was taken at a wavelength of 280 nm to determine the approximate amount of protein in solution. Ara h 3 (40 kDa) in 50 ml of SJM - 0 was loaded onto a High Prep Q column (2.5 x 12 cm) (Bio-Rad Laboratories) and washed with approximately 200 ml of SJM - 0 buffer and then 200 ml of SJM - 100 buffer. A linear salt gradient (200 ml total volume) of SJM - 100 buffer to SJM - 500 buffer was applied and 80 two ml fractions were collected. A flow rate of five ml/min was maintained for sample loading, washing, and gradient elution. Collected

fractions were assayed for protein by absorbance at 280 nm on a spectrophotometer and strongly absorbing fractions were examined by SDS-PAGE.

Subsequent purification attempts used new precipitated 50 % ammonium sulfate pellets containing recombinant Ara h 3 acidic subunits. The pellet was solubilized in 75 ml of SJM - 0 salt buffer at pH value of 8.4 and loaded onto a High Prep Q column (2.5 x 12 cm) and a linear salt gradient (200 ml total volume) of SJM - 250 buffer to SJM - 500 buffer was applied. In another purification, the pellet solubilized in 50 ml of SJM - 0 salt buffer at pH value of 9.0 and loaded onto a High Prep Q column (2.5 x 12 cm) and a linear salt gradient (200 ml total volume) of SJM - 50 buffer to SJM - 500 buffer was applied. The fractions and SJM - 500 buffer wash (approximately 248 ml) containing Ara h 3 (40 kDa) were pooled and diluted 1:2 with SJM - 0 buffer to reduce the NaCl concentration to approximately 250 mM from this purification at pH 9.0. This solution was re-loaded onto the High Prep Q column (2.5 x 12 cm) and washed with 200 ml of SJM - 250 buffer. A linear salt gradient (200 ml total volume) of SJM - 250 buffer to SJM - 520 buffer was used to elute Ara h 3 (40 kDa). Fractions were collected and assayed as described above. Confirmation of protein in fractions was achieved by SDS-PAGE and western blot analysis using anti-Ara h 3 (40 kDa) antibody.

A cation exchange column was utilized and a 50 % ammonium sulfate pellet containing recombinant Ara h 3 (40 kDa) was solubilized in 50 ml of SJM - 0 salt buffer at pH value of 6.5. Ara h 3 (40 kDa) in 50 ml of SJM - 0 was loaded onto a High Prep S column (2.5 x 12 cm) and washed with approximately 200 ml of SJM - 0 buffer and then 200 ml of SJM - 50 buffer. A linear salt gradient (200 ml total volume) of SJM - 50 buffer to SJM - 500 buffer was applied and 80 two ml fractions were collected. Collected fractions were assayed for protein by absorbance at 280 nm on a spectrophotometer and absorbing fractions were examined by SDS-PAGE.

Inclusion Body Purification of the Recombinant Ara h 3 Acidic and Basic Subunits

One liter cell pellets containing Ara h 3 subunits were re-suspended in 20 ml of 50 mM Tris, 100 mM NaCl, 1 mM EDTA pH 8.0 and 2 mg/ml of lysozyme was added. The cell lysates were sonicated[ii] briefly on ice and incubated for 20 minutes at room temperature. Bacterial lysates were centrifuged at 5,000 *g* for 10 minutes and the supernatant was removed and discarded. The remaining cell pellets were transferred to ice and re-suspended in 50 ml of cold 50 mM Tris, 100 mM NaCl, 1 mM EDTA, and 0.1 % sodium deoxycholate pH 8.0[iii]; and incubated on ice for 10 minutes occasionally mixing the cell lysates to prevent settling. A final concentration of 8 mM of $MgCl_2$ was added to each of the tubes and DNAse I to a final concentration of 10 µg/ml and incubated at 4 °C overnight with

[ii] 3x 30 seconds with 60 seconds of rest between sonication on ice
[iii] Buffer was made while and adjusted while on ice to pH 8.0 and stored at 4°C until use.

occasional mixing until all viscosity disappeared. The inclusion bodies were removed the following day by centrifugation at 10,000 *g* for 10 minutes. The supernatant was removed and the pellets were re-spun at 6,000 *g* to reduce background and tighten the pellets. The inclusion body pellets were washed by re-suspension in 25 ml 1 % NP-40, 50 mM Tris, 100 mM NaCl, 1 mM EDTA, 2 M urea pH 8.0, and centrifuged at 6,000 *g* for 10 minutes. The Ara h 3 (20 kDa) inclusion body pellet was washed again by re-suspension in 25 ml of 50 mM Tris, 2 M urea pH 8.0, centrifuged at 6,000 *g* for 10 minutes whereas Ara h 3 (40 kDa) inclusion body pellet was treated the same way except re-suspended in 10 ml of buffer. Both remaining inclusion body pellets were washed by re-suspension with 20 ml of 50 mM Tris, 100 mM NaCl, 1 mM EDTA pH 8.0, centrifuged at 10,000 *g* for 10 minutes. The pellets were washed once more with 20 ml of 50 mM Tris, 100 mM NaCl, 1 mM EDTA pH 8.0, and centrifuged at 6,000 *g* for 20 minutes.

The inclusion bodies of both subunits needed to be cleaned up further. The pellets were re-suspended in 4 M urea and incubated overnight at room temperature. The pellets were centrifuged at 15,000 rpm for 30 minutes and subjected to a 4 - 20 % Tris-Glycine SDS-PAGE (Bio-Rad Laboratories). The protein bands corresponding to the subunits were excised from the SDS-PAGE gels, placed into a 1.5 ml microcentrifuge tube, crushed into a paste, and re-suspended in 1 ml of 3x SDS-sample dye plus reducing agent DTT. The tubes were vortexed, placed in a 65 °C water bath for overnight (approximately 12 hr), and centrifuged the next day at 15,000 rpm for 15 minutes. The supernatant was removed and the process was repeated two more times. After removal of the supernatant, the supernatants containing the purified inclusion body subunits were stored at -20 °C for use in subsequent SDS-PAGE and western blot analysis.

Chicken Recombinant Ara h 3 (20 kDa) Antibody Production

The recombinant Ara h 3 basic subunit inclusion body was subjected to SDS-PAGE and the bands that corresponded to the 20 kDa subunit were cut out and sent for custom antibody production. The proteins in the SDS-PAGE matrix were crushed and mixed with saline. Addition of Freund's adjuvant was mixed in to suspend the gel particles containing Ara h 3 (20 kDa). The mixture was used to inoculate chickens and yolks from the chicken eggs are collected and tested for antibody production.

In vitro Maillard Reaction of Purified Native Raw Ara h 3

Purified Ara h 3 from raw peanut (native Ara h 3) (0.653 mg/ml) was added to in vitro Maillard reactions at a final concentration of 0.5 mg/ml. This reaction consisted of native Ara h 3 incubated at 55 °C in the presence of 0.25 mol/L glucose or xylose and 0.02 % sodium azide for a period of 72 hours (3 days). Samples were collected after immediately adding sugar to the reaction, at 24 hours (day 1) and 72 hours (day 3) and used in immunoblot analysis experiments.

Normalization of Samples and IgE Western Blot Analyses

Samples subjected to the Maillard reaction, cell lysates, in which recombinant Ara h 3 (40 kDa) and (20 kDa) subunits were expressed and purified via gel-inclusion body method as described previously, raw and roasted crude peanut protein, and native raw and roasted Ara h 3 were subjected to SDS-PAGE for normalization of protein loads and western blot analysis using anti-Ara h 3 (40 kDa) and serum IgE from peanut allergic patients. After normalization, SDS-PAGE gels were transferred to a 0.45 µm pore size PVDF membrane electrophoretically using iBlot® Gel Transfer Device (Invitrogen, Carlsbad, CA, USA). The membranes were blocked in 2 % Blotto for 30 minutes. After blocking, the membranes were washed in PBST and each individual membrane was incubated overnight (approximately12 hr) at 4 °C with a different dilution of human serum (dilutions listed in Table 2-5). The following day, the membranes were washed with PBST. Detection of bound IgE was completed by using a polyclonal mouse anti-human IgE-HRP conjugated secondary antibody at a 1:10,000 dilution. The immunoblots were incubated in the presence of the secondary antibody for 30 minutes, washed with PBST, and incubated with ECL Plus, a chemiluminscent substrate according to the manufacturer's instructions. Multiple images were captured at various exposure times using an incremental time setting on the FujiFilm LAS-4000 (GE Healthcare, Piscataway, NJ, USA).

Table 2-5 Patient Serum Dilutions Used in Immunoblot Assays

Patient Identification	Patient Serum Dilution Factor
P001	1:40
P002	1:5
P003	1:5
P004	1:5
P005	1:10
P006	1:2
P007	1:2
P008	1:5
P009	1:2
P010	1:2
P011	1:2
P012	1:2
P013	1:10
P014	1:10

Results

PCR of Recombinant Ara h 3 (40 kDa, Acidic Subunit) and Ligation into pET-9a, A Low-Copy Number Expression Vector

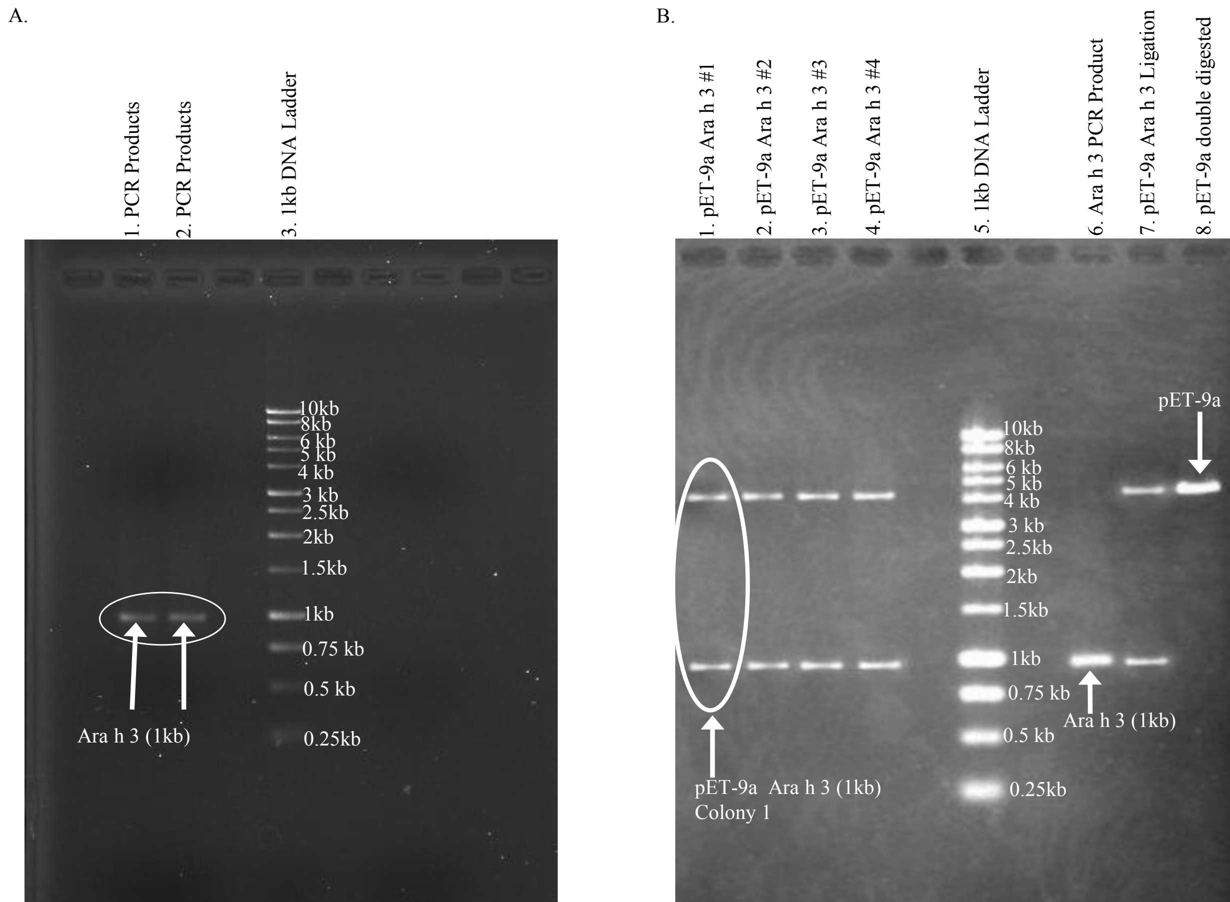


Figure 3-1 PCR Amplification of Ara h 3 1 kb cDNA and ligation into pET-9a vector. (Figure A) Ara h 3(1 kb) cDNA was PCR amplified (lanes 1 and 2) then (Figure B) ligated into a low-copy expression vector pET-9a. Analysis for correct band size was carried out by agarose gel electrophoresis. Double restriction digests were done with Nde I and Bam HI. Ara h 3 (1 kb) insert (Figure A) and pET-9a Ara h 3 (1 kb) Colony #1 (Figure B) are indicated with a circle and arrow. 1 kb DNA ladders are labeled in kilobase pairs (kb).

Ara h 3 (1 kb) cDNA was PCR amplified from a pre-existing clone (Figure 3-1 (A) lanes 1 and 2) used by Hurlburt et.al. (see Materials and Methods). The PCR products were subjected to a 1 % agarose gel prior to ligation to check for correct band size at 1 kb. Ligation reactions of Ara h 3 (1 kb) and pET-9a proceeded overnight and four colonies were picked, DNA purified with a mini-prep, restriction enzyme digested, and subjected to agarose gel electrophoresis (Figure 3-1 (B) lanes 1-4). Lane 6 is Ara h 3 (1 kb) insert from the PCR reaction, Lane 7 is the 250 ng : 20 ng insert to vector ligation reaction, and lane 8 is

pET-9a expression vector digested with Nde I and Bam HI. Colony 1 out of 4 (Figure 3-1 (B) lane 1) was chosen to proceed with due to its highest DNA concentration out of the four.

Ligation of Synthesized Ara h 3 Basic Subunit ((0.5 kb), 20 kDa) Into Low-Copy Number Expression Vector, pET-9a

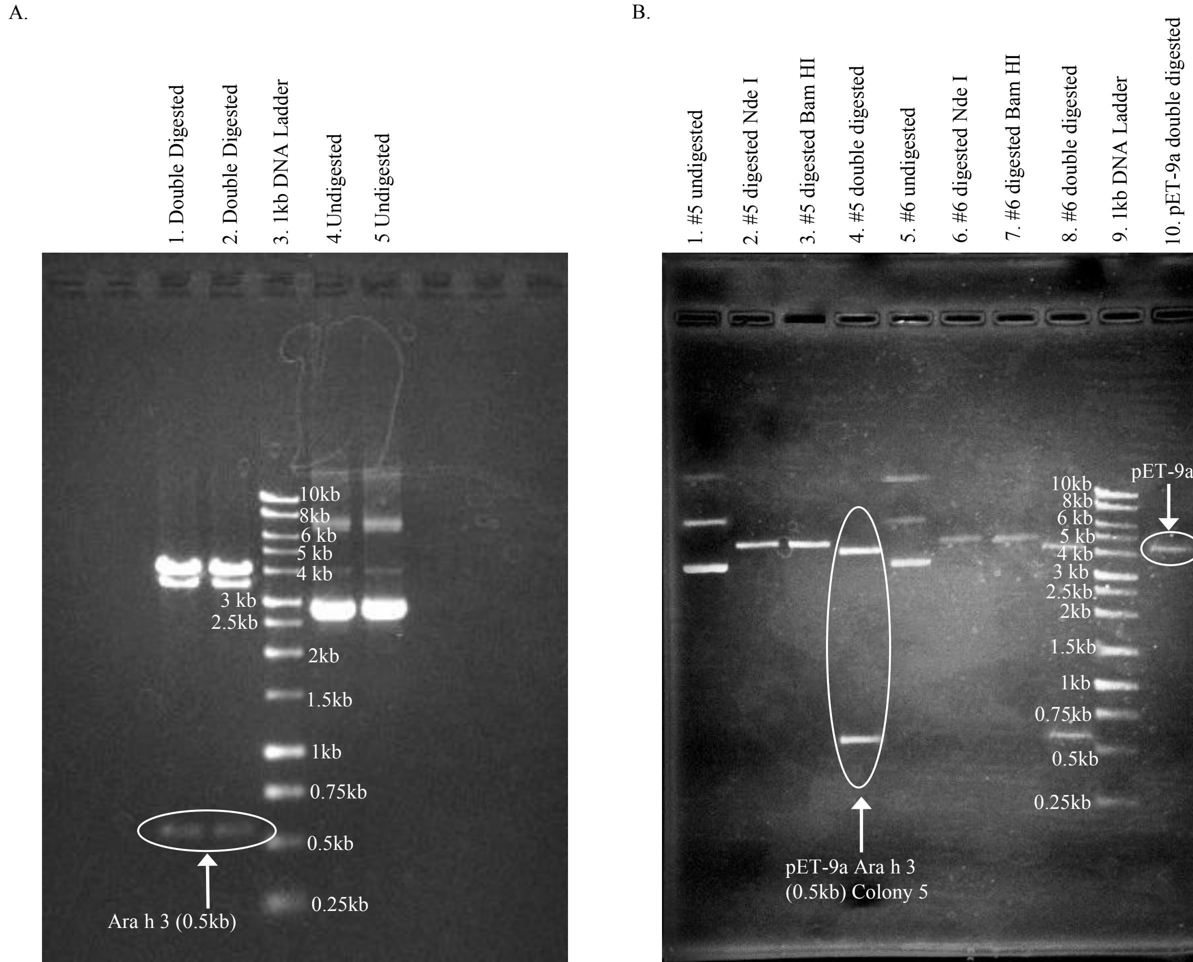


Figure 3-2 Ara h 3 (0.5 kb) cDNA was commercially synthesized (Figure A), then subsequently ligated into low copy number, pET-9a, expression vector (Figure B). Analysis for correct band size (Figure A) and successful ligation (Figure B) was carried out by agarose gel electrophoresis. Double restriction digestions were done with Nde I and Bam HI. Ara h 3 (0.5 kb) (Figure A) and pET-9a Ara h 3 (0.5 kb) Colony 5 (Figure B) are indicated by a circle and arrow. 1 kb DNA ladders are labeled in kilobase pairs (kb).

Ara h 3 (0.5 kb) subunit was commercially synthesized by Retrogen to have codons preferred by *E. coli* and double restriction digested with Nde I and Bam HI (Figure 3-2 (A) lanes 1 and 2). The digested products were subjected to a 1 % agarose gel to check for correct band size at 500 bp (0.5 kb) prior to proceeding with ligation reactions into to pET-9a. Ligation reactions proceeded overnight and 6 colonies were chosen from the 500 ng insert plate for DNA mini-preps, restriction digests, and were subjected to agarose gel electrophoresis (Figure 3-2 (B)). Figure 3-2 (B) displays colonies #5 and #6, both of which had the correct 500 bp insert, and colony #5 (Figure 3-2 (B) lane 4) was chosen to proceed.

Protein Expression of Recombinant Ara h 3 Acidic Subunit

Recombinant Ara h 3 (1 kb) DNA from plasmid #1 was transformed into BL-21 (DE3) protein expression *E. coli* competent cells and expressed at 37 °C. Samples were subjected to SDS-PAGE and anti-Ara h 3 (40 kDa) western blot for determination of time for optimal protein expression. Positive and negative controls, recombinant Ara h 3 (40 kDa) inclusion body and untransformed BL-21 (DE3) competent cells respectively, were included.

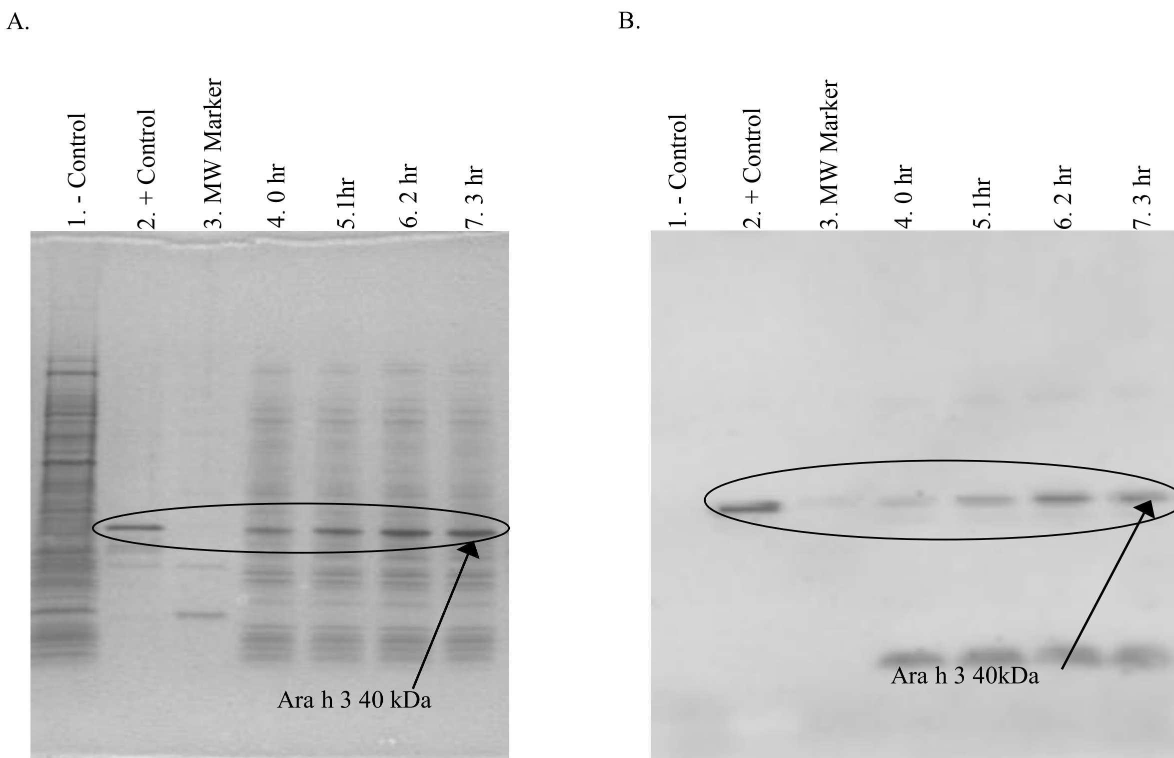


Figure 4-1 Expression of Ara h 3 (40 kDa) from BL-21 (DE3) transformant #1 at 37°C. Samples of Ara h 3 (40 kDa) in BL-21 (DE3) cells at 0 hr (uninduced), 1 hr, 2hr, and 3 hr after induction at 37°C. All samples were subjected to SDS-PAGE (Novex) (Figure A) and anti-Ara h 3(40 kDa) western blot (Figure B). Negative Control (-): Untransformed BL-21 (DE3) cells, Positive (+) Control: recombinant Ara h 3 (40 kDa) inclusion body. MW Marker: MagicMark

As shown in Figure 4-1 (A and B), protein expression was occurring prior to induction with IPTG. The protein levels from the uninduced fraction (Figure 4-1 (A) lane 4) are equivalent to the post-induction fractions at time points 1 hr, 2 hr, and 3 hr (Figure 4-1 (A) lanes 5-7). The induced fractions from the anti-Ara h 3 (40 kDa) western blot (Figure 4-1 (B) lanes 5-7) all appear to have approximately the same amount of protein expression.

Another bacterial cell line for protein expression was investigated to address leaky protein expression prior to induction with IPTG. In previous work, it was found that growth at lower temperatures and induction at a lower absorbance would assist in proper protein folding, minimize

inclusion body formation, and increase protein expression post-IPTG induction. Two one liter cultures of Ara h 3 (40 kDa) in two different cell lines, BL-21 (DE3) and BL-21 (DE3) pLysS were expressed at 37 °C until an induction point of 0.35, the cultures were removed from the incubator to 20 °C, and the induced cultures of Ara h 3 (40 kDa) continued to express overnight at 20 °C (approximately 12 hr) for comparison. In Figure 4-2, protein expression levels of recombinant Ara h 3 (40 kDa) in the expression hosts are shown by SDS-PAGE. Pre-induction (0 hr, uninduced) and post-induction (expressed for 12 hr) of recombinant Ara h 3 (40 kDa) in *E. coli* cells BL-21 (DE3) are illustrated in Figure 4-2 lanes 1 and 2 respectively and recombinant Ara h 3 (40 kDa) in *E. coli* cells BL-21 (DE3) pLysS are represented in lanes 5 and 6 respectively.

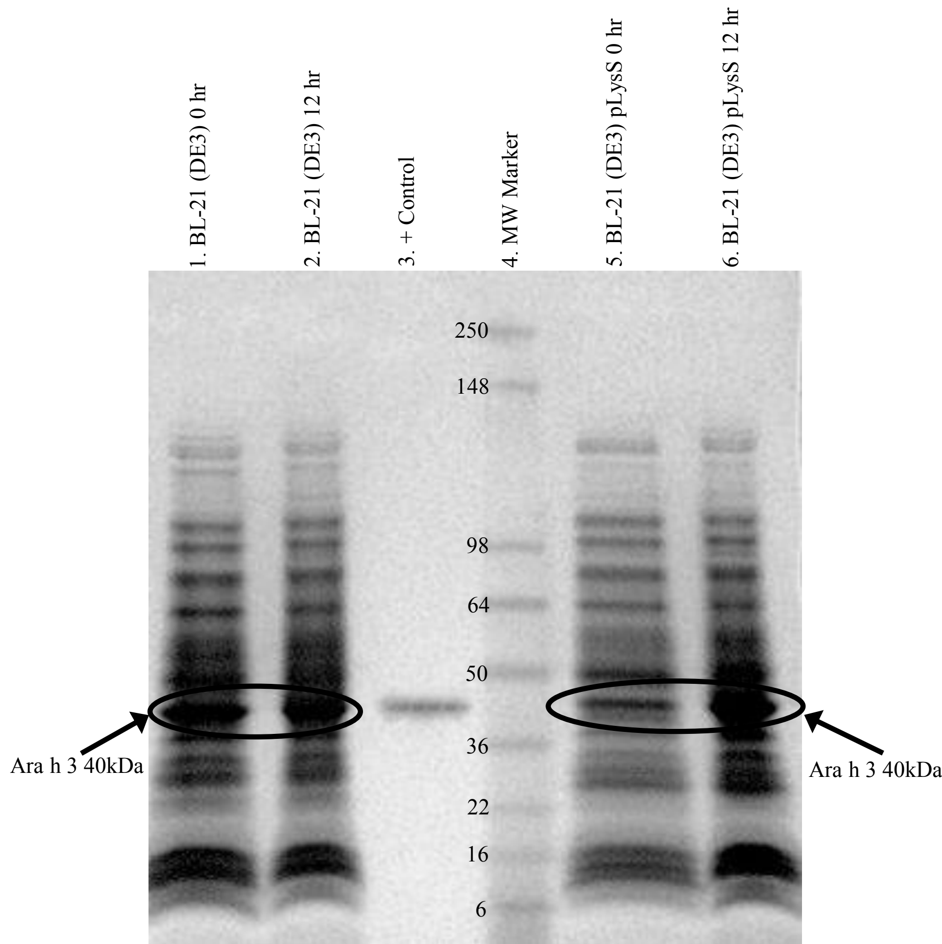


Figure 4-2 Comparison between protein expression levels of recombinant Ara h 3 (40 kDa) in *E. coli* cells, BL-21 (DE3) and BL-21 (DE3) pLysS at expressed 20°C. Samples were subjected to a 4 - 20 % Tris-Glycine SDS-PAGE (Bio-Rad) for analysis. Ara h 3 (40 kDa) is circled with an arrow and labeled. Positive (+) Control: recombinant Ara h 3 (40 kDa) inclusion body.

When Ara h 3 (40 kDa) was expressed in BL-21 (DE3) *E. coli* cells, inducing the bacterial culture at an O.D. of 0.35 versus 0.6 and the temperature drop from 37 °C to 20 °C after the addition of IPTG increased the amount of protein expressed (compare Figure 4-2 lane 2 to Figure 4-1 (A) lane 7).

Nevertheless, protein expression levels after induction of Ara h 3 (40 kDa) at O.D. 0.35, temperature drop to 20 °C, and induction overnight in *E. coli* cells BL-21 (DE3) (Figure 4-2 lanes 1 and 2) had no effect on leaky protein expression occurring pre-induction (Figure 4-2 lane 1). Pre-induction and post-induction samples of Ara h 3 (40 kDa) in BL-21 (DE3) cells (Figure 4-2 lanes 1 and 2) appear to have approximately the same amount of expressed protein.

In *E. coli* cells, BL-21 (DE3) pLysS, Ara h 3 (40 kDa) had an increase in protein expression after the addition of IPTG (Figure 4-2 lanes 5 and 6). There is less evidence of leaky protein expression occurring than in BL-21 (DE3) cells (compare Figure 4-2 lanes 2 and 5). Post-induction with IPTG (Figure 4-2 lane 6), there is a substantial amount of Ara h 3 (40 kDa) visible and protein expression is easily distinguishable from the uninduced fraction (lane 5). It was concluded that *E. coli* BL-21 (DE3) pLysS competent cells were the better option to eliminate leaky protein expression pre-IPTG induction and still be able to achieve maximal protein expression.

Protein Expression of Recombinant Ara h 3 Basic Subunit

With respect to recombinant Ara h 3 (40 kDa) in BL-21 (DE3) pLysS cells, there were significant impacts on the amount of protein expressed (Figure 4-2 lane 6). Therefore, it was concluded to use the same *E. coli* cells BL-21 (DE3) pLysS for protein expression and same conditions for the recombinant Ara h 3 basic subunit.

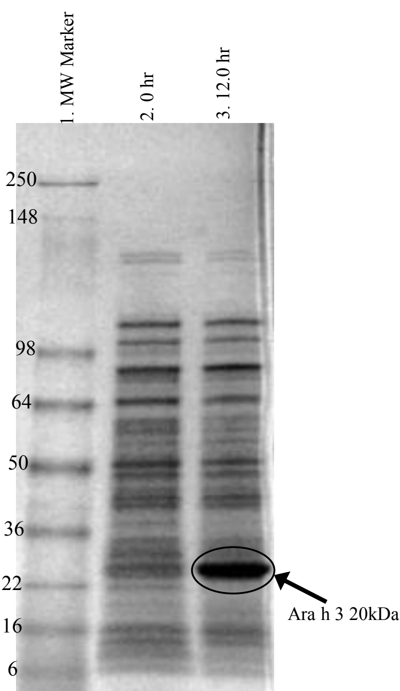


Figure 4-3 Protein expression of recombinant Ara h 3 basic subunit in *E. coli* cells BL-21 (DE3) pLysS. The uninduced (0 hr) and induced (overnight, approx. 12 hr) were subjected to a 4 - 20 % Tris-Glycine SDS-PAGE (Novex). Ara h 3 (20 kDa) is circled with an arrow pointing to it.

In *E. coli* cells BL-21 (DE3) pLysS, recombinant Ara h 3 (20 kDa) does not appear to have significant leaky protein expression prior to the addition of IPTG (Figure 4-3 lane 2). Recombinant Ara h 3 (20 kDa) had an increase in protein expression after induction of the bacterial culture at an O.D. of 0.35 and the temperature was dropped from 37 °C to 20 °C (Figure 4-3 lanes 3).

Solubility and Ammonium Precipitation of Recombinant Ara h 3 (40 kDa)

Recombinant Ara h 3 (40 kDa) was expressed in BL-21(DE3) pLysS *E. coli* cells and tested for solubility under various conditions and pH values. Figure 5-1 (A) shows expression of Ara h 3 (40 kDa) pellets that were re-suspended in buffers at the pH values indicated above each lane (6.5, 7.0, and 8.0), sonicated, and centrifuged at 6,000 rpm at 4 °C. The soluble and insoluble fractions were subjected to SDS-PAGE and stained. It was found that recombinant Ara h 3 (40 kDa) was solubilized in 0.5 M Tris buffer containing 300 mM NaCl, 5 mM EDTA, and 1 mM PMSF at a pH value of 8.0 (Figure 5-1 (A) lanes 9 and 10).

Following solubility testing, recombinant Ara h 3 (40 kDa) was precipitated by ammonium sulfate. Figure 5-1 (B) lanes 7-9 shows the precipitates following each percentage of saturation with ammonium sulfate of expressed and solubilized recombinant Ara h 3 (40 kDa). Ara h 3 (40 kDa) was saturated by 25 %, 50 %, and 60 % of ammonium sulfate and all precipitates were subjected to SDS-PAGE for analysis. As shown in Figure 5-1 (B) lane 8, a majority of Ara h 3 (40 kDa) precipitated at 50 % ammonium sulfate saturation.

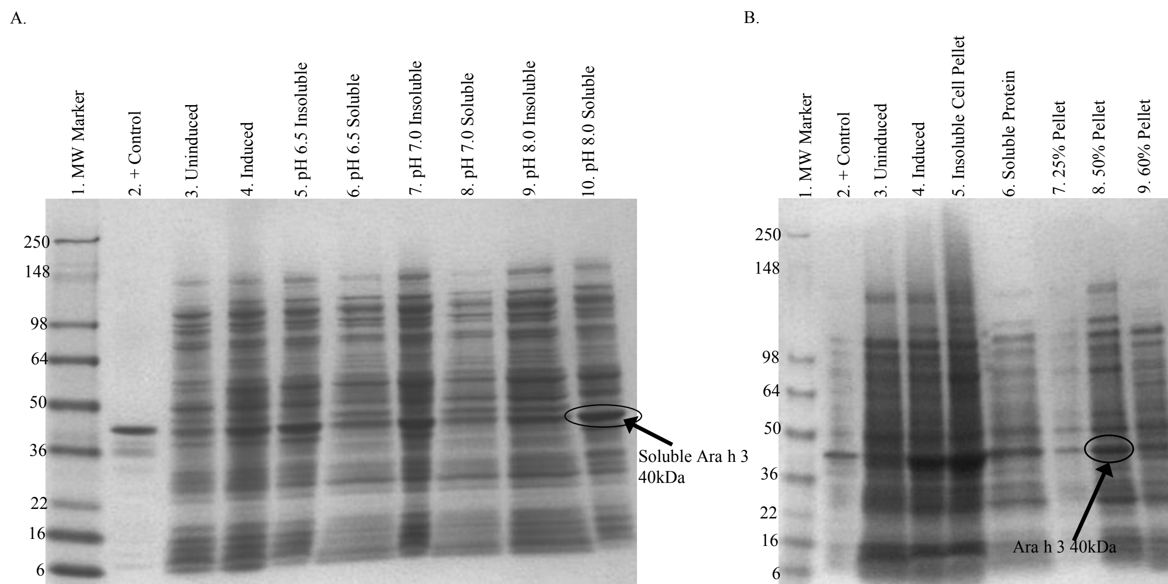


Figure 5-1 Expressed recombinant Ara h 3 (40 kDa) subjected to solubility testing and ammonium sulfate precipitation. The pellets (insoluble fraction) and supernatants (soluble fractions) are shown at the indicated pH values above each lane were subjected to a 4 - 20 % Tris-Glycine SDS-PAGE (Novex) for analysis (Figure A). (Figure B) Solubilized Ara h 3 (40 kDa) was saturated by percentage of ammonium sulfate (25 %, 50 %, 60 %). The precipitates at the indicated percentage are shown (lanes 7-9). Positive (+) Control: recombinant Ara h 3 (40 kDa) inclusion body.

Solubility Testing of Recombinant Ara h 3 Basic Subunit

It was determined that the plasmid expressed Ara h 3 (20 kDa) at high levels and maximum protein expression was achieved at 20 °C when expressed overnight (see Figure 4-3) and when recombinant Ara h 3 (20 kDa) was induced with IPTG. Following protein expression of Ara h 3 (20 kDa), several buffers were tested in order solubilize the protein (Figure 5-2 (A)).

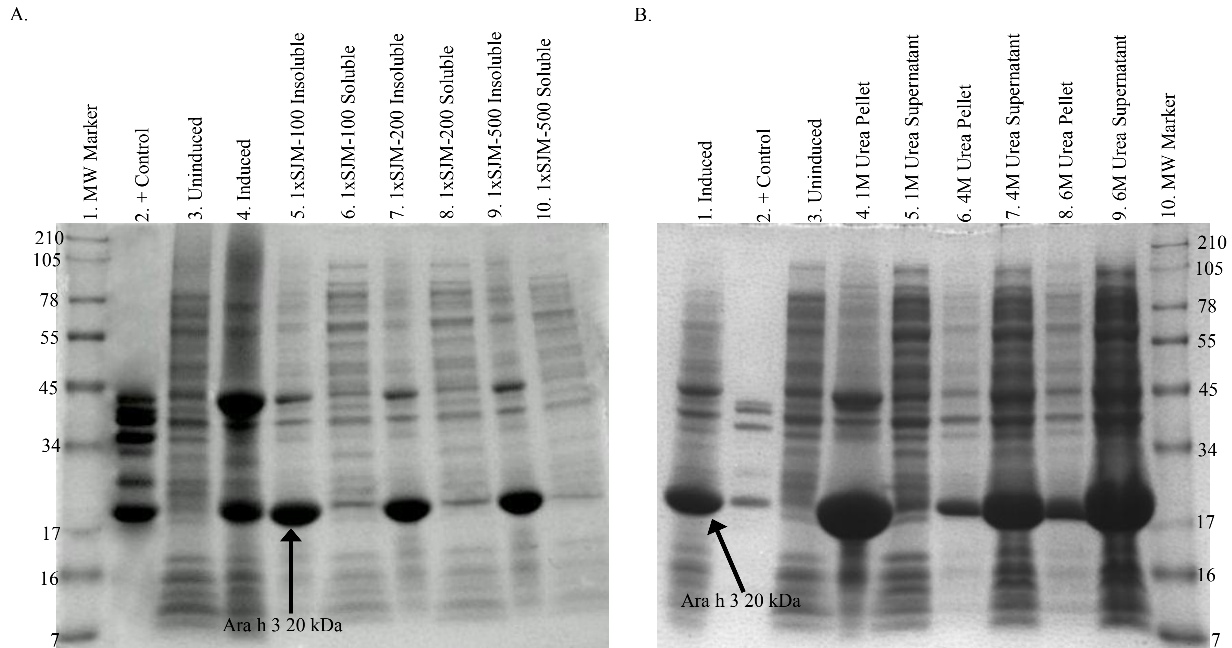
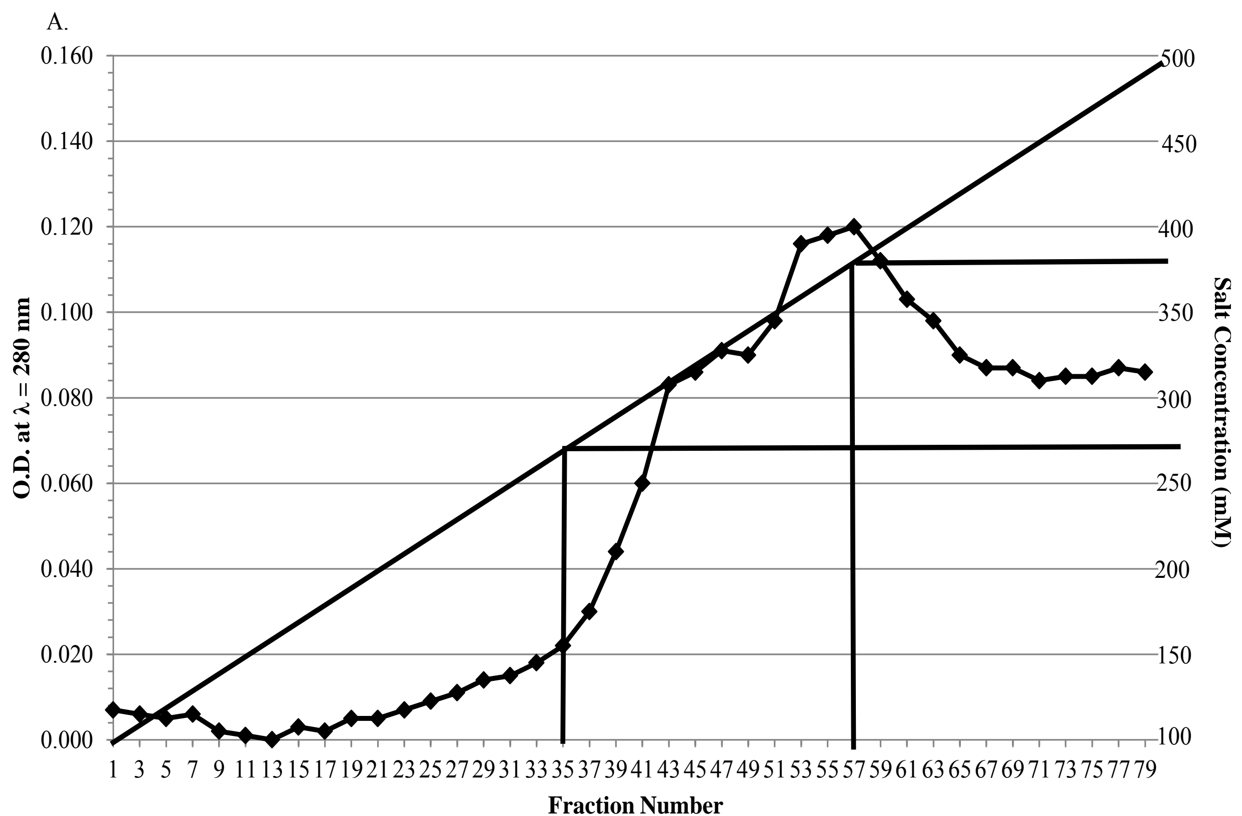


Figure 5-2 Solubility testing of recombinant Ara h 3 basic subunit. Pellets (insoluble) and supernatants (soluble) fractions were subjected to a 10 - 20 % Tris-Glycine SDS-PAGE (Novex). (Figure A) Expressed recombinant Ara h 3 (20 kDa) pellets solubilized in SJM-buffer with increasing concentrations of NaCl at pH value of 8.0 at room temperature. (Figure B) Expressed recombinant Ara h 3 (20 kDa) pellets were incubated in different concentrations of urea for 24 hrs while shaking at room temperature. Positive (+) control: native Ara h 3. MW Marker: SeeBlue Plus 2.

Following lysis, a majority if not all of Ara h 3 (20 kDa) protein was found in the insoluble pellet (Figure 5-2 (A) lanes 5, 7, and 9). It appeared that the basic subunit of Ara h 3 was insoluble. To determine if Ara h 3 (20 kDa) could be solubilized from the cell pellet following expression, expressed protein pellets were incubated in the presence of different concentrations of urea for 12 hours at room temperature. In Figure 5-2 (B) lane 9, 6 M urea was found to solubilize the most amount of Ara h 3 (20 kDa) although some of Ara h 3 (20 kDa) protein still remained insoluble and is still present in the pellet (Figure 5-2 (B) lane 8). It was concluded that Ara h 3 (20 kDa) was most likely improperly folded inclusion bodies due to the high concentration of urea it took to solubilize the protein from the cell pellet. When urea was subsequently removed, it resulted in precipitation of the protein.

Purification of Recombinant Ara h 3 Acidic Subunit

Following solubilization and ammonium sulfate precipitation of the recombinant acidic subunit, ion exchange chromatography was utilized as the next steps in purification. The first chromatographic step to purify recombinant Ara h 3 (40 kDa) used a strong anion-exchange resin High-Prep Q (Bio-Rad). The 50 % ammonium sulfate pellet was re-suspended in 50 ml SJM - 0 buffer at pH value of 9.0 and applied to the column. Figure 6 (A-C) depicts the chromatogram of the linear salt gradient from SJM - 100 buffer – SJM - 500 buffer and SDS-PAGE analysis of wash fractions and selected fraction numbers from the salt gradient.



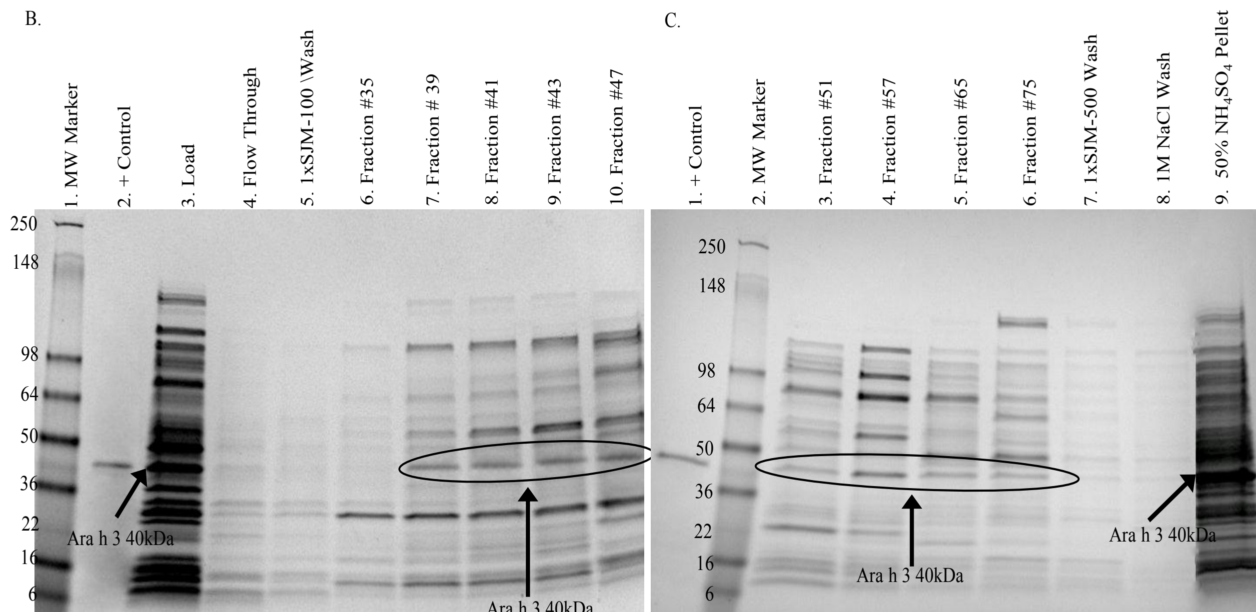


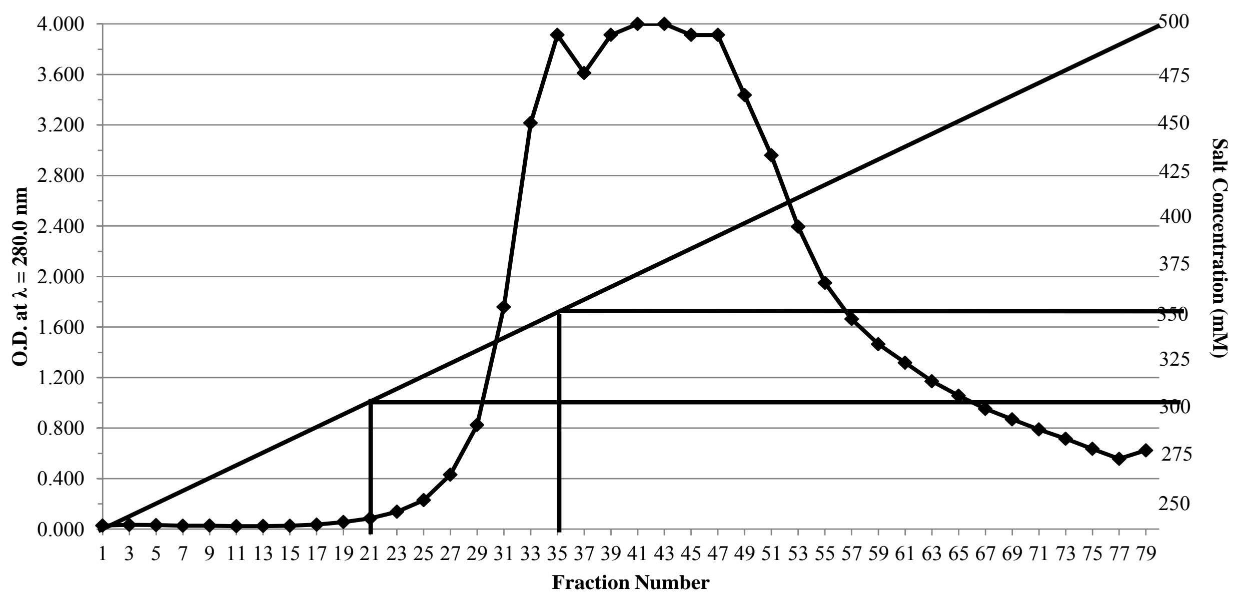
Figure 6 (A) Ion exchange chromatography of recombinant acidic subunit of Ara h 3 using a strong High-Prep Q resin. A fifty percent ammonium sulfate pellet was subjected to standard anion exchange chromatography and fractions collected (x-axis) were assayed for protein concentration by absorbance at 280 nm (left axis). The salt gradient is shown as the diagonal line and corresponds to the concentrations 100 mM -500 mM (right axis). Figure (B and C). Selected fractions were collected from anion exchange chromatography following a linear salt gradient elution shown in Figure (A). Fraction numbers that correspond to Figure (A) are shown at the tops of Figure (B) and (C) were subjected to a SDS-PAGE (Bio-Rad). Ara h 3 (40 kDa) is indicated by a circle and arrow. Positive (+) Control: recombinant Ara h 3 (40 kDa) inclusion body. MW Marker: SeeBlue Plus 2

Most of Ara h 3 (40 kDa) and contaminating *E. coli* proteins (approximately 420 mg of protein) bound to this resin when loaded in SJM - 0 buffer (Figure 6 (B) lane 4) and when washed with SJM - 100, SJM - 500 at pH 9.0, and 1 M NaCl, Ara h 3 (40 kDa) was not present in the wash fractions (Figure 6 (B) lane 5 and (C) lane 7 and 8).

Linear gradient of SJM - 100 – SJM - 500 was used to elute bound Ara h 3 (40 kDa) (Figure 6 (A). Ara h 3 (40 kDa) eluted during the NaCl gradient as a moderately-wide peak ranging between approximately 275 mM – 375 mM NaCl (Figure 6 (A)). There were obvious contaminating proteins that eluted in the shoulder peaks of Ara h 3 (40 kDa) (Figure 6 (B) lanes 7-10 and (C) lanes 3-6). A narrower NaCl gradient would better resolve these peaks and a possible slight decrease in pH would eliminate some of the contaminating proteins.

A new 50 % precipitated ammonium sulfate pellet containing Ara h 3 (40 kDa) was loaded onto a High-Prep Q column towards developing a method for purifying recombinant Ara h 3 (40 kDa) using the data collected from the previous purification and the pH was decreased to 8.4. The pH value of 8.4 was chosen in an attempt to keep more protein in solution prior to loading it onto the column. A pH value of 8.4 is closer to the pH value that Ara h 3 (40 kDa) is solubilized. Approximately 960 mg of protein in 75 ml of SJM - 0 buffer was loaded onto the column. Figure 7 (A-C) depicts the chromatogram of the linear salt gradient from SJM - 250 buffer – SJM - 500 buffer and SDS-PAGE analysis of wash fractions and selected fraction numbers from the salt gradient.

A.



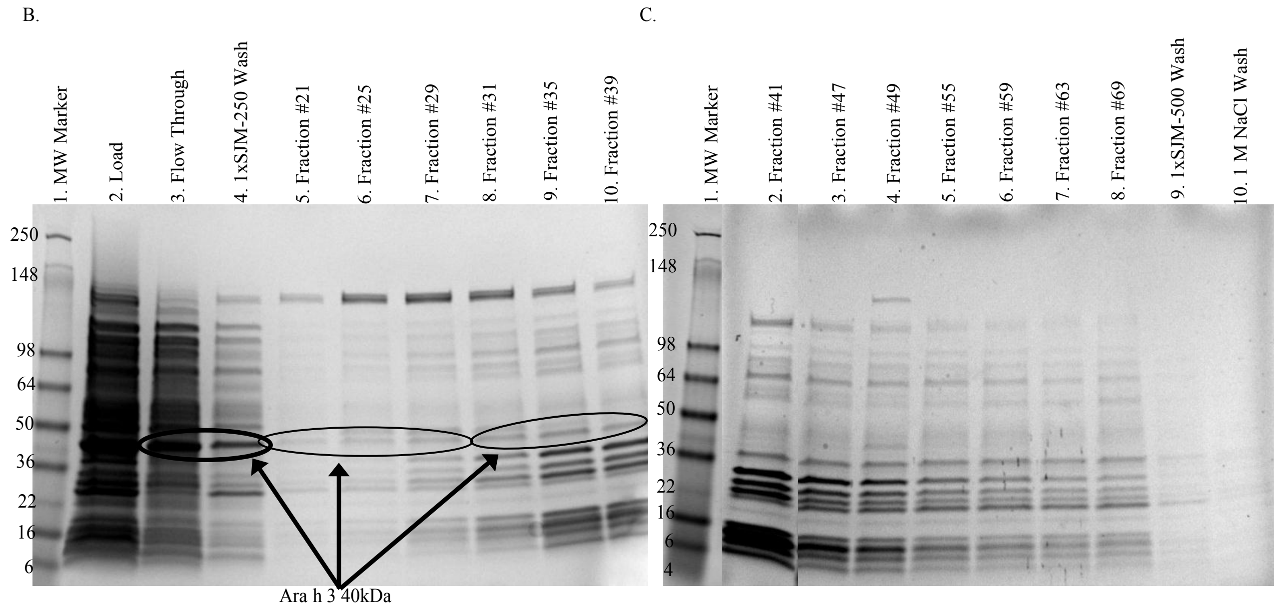
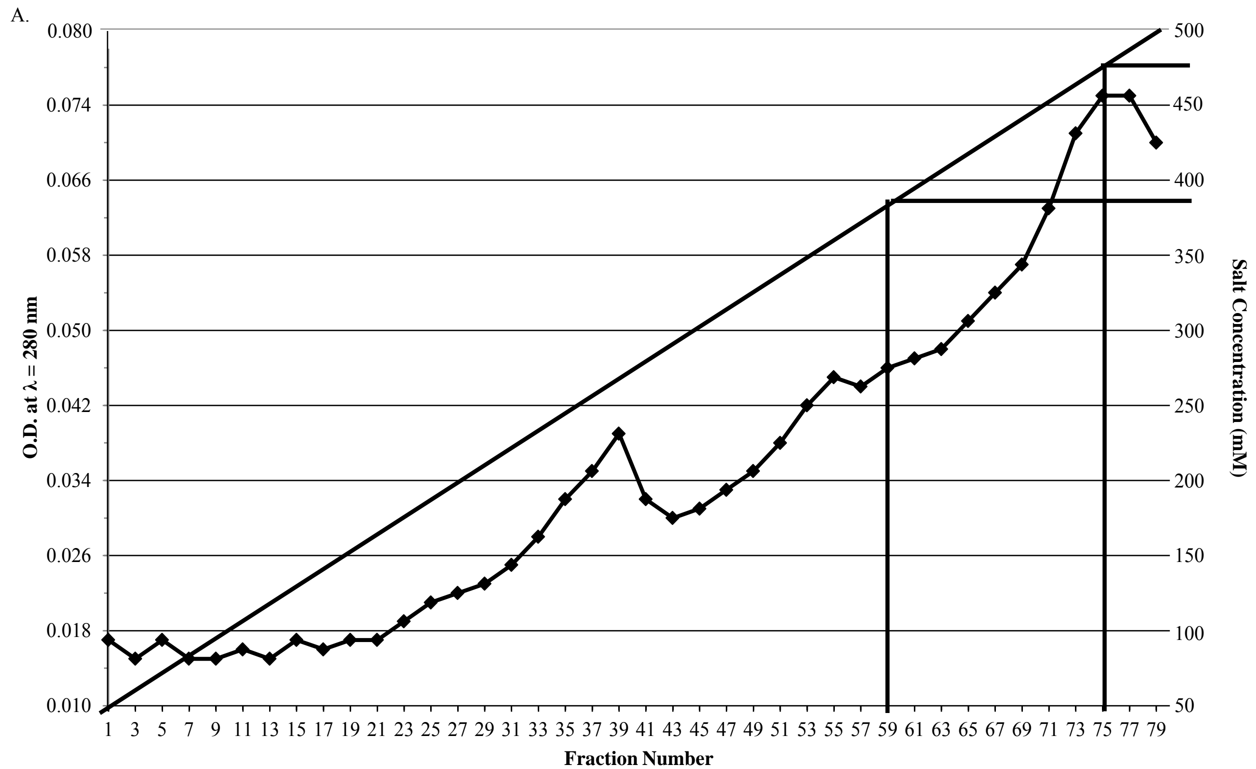


Figure 7 (A) Ion exchange chromatography of recombinant Ara h 3 (40 kDa) with High-Prep Q resin. A fifty percent ammonium sulfate pellet was subjected to anion exchange chromatography and fractions collected (x-axis) were assayed for protein concentration by absorbance at 280 nm (left axis). The salt gradient is shown as the diagonal line and corresponds to the concentrations 250 mM – 500 mM (right axis). Figure (B and C). Selected fractions collected from anion exchange chromatography following a linear salt gradient elution shown in Figure (A) were subjected to SDS-PAGE (Bio-rad). Fraction numbers that correspond to Figure (A) are shown at the top of Figures (B and C). Ara h 3 (40 kDa) is indicated by a circle and arrow. MW Marker: SeeBlue Plus 2.

Most of Ara h 3 (40 kDa) and contaminating proteins eluted when loaded in SJM-0 (Figure 7 (B) lane 3) and washed with SJM - 250 at a pH value of 8.4 (Figure 7 (B) lane 4). A linear NaCl gradient of SJM - 250 buffer – SJM - 500 buffer was used to elute any remaining bound Ara h 3 (40 kDa). Even though, most of Ara h 3 (40 kDa) eluted off the column prior to the NaCl gradient, approximately 75 mg of protein containing some Ara h 3 (40 kDa) remained bound to the resin and eluted in a narrow peak during the NaCl gradient between approximately 315 mM – 360 mM NaCl (Figure 7 (A)). Contaminating proteins eluted off the column in the same peak as Ara h 3 (40 kDa) (Figure 7 (B) lanes 5-10) and only a minimal amount of protein was eluted in the collected fractions compared to approximately 370 mg of total protein in the Ara h 3 (40 kDa) containing cell lysate in the eluate of the flow through and SJM - 250 wash (Figure 7 (B) compare lanes 5-10 to lanes 3 and 4). Moreover, most contaminating proteins seemed to have bound this resin at a pH value of 8.4 and salt concentration of 250 mM (Figure 7 (B and C)) despite what appeared to look as though most eluted from the column (Figure 7 (B) lanes 3 and 4). The high absorbances of the collected fractions (Figure 7 (A) indicated the presence of protein. Analysis of selected fractions from the high absorbance readings in Figure 7 (A) by SDS-PAGE revealed the presence of contaminating proteins and the absence of Ara h 3 (40 kDa) (Figure 7 (C) lanes 2-8).

A new precipitated 50 % ammonium sulfate pellet was used to continue with deciphering a purification protocol. This purification used all data collected from previous purifications and again the strong anion-exchange resin, High-Prep Q was used. The 50 % ammonium sulfate pellet containing recombinant Ara h 3 (40 kDa) was re-suspended in 50 ml of SJM - 0 buffer and approximately 410 mg of protein was loaded onto the strong-anion exchange column at a pH value of 9.0. Figure 8 (A-E) depicts the chromatogram of the linear salt gradient from SJM - 50 buffer – SJM - 500 buffer, SDS-PAGE gels, and anti-Ara h 3 (40 kDa) western blot analyses of wash fractions and selected fraction numbers from the salt gradient.



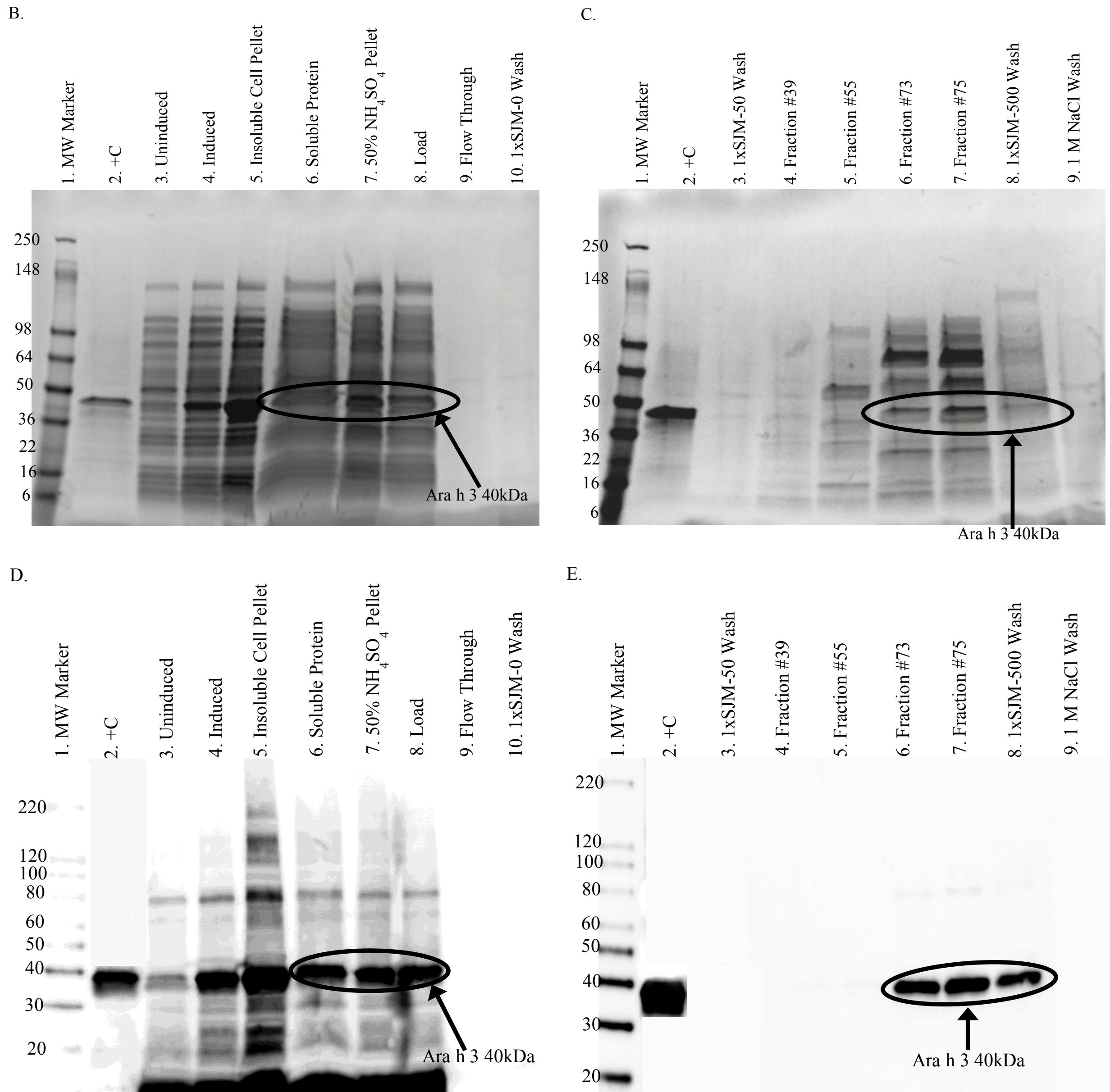


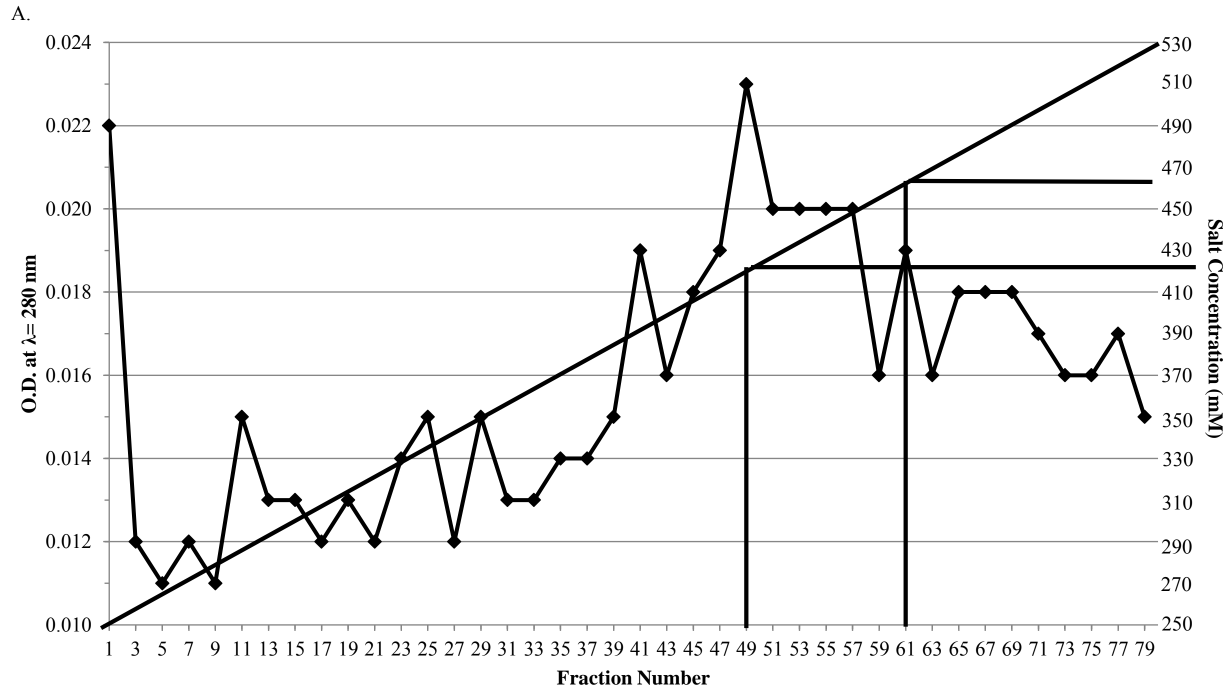
Figure 8 (A) Ion exchange chromatography of recombinant Ara h 3 acidic subunit using High-Prep Q resin at a pH 9.0. Fifty percent ammonium sulfate pellet was subjected to standard anion exchange chromatography and fractions collected (x-axis) were assayed for protein concentration by absorbance at 280 nm (left axis). The salt gradient is shown as the diagonal line and corresponds to the concentrations 50 mM -500 mM (right axis). Figure (B and C) Selected fractions were collected from anion exchange chromatography following a linear salt gradient elution shown in Figure (A). Fraction numbers that correspond to Figure (A) are shown at the top of (Figure B) and (Figure C) were subjected to SDS-PAGE (Bio-Rad) gel separation. Ara h 3 (40 kDa) is indicated by a circle and arrow. MW marker: SeeBlue Plus 2. Figure (D and E) Anti-Ara h 3 (40 kDa) western blot of SDS-PAGEs shown in Figures (B and C) to confirm presence of Ara h 3 (40 kDa) in selected fractions numbers shown at the top of the figure from Figure (A). Positive (+) Control: recombinant Ara h 3 (40 kDa) inclusion body. MW Marker: MagicMark.

Most, if not all, of recombinant Ara h 3 (40 kDa) bound to this resin when loaded in SJM - 0 buffer (Figure 8 (B) lane 9). When washed with SJM - 0 and SJM - 50 buffers, Ara h 3 (40 kDa) nor any contaminating proteins were present in any of the wash fractions (Figure 8 (B) lane 10 and (C) lane 3). Linear NaCl gradient of SJM - 50 – SJM - 500 was used to elute bound Ara h 3. As shown in Figure 8 (A), Ara h 3 eluted as a narrow peak between 380 mM – 500 mM NaCl. Using the information collected from a previous purification and since there were contaminating proteins that eluted in the same peak as Ara h 3 (40 kDa) (Figure 8 (C) lanes 5-8), widening the gradient to 250 mM – 520 mM NaCl would better resolve the peak of Ara h 3 (40 kDa).

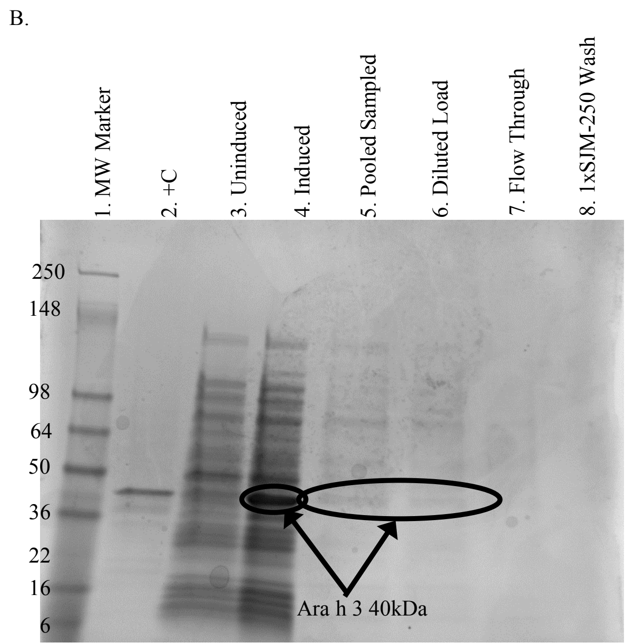
Prior to proceeding with the purification, binding to the Q column, absence of Ara h 3 in the wash fractions, and presence of Ara h 3 in collected fractions was confirmed by anti-Ara h 3 (40 kDa) western blots as shown in Figure 8 (D and E). In Figure 8 (D) lanes 6 and 7 confirmed recombinant Ara h 3 (40 kDa) was soluble and precipitated as expected at 50 % ammonium sulfate saturation. The load onto the strong anion-exchange resin (Figure 8 (D) lane 8) indicated that Ara h 3(40 kDa) was present after re-solubilization in SJM - 0 buffer. Lanes 9 and 10 in Figure 8 (D) and lane 3 in Figure 8 (E) confirmed the absence of Ara h 3 (40 kDa) in the wash fractions. Ara h 3 (40 kDa) eluted in fraction numbers 73 and 75, and appeared in the SJM - 500 wash fraction (Figure 8 (E) lanes 6, 7, and 8), but not in fraction number 55 (Figure 8 (E) lane 5) which is contrary to the appearance of a band at approximately 40 kDa on the SDS-PAGE (Figure 8 (C) lane 5). This concluded that the western blot was needed in order to confirm the presence or absence of Ara h 3 (40 kDa) protein in collected fractions, in order to pool correct fractions from the gradient.

Fraction numbers 59 - 80 and SJM - 500 wash were pooled and diluted to approximately 250 mM NaCl using SJM - 0 buffer at pH 9.0. The diluted sample contained approximately 30 mg of protein and was re-loaded onto the strong anion-exchange resin High-Prep Q (Figure 9). The pH was kept at 9.0 during this second purification since Ara h 3 (40 kDa) had bound tightly to this resin at this pH and only the NaCl concentrations of linear gradient was altered.

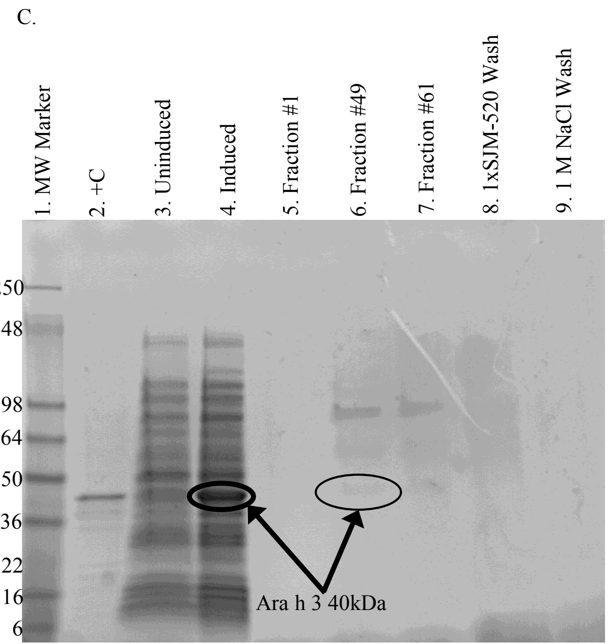
A.



B.



C.



D.

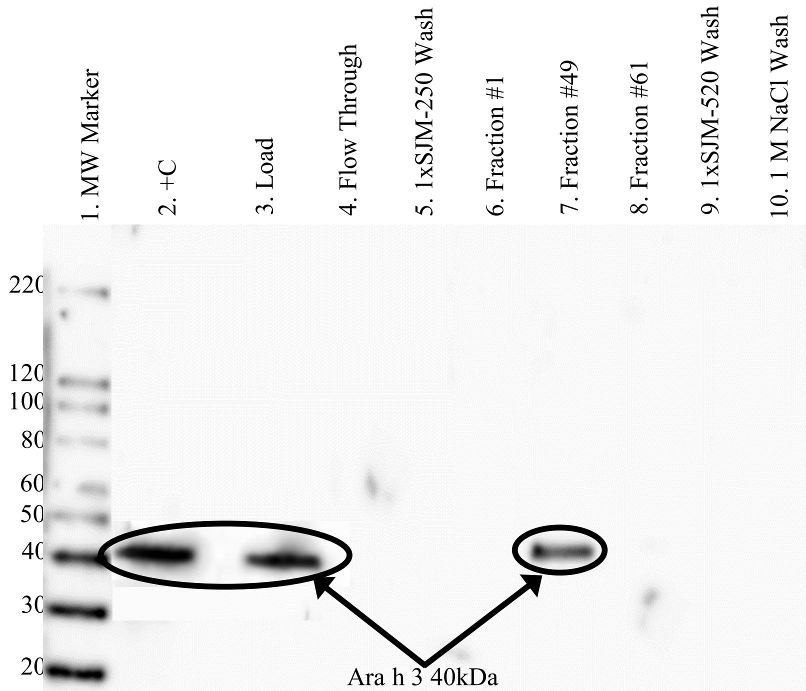


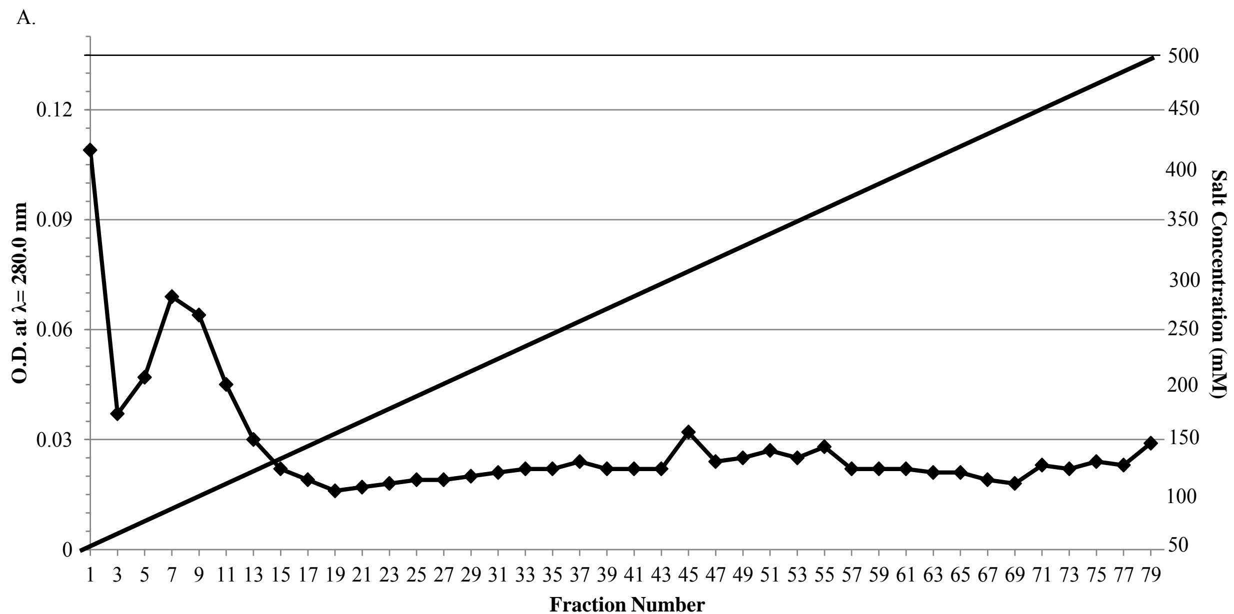
Figure 9 (A) Ion exchange chromatography of pooled fractions from Figure 8 subjected to High-Prep Q resin. Pooled fractions 59-80 and SJM-500 wash from Figure 8 were subjected to another round of anion exchange chromatography and fractions collected (x-axis) were assayed for protein concentration by absorbance at 280 nm (left axis). The salt gradient is shown as the diagonal line and corresponds to the concentrations 250 mM -520 mM (right axis). Figure (B and C) Selected fractions were collected from anion exchange chromatography following a linear salt gradient elution shown in Figure (A). Fractions numbers that correspond to Figure (A) are shown at the top of Figure (C) were subjected to SDS-PAGE (Bio-Rad). Ara h 3 (40 kDa) is indicated by a circle and arrow. Positive (+) control: recombinant Ara h 3 (40 kDa) inclusion body. MW Marker: SeeBlue Plus 2. Figure (D). Anti-Ara h 3 (40 kDa) western blot of selected samples from Figures (B and C). Selected samples are the diluted load (Figure (B), lane 6), flow through (Figure (B) lane 7), column washes, and fractions from SDS-PAGE shown in Figures (B and C). Confirmation of Ara h 3 (40 kDa) in selected fractions numbers shown at the top of Figure (D). Positive (+) Control: recombinant Ara h 3 (40 kDa) inclusion body. MW Marker: MagicMark.

A linear gradient of NaCl (250 mM – 520 mM) in SJM buffer was used to elute the bound Ara h 3 (40 kDa). According to the chromatogram (Figure 9 (A)), there was not a specific defined peak that could be assigned to Ara h 3. Three fractions that had the highest absorbances were selected to for SDS-PAGE analysis and staining (Figure 9 (C) lanes 5, 6, and 7). The SDS-PAGE was inconclusive of any protein being collected in fractions. Therefore, a western blot analysis using the anti-Ara h 3 (40 kDa) antibody (Figure 9 (D)) was performed and results confirmed the presence of Ara h 3 (40 kDa) in the pooled protein load (Figure 9 (D) lane 3). SDS-PAGE analysis indicated a very faint band at the molecular weight of 40 kDa in the pooled load (Figure 9 (B) lanes 5 and 6). There was not any visible evidence revealed by SDS-PAGE of Ara h 3 (40 kDa) eluting from the column and the chromatogram of the linear salt gradient (Figure 9 (A)) had low absorbance readings of the collected fractions.

The anti-Ara h 3 (40 kDa) western blot confirmed that Ara h 3 (40 kDa) eluted from the column in fraction number 49 (Figure 9 (D) lane 7). Fraction number 49 had the highest absorbance reading off all fractions collected (Figure 9 (A)) according to the chromatogram. The appearance of an extremely faint band that is approximately the molecular weight of Ara h 3 (40 kDa) was seen on the SDS-PAGE when examined carefully (Figure 9 (C) lane 6).

The strong cation-exchange resin High-Prep S was utilized as a final attempt to purify recombinant Ara h 3 (40 kDa) before proceeding to other methods of purification (Figure 10). A 50 % ammonium sulfate pellet containing recombinant Ara h 3 (40 kDa) was re-suspended in 50 ml of SJM-0 pH 6.5 buffer and loaded onto the column. In Figure 10 (A and B), the chromatogram from the linear salt gradient from SJM - 50 buffer – SJM - 500 buffer and SDS-PAGE analysis of selected fractions are shown.

A.



B.

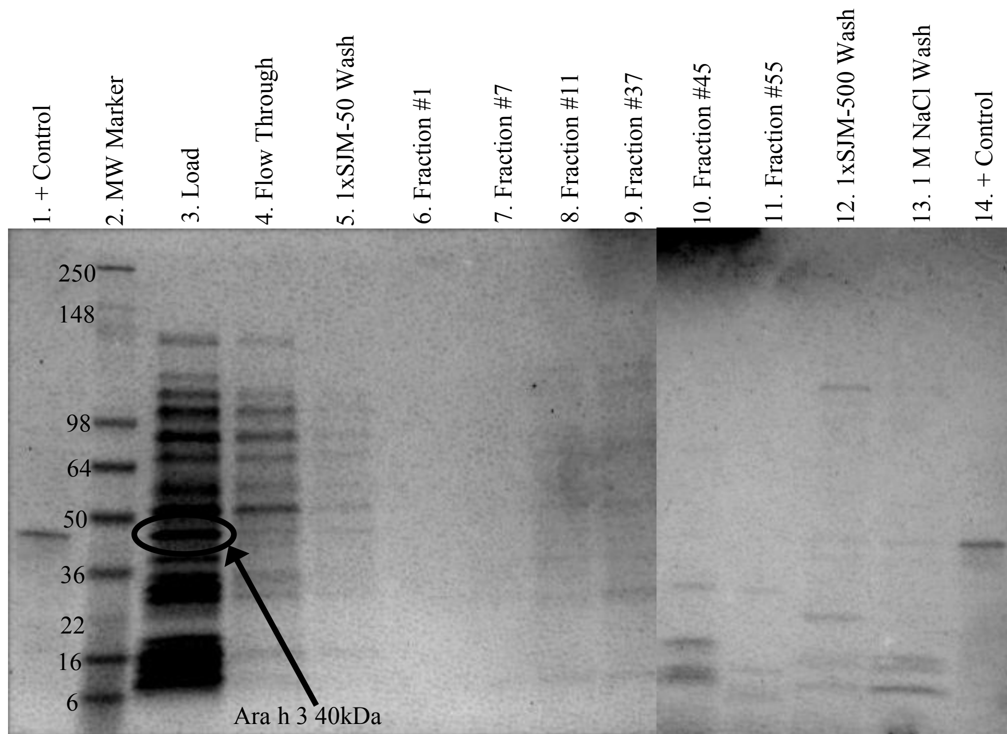


Figure 10 (A) Ion exchange chromatography of recombinant Ara h 3 (40 kDa) using a strong cation-exchange resin, High-Prep. Fifty percent ammonium sulfate pellet was subjected to standard anion exchange chromatography and fractions collected (x-axis) were assayed for protein concentration by absorbance at 280 nm (left axis). The salt gradient is shown as the diagonal line and corresponds to the concentrations 50 mM - 500 mM (right axis). Figure (B) Selected fractions collected from anion exchange chromatography following a linear salt gradient elution shown in Figure (A). Fraction numbers that correspond to Figure (A) are shown at the top of the figure were subjected to a 4-20% Tris-Glycine SDS-PAGE (Bio-Rad) gel separation. Ara h 3 (40 kDa) is indicated by a circle and arrow. Positive (+) Control: recombinant Ara h 3 (40 kDa) inclusion body. MW Marker: SeeBlue Plus 2.

Most contaminating proteins did not bind to this resin and Ara h 3 (40 kDa) presumably bound when loaded in SJM - 0 buffer at pH 6.5 (Figure 10 (B) lane 4). Ara h 3 was not present in any of the wash fractions of SJM - 50 and SJM - 500 (Figure 10 (B) lanes 5 and 12 respectively). A linear NaCl gradient of SJM - 50 – SJM - 500 was used to elute bound Ara h 3 (Figure 10 (A)) from the column. The absorbance readings for all the fractions collected (Figure 10 (A)) were extremely low and analysis by SDS-PAGE (Figure 10 (B) lanes 6-11) confirmed the low absorbance readings in Figure 10 (A) and concluded that minimal protein was collected in fractions. A significant amount of protein containing Ara h 3 (40 kDa) (approximately 360 mg) was loaded onto the column (Figure 10 (B) lane 3) and approximately 170 mg of protein did not bind (Figure 10 (B) lane 4). None of the unbound protein in lane 4 was Ara h 3 (40 kDa). Ara h 3 (40 kDa) appeared to have bound to the High-S column at a pH 6.5, but never eluted from the column even after a 1 M NaCl wash (Figure 10 (B) lane 13). It was concluded that recombinant Ara h 3 (40 kDa) was unfolded, aggregated, inclusion bodies, and had precipitated on the column.

Inclusion Body Isolation and SDS-PAGE Purification of Recombinant Ara h 3 Acidic and Basic Subunits

Other approaches were considered since recombinant Ara h 3 acidic subunits were unable to be separated and purified through ion exchange chromatography protocols at high enough concentrations. It was determined recombinant Ara h 3 acidic and basic subunits were to be purified through an inclusion body isolation followed by gel purification (Figure 11) (see Materials and Methods for detailed protocol).

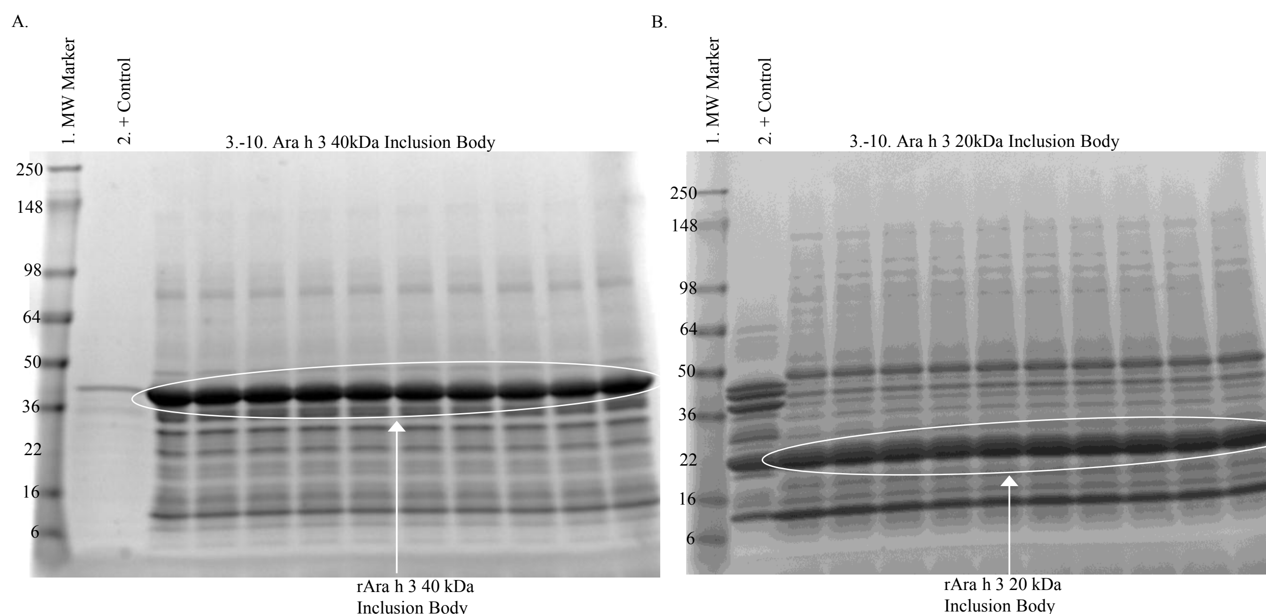


Figure 11 (A and B) Recombinant Ara h 3 acidic and basic subunit gel inclusion body purification. (Figure A) Recombinant Ara h 3 acidic subunit. (lane 2) Positive (+) control: previous recombinant Ara h 3 (40 kDa) inclusion body. (Figure B) Recombinant Ara h 3 basic subunit. (lane 2) Positive (+) control: native Ara h 3 (lane 2). In both panels, inclusion bodies excised from the 4 - 20 % Tris-Glycine SDS-PAGE (Bio-Rad) are indicated by a circle and arrow. MW marker: SeeBlue Plus 2.

Ara h 3 (40 kDa) and (20 kDa) inclusion bodies were subjected to SDS-PAGE gel, stained, and the corresponding bands were excised from the SDS-PAGE gels and eluted (Figure 11 A and B). Figure 11 (A) lanes 3-10 correspond to the recombinant Ara h 3 (40 kDa) inclusion body. Figure 11 (B) lanes 3-10 corresponds to recombinant Ara h 3 (20 kDa) inclusion body and was sent for custom-made polyclonal chicken antibodies as shown in Figure 13.

After excision from the SDS-PAGE gels, the recombinant Ara h 3 subunits were subjected to SDS-PAGE to determine load volume and purity. In Figure 12, 4 - 20 % Tris-Glycine SDS-PAGE analysis of the recombinant subunits is depicted after purification.

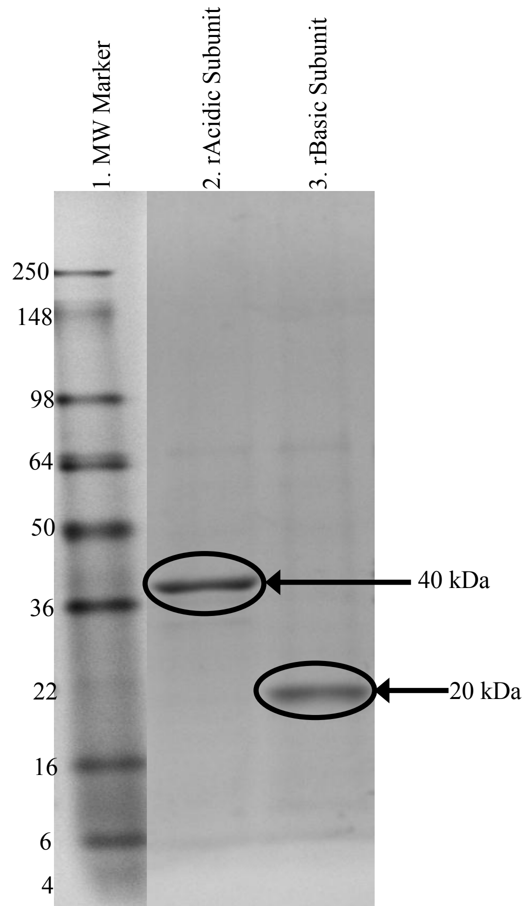


Figure 12 SDS-PAGE (Novex) analysis of recombinant Ara h 3 40 kDa and 20 kDa subunits after gel purification. MW Marker:SeeBlue Plus 2.

Both recombinant subunits obtained were highly pure and concentrated as shown in Figure 12 lanes 2 and 3. The volume loaded in lanes 2 and 3 was 5 µl per well. A volume of more than 5 µl produced a band that appeared to look overloaded, therefore, it was determined that only 5 µl of each recombinant subunit was to be loaded per well. Lane 3 was the recombinant Ara h 3 (20 kDa) inclusion body sent for chicken antibodies.

Anti-Ara h 3 (20 kDa) Antibody Specificity

An anti-Ara h 3 (20 kDa) western blot was performed to test the purity and specificity of the custom-made chicken anti-Ara h 3 (20 kDa) antibody (Figure 13). Native raw Ara h 3 was used to test the Ara h 3 (20 kDa) antibody specificity. After subjecting the sample to SDS-PAGE, the gel was transferred to a PVDF membrane for western blotting.

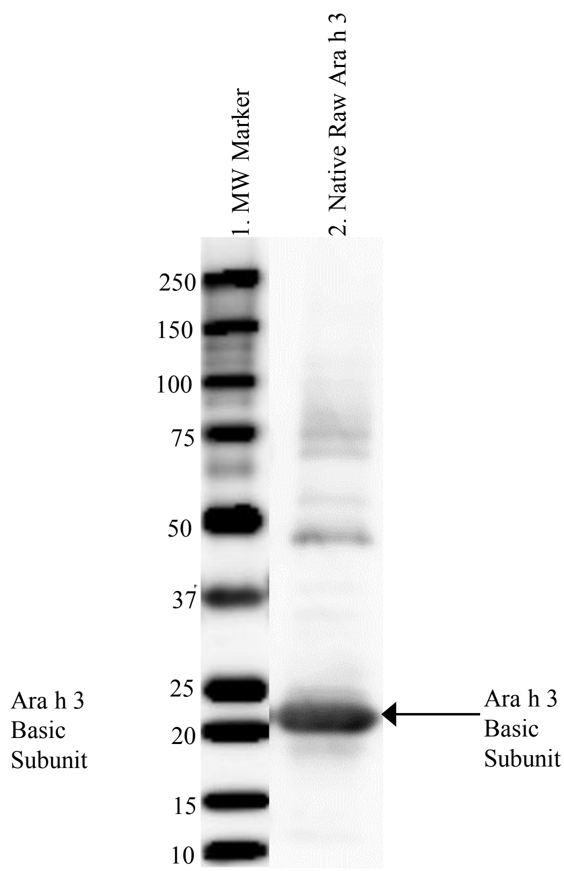


Figure 13 Anti-Ara h 3 (20 kDa) western blot. Lane 2: native raw Ara h 3 sample Q80107. Ara h 3 (20 kDa) has an arrow pointing to it and is labeled. MW Marker: Precision Plus Protein WesternC

In Figure 13, native raw Ara h 3 was used to determine if the antibody, which was made from the recombinant form, was still highly specific and would recognize the 20 kDa subunit in the native protein. As seen in lane 2 of Figure 13, anti-Ara h 3 (20 kDa) antibody still is very highly specific and recognized the 20 kDa subunit in native Ara h 3.

Patient Identification Chart, List of Clinical Symptoms and Peanut Specific IgE Levels

Table 3-1 Patient Identification, Clinical Symptoms, and Peanut Specific IgE Levels

Patient ID	Clinical Symptoms	Peanut Specific IgE Levels
P001	Watery eyes, throat, face, and lips swelling. Itchy general mouth, throat, and hives, sense of fear.	>100
P002	Anaphylaxis	14.4
P003	Eczema	27.5
P004	Anaphylaxis	21.5
P005	N/A	369.51
P006	Swelling and itchy throat and mouth and face swelling. General itchiness hives, flushing.	N/A
P007	Anaphylaxis	23.1
P008	Peanut: Severe- Eczema, hearing loss, itchy mouth/throat, eyes burn, puffiness	>17.5
P009	Swelling of throat, itchy throat, hives, wheezing, and chest tightness for all allergic foods. Peanut: includes sense of doom, fear.	N/A
P010	Face and lip swelling, itchy mouth	N/A
P011	Nose congestion and diarrhea	N/A
P012	Gastrointestinal upset	N/A
P013	N/A	N/A
P014	N/A	N/A

Table 3-1 Patient identification chart. Includes clinical symptoms and specific IgE levels for peanut. Some patient data was not available and is indicated by N/A.

Immunoblots of Three Patients Using Only Native Raw Ara h 3 and Recombinant Ara h 3 Subunits

After purifying the recombinant subunits, three patients were randomly chosen to test for serum IgE binding to the recombinant Ara h 3 inclusion body subunits prior to immunoblot testing of other patients. Raw native Ara h 3 was used as the positive control to the recombinant subunits.

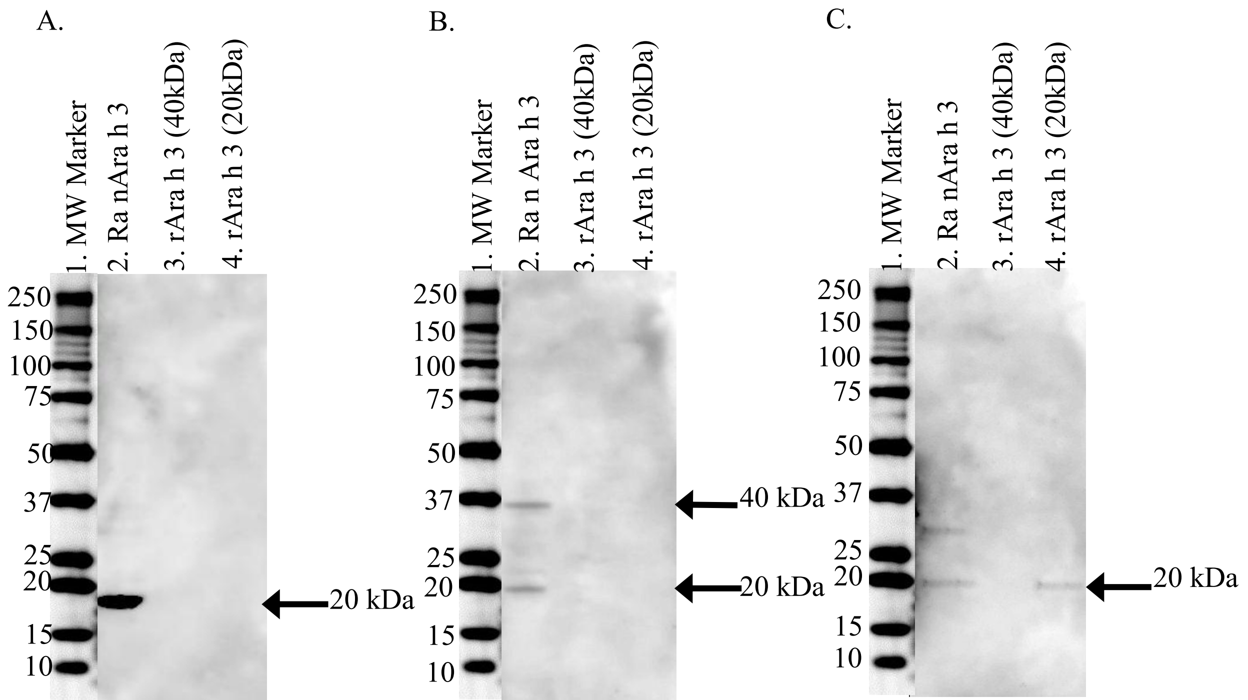


Figure 14 (A-C) Immunoblot analysis using native raw Ara h 3, recombinant Ara h 3 (40 kDa) and (20 kDa) subunits to determine if serum IgE from peanut allergic patients recognized the recombinant Ara h 3 subunits prior to using them in subsequent immunoblots. Samples are indicated above each lane in the figure. Ara h 3 (40 kDa) and (20 kDa) are indicated by arrows. (Figure A) P012. (Figure B) P013. (Figure C) P014. MW Marker: Precision Plus Protein WesternC.

Figure 14 shows western blots using serum IgE from the three randomly chosen patients, P012, P013, and P014. It is shown in Figure 14 (A) that serum IgE from P012 only recognized the basic subunit in the native form (lane 2). Serum IgE from P013 is depicted in Figure 14 (B) and recognized both the acidic and basic subunits in the native form (lane 2) but not in either of the recombinants (lanes 3 and 4). Additionally, there may be some slight recognition of the truncated form of the Ara h 3 acidic subunit at approximately 27 kDa (lane 2). Serum IgE from P014, as shown in Figure 14 (C), bound the basic subunit in the native form of Ara h 3 (lane 2) and with a possible binding to a truncated form of the acidic subunit in the native Ara h 3 (lane 2). It is interesting to note that P014 was the only patient of all tested to recognize and bind the recombinant basic subunit (Figure 14 (C) lane 4).

SDS-PAGE of Protein Profiles Used in Immunoblots

It was important that all the protein loads were normalized. Figure 15 illustrates all protein samples used in the immunoblots analyses and normalization of the protein loads. Raw and roasted native Ara h 3 protein, recombinant Ara h 3 subunits, and simulated roasted (SR) Ara h 3 profiles are also shown and indications of covalent crosslinking are depicted.

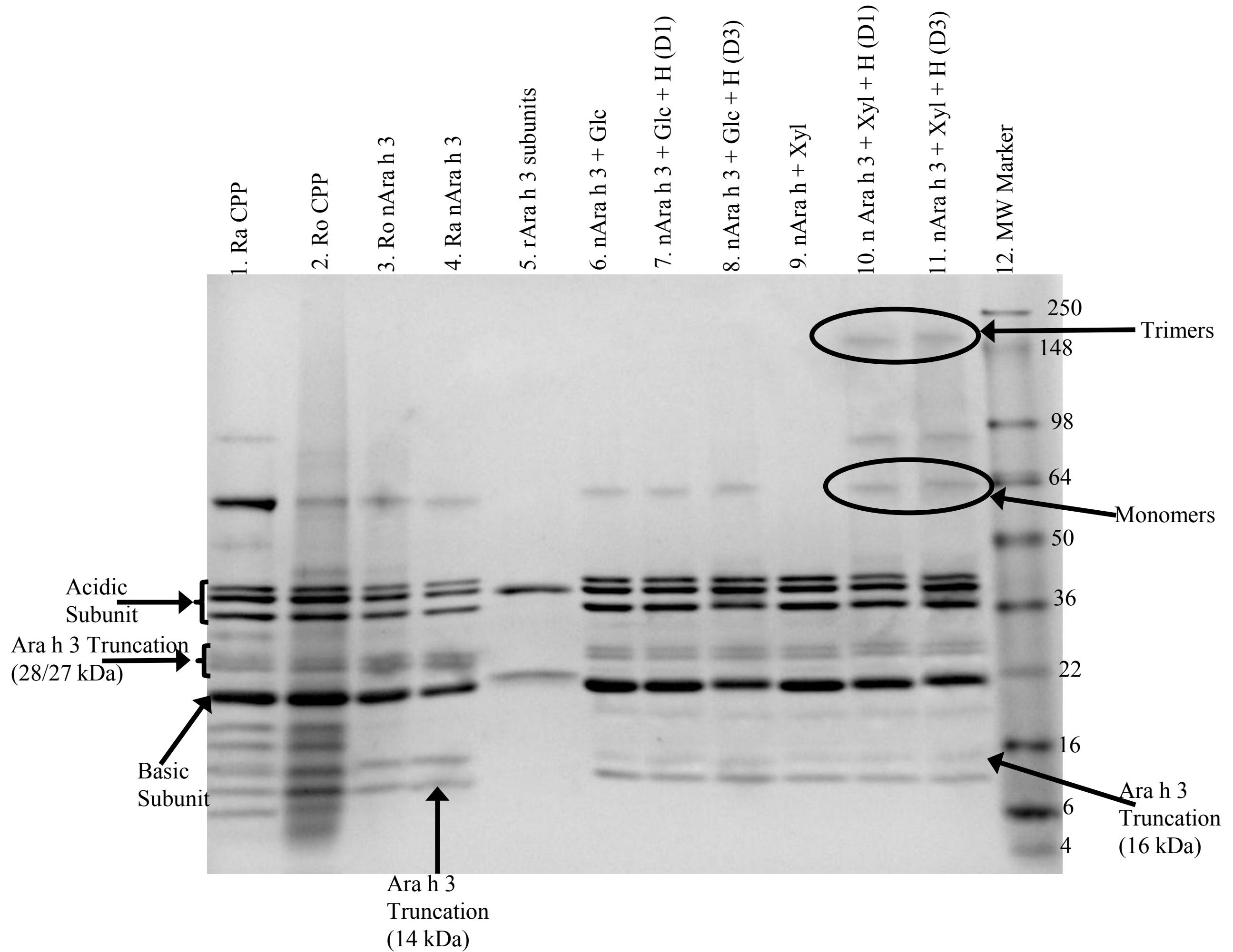


Figure 15 Normalization of protein loads prior to immunoblot analysis as shown by a 4 - 20 % Tris-Glycine SDS-PAGE (Novex). CPP refers to crude peanut protein (lanes 1 and 2). Recombinant Ara h 3 subunits were loaded in the same lane (lane 5). Lanes 6-11: SR Ara h 3 Glc: glucose (lanes 6-8) Xyl: xylose (lanes 9-11). Acidic and basic subunits, formation of Ara h 3 trimers (lanes 10-11) and monomers (lanes 6-8,10-11), and truncated acidic subunit bands are shown and indicated by arrows and labeled. MW Marker: SeeBlue Plus 2

In Figure 15, the migration patterns of Ara h 3 in raw and roasted crude peanut protein (CPP), purified native roasted and raw Ara h 3, recombinant Ara h 3 subunits, and raw Ara h 3 subjected to the Maillard reaction or simulated roasted model (SRM) with glucose (Glc) or xylose (Xyl) from zero to three days are shown in the SDS-PAGE. As seen more in raw CPP (lane 1), raw Ara h 3 (lane 4), and

recombinant Ara h 3 subunits (lane 5), the individual bands of the proteins are clearer, slightly more distinct, and there is not any clear evidence of higher order structures or smearing when comparing those lanes to roasted CPP (lane 2), roasted Ara h 3 (lane 3), and simulated roasted (SR) Ara h 3 (lanes 6-11).

SR Ara h 3 in the presence of xylose (lanes 9-11) had the presence of higher order structures and smearing after 24 hours of heating (compare lane 9 and 10). Smearing and higher order structures become more apparent and significant after 72 hours of heating SR Ara h 3 in the presence of xylose (compare lane 10 and 11).

The presence of monomer formation is seen (Figure 15 lane 6), which is the association of the acidic and basic subunit, immediately after glucose and protein are incubated together in the absence of heat in the SR Ara h 3 sample. Comparing SR Ara h 3 samples in the presence of glucose (lanes 6-8) smearing and higher order structure formation are not seen aside from the monomer formation from 0 hours to 72 hours. When SR Ara h 3 samples in the presence of glucose (lanes 7 and 8) are compared to the reactions with xylose (lanes 10 and 11), the occurrence of smearing and higher order structures above 60 kDa happened faster when Ara h 3 was incubated with xylose.

The simulated roasted proteins (Figure 15 lanes 10 and 11) took on characteristics that are seen in dry roasted peanuts and these observations are illustrated in Figure 15 when SR Ara h 3 (lanes 10 and 11) are compared to roasted peanuts (lane 2) and native roasted Ara h 3 (lane 3).

Anti-Ara h 3 (40 kDa) Western Blot of Raw and Roasted CPP, Raw and Roasted Native Ara h 3, and Day 3 Samples of Simulated Roasted Ara h 3

An anti-Ara h 3 (40 kDa) western blot of raw and roasted CPP, native raw and roasted Ara h 3, and day three sample (72 hour) of SR Ara h 3 in the presence of glucose or xylose was performed to determine that SR Ara h 3 mimics dry roasting. The presence of higher order structures and smearing that occurred only in SR Ara h 3 in the presence of both glucose and xylose and was equivalent to what is seen in roasted peanuts and roasted Ara h 3.

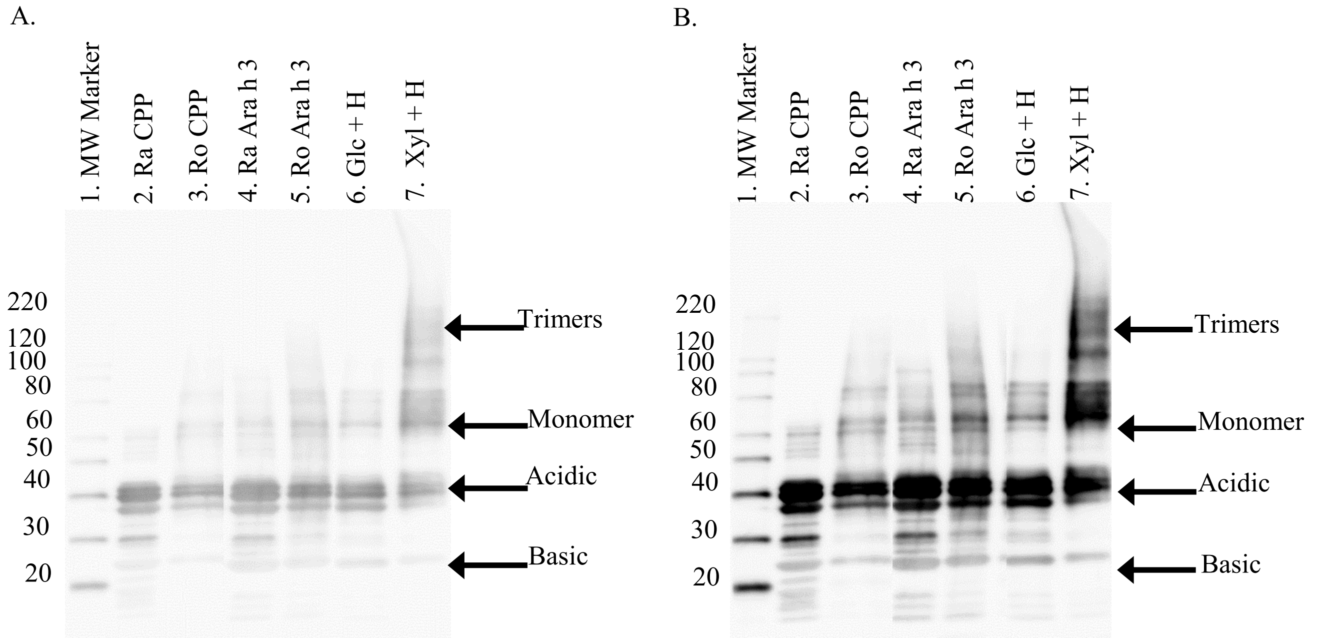


Figure 16 (A-C) Anti-Ara h 3 (40 kDa) western blot. Samples are raw and roasted CPP, raw and roasted Ara h 3, and SR Ara h 3 for 72 hours (D3) in the presence of Glc (lane 6) or Xyl (lane 7). (Figure A) Exposure time: 30 seconds (Figure B) Exposure time: 120 seconds. Acidic and basic subunits, monomer, and trimers are labeled with an arrow. MW Marker: Magic Mark

Figure 16 (A and B) shows different exposure times of the anti-Ara h 3 (40 kDa) western blot membrane. As seen in Figure 16 (A), the presence of smearing in lanes 3, 5, 6, and 7 was present after a 30 second exposure and is indicative of the presence of higher order structures, peptide crosslinking, and chemical modifications.

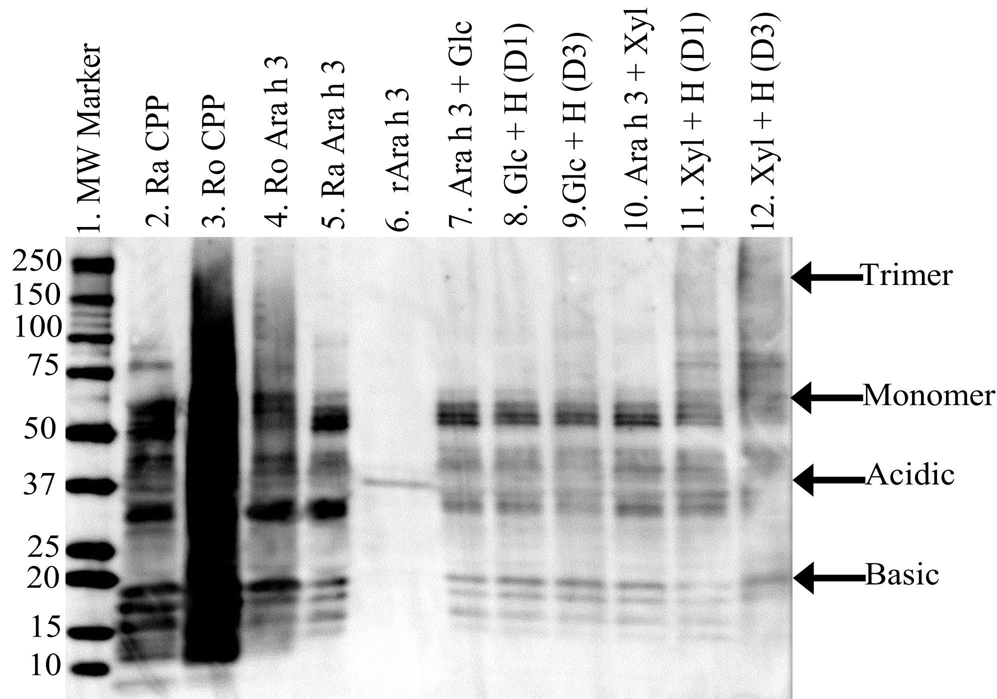
After an exposure time of 120 seconds (Figure 16 (B)), it is clear that raw CPP (lane 2) and raw Ara h 3 (lane 4) have significantly less smearing and clearer more defined bands than roasted CPP (lane 3), roasted Ara h 3 (lane 5), SR Ara h 3 in the presence of glucose for 72 hours (lane 6), or SR Ara h 3 in the presence of xylose for 72 hours (lane 7). All roasted samples (lane 3 and 5) and SR samples (lane 6 and 7) have less defined bands and smearing mainly starting above the 40 kDa molecular weight markers (lane 1). Lower molecular weight proteins are less prominent and defined in roasted samples, especially in roasted CPP (lane 3) and SR Ara h 3 in the presence of xylose (lane 7).

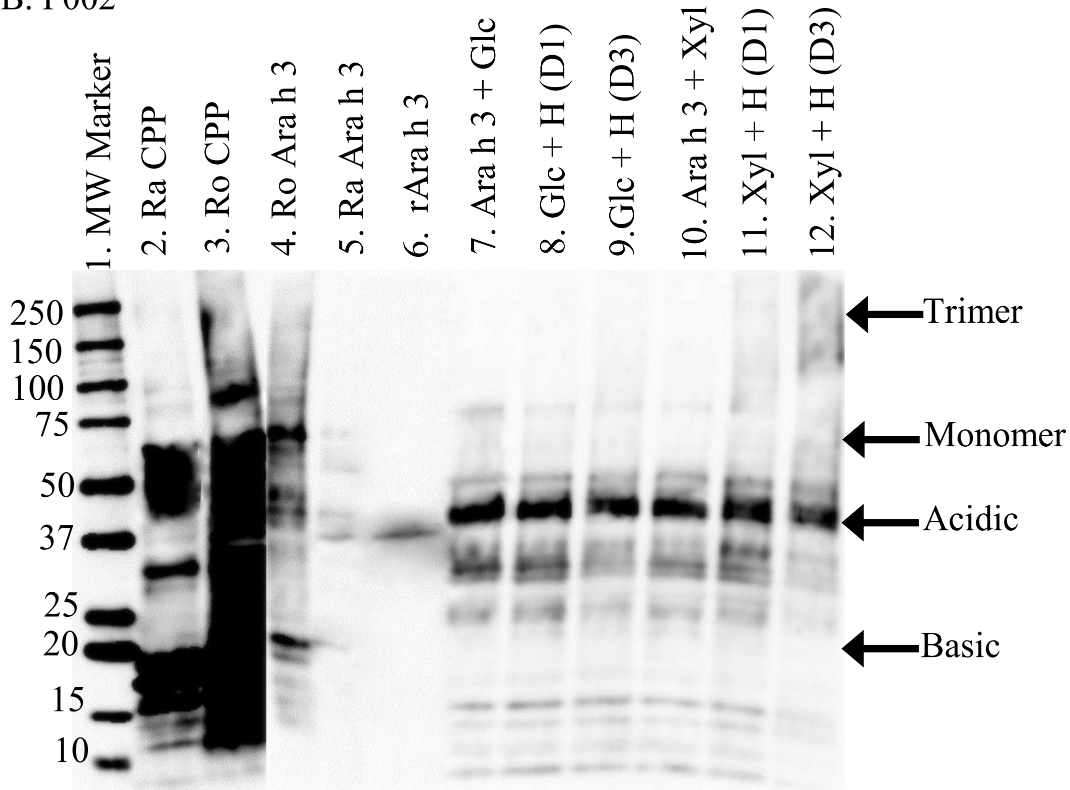
Comparing SR Ara h 3 samples (lane 6 and 7) to each other, the Maillard reaction occurred faster in the presence of xylose (lane 7) than glucose (lane 6). This observation is more obvious in Figure 16 than the SDS-PAGE (Figure 15). SR Ara h 3 in the presence of xylose (lane 7) had the most significant appearance of higher molecular weight proteins as shown by the most amount of smearing. SR Ara h 3 in the presence of glucose (lane 6) also had the presence of smearing and higher order structures after 72 hours of heating which was not visible in Figure 15 lane 8. This indicates that proteins subjected to the Maillard reaction undergo chemical and structural modifications that are analogous to those seen in roasted peanuts (lane 3 and 5).

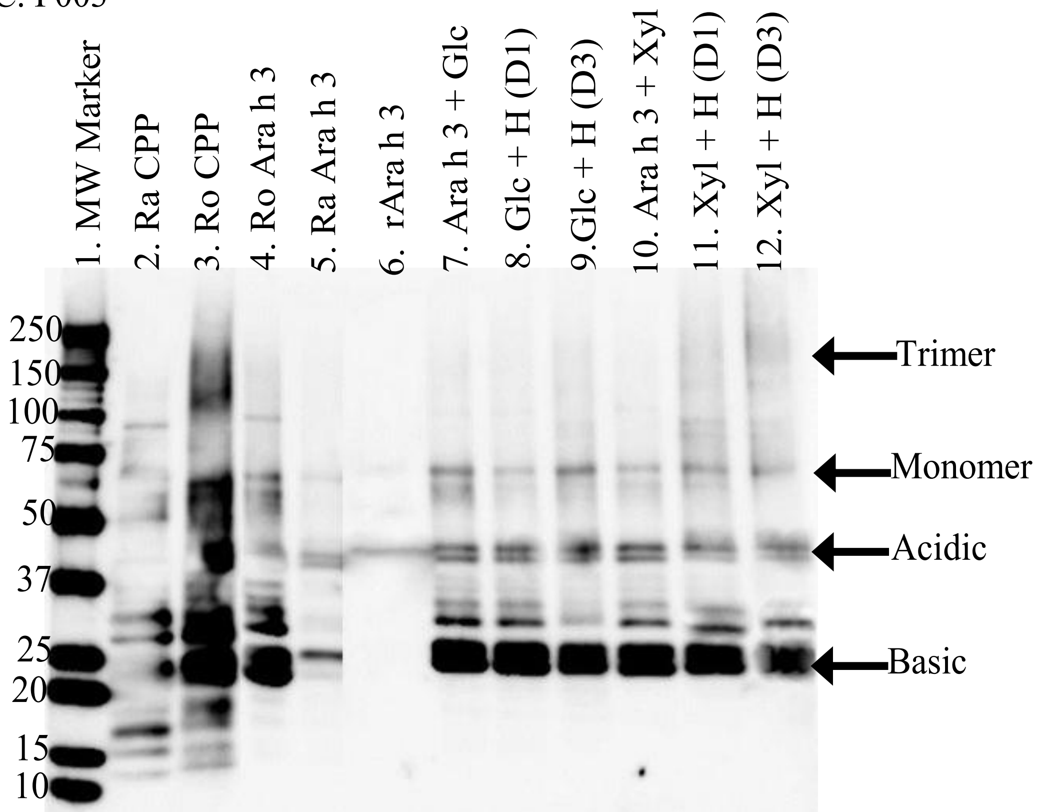
Serum IgE Recognition to Raw, Roasted Ara h 3, Recombinant Ara h 3 Acidic and Basic Subunits, and Simulated Roasted Ara h 3

Immunoblot analyses (Figure 17 (A-K)) using peanut allergic patient sera (see Table 3-1 for patient ID and clinical reactions) were performed to determine the answers to three different questions. First, does patients' serum IgE recognize the recombinant subunits with as much efficiency as it does purified native raw and roasted Ara h 3? Secondly, immunoblots were performed to identify the differences in patient recognition of the acidic and basic subunits in raw and roasted Ara h 3. Lastly, patient serum IgE immunoblots were performed to determine if an in vitro Maillard reaction on native raw Ara h 3 in the presence of glucose or xylose could mimic dry roasting as seen in roasted peanuts and compare patient IgE binding to the SR proteins. IgE binding of SR Ara h 3 in the presence of glucose and xylose was compared to roasted CPP and native roasted Ara h 3 (Figure 17 (A-K) lanes 3, 4, 7-12).

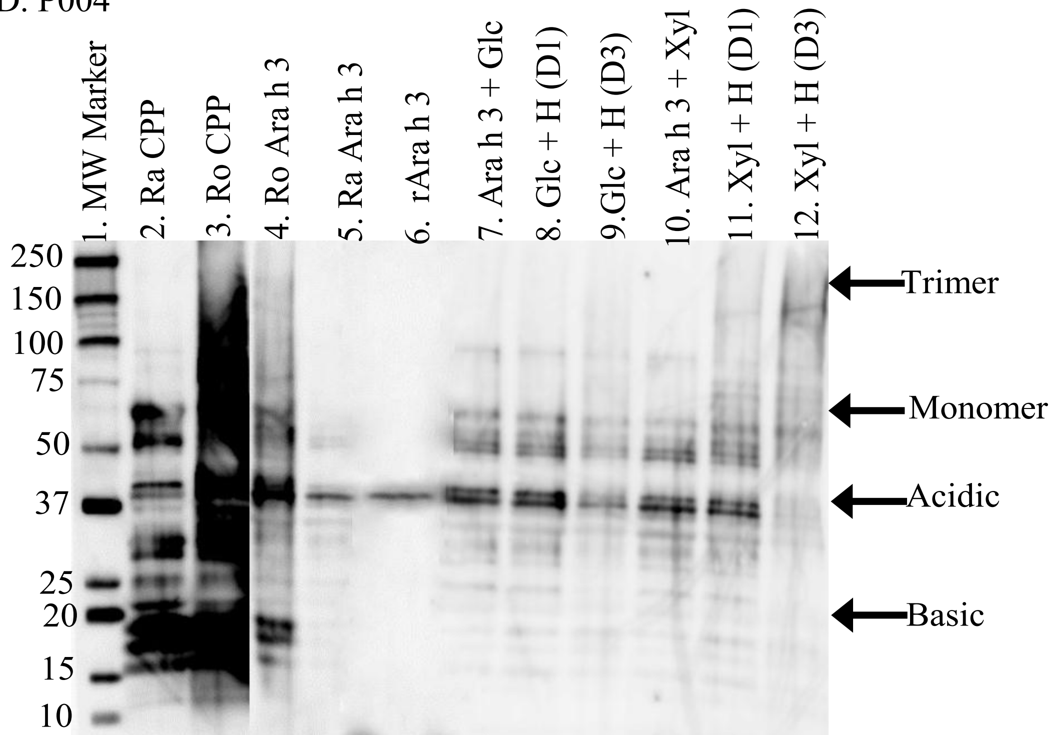
A. P001



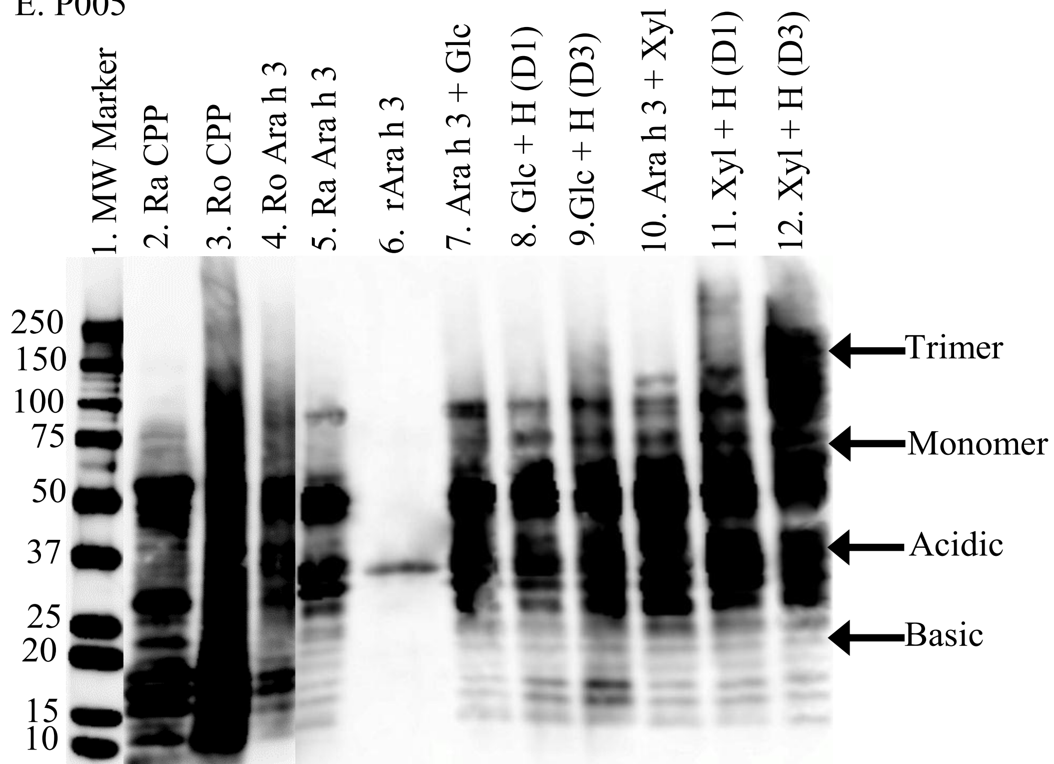

B. P002
1. MW Marker
2. Ra CPP
3. Ro CPP
4. Ro Ara h 3
5. Ra Ara h 3
6. rAra h 3
7. Ara h 3 + Glc
8. Glc + H (D1)
9.Glc + H (D3)
10. Ara h 3 + Xyl
11. Xyl + H (D1)
12. Xyl + H (D3)
250
150
100
75
50
37
25
20
15
10
Trimer
Monomer
Acidic
Basic


C. P003
1. MW Marker
2. Ra CPP
3. Ro CPP
4. Ro Ara h 3
5. Ra Ara h 3
6. rAra h 3
7. Ara h 3 + Glc
8. Glc + H (D1)
9.Glc + H (D3)
10. Ara h 3 + Xyl
11. Xyl + H (D1)
12. Xyl + H (D3)
250
150
100
75
50
37
25
20
15
10
Trimer
Monomer
Acidic
Basic

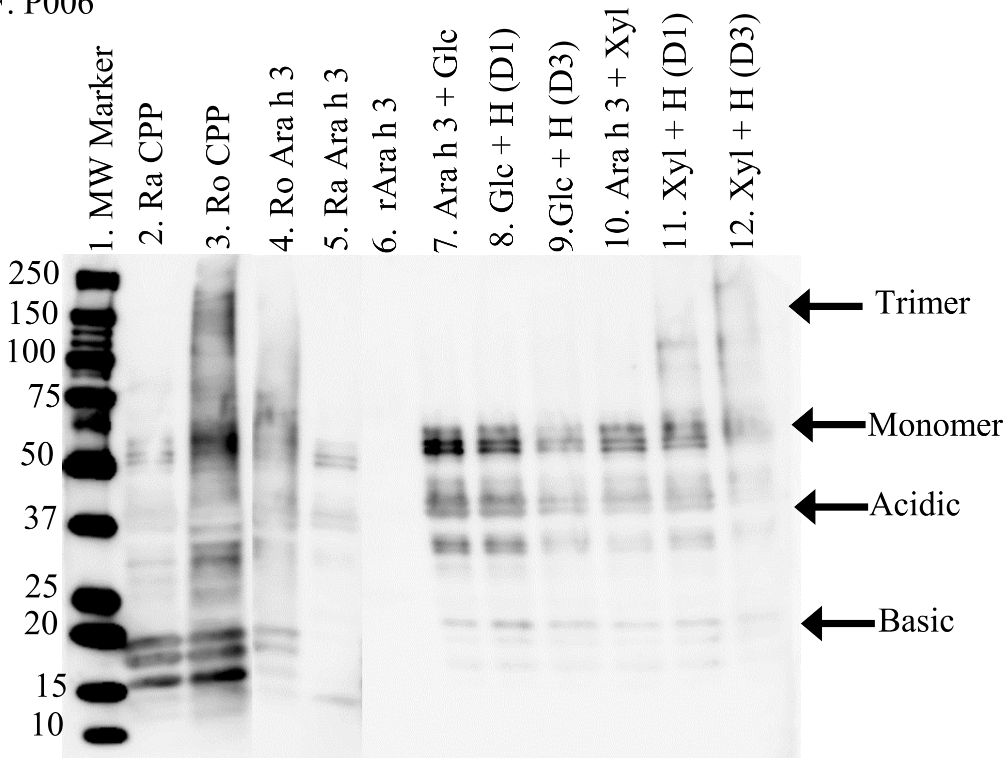
D. P004



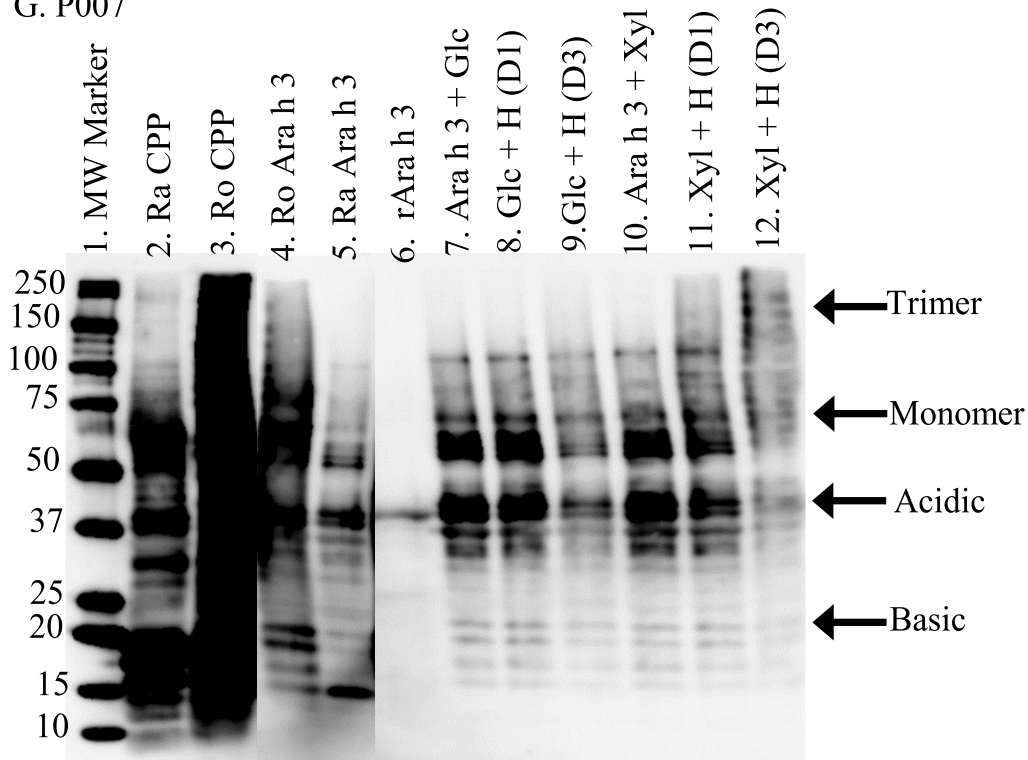
E. P005



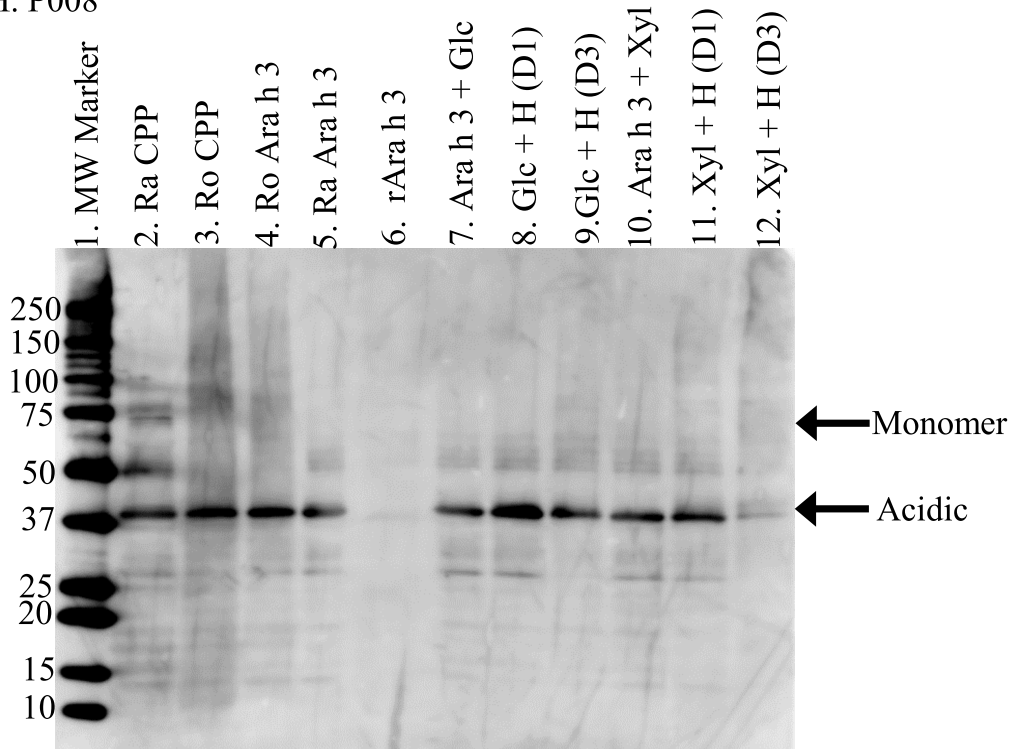
F. P006



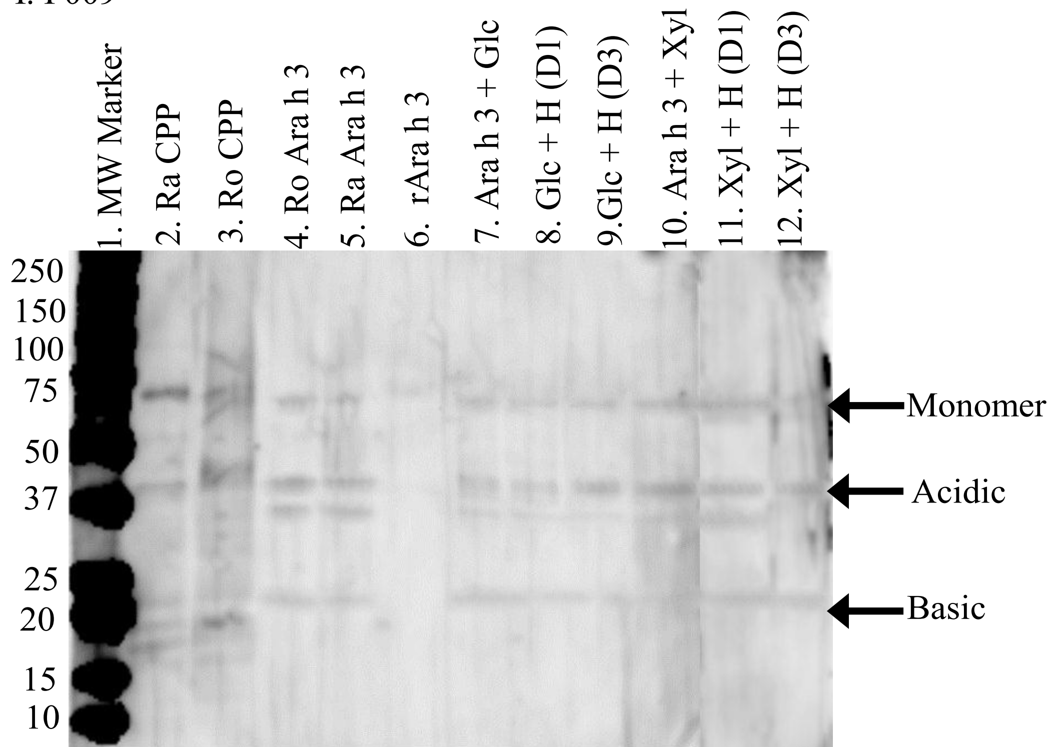
G. P007

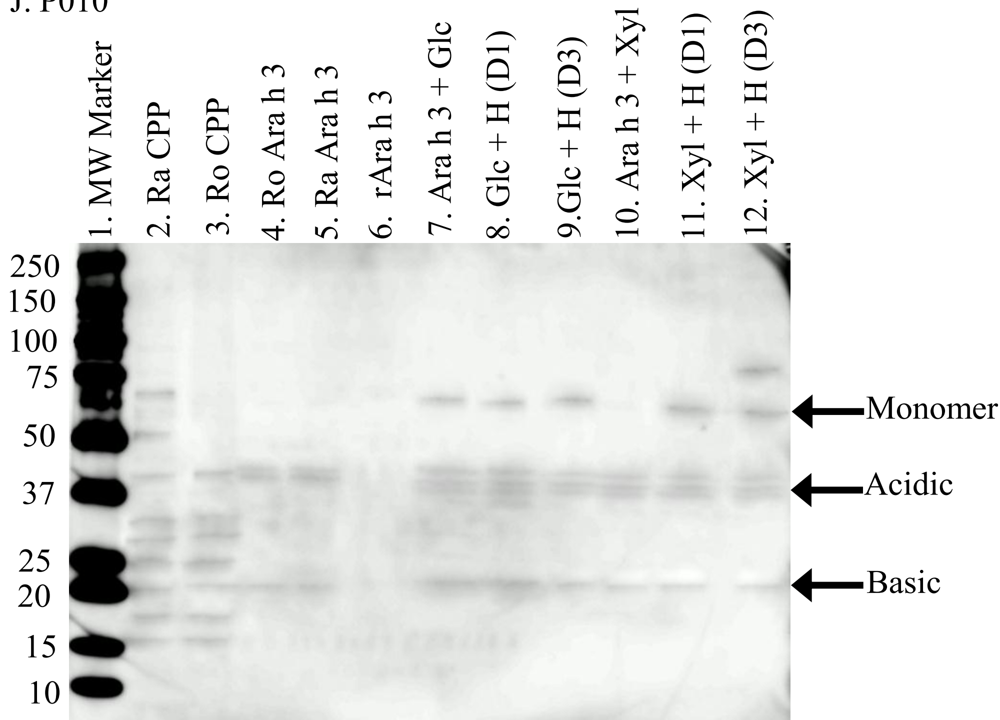


H. P008



I. P009





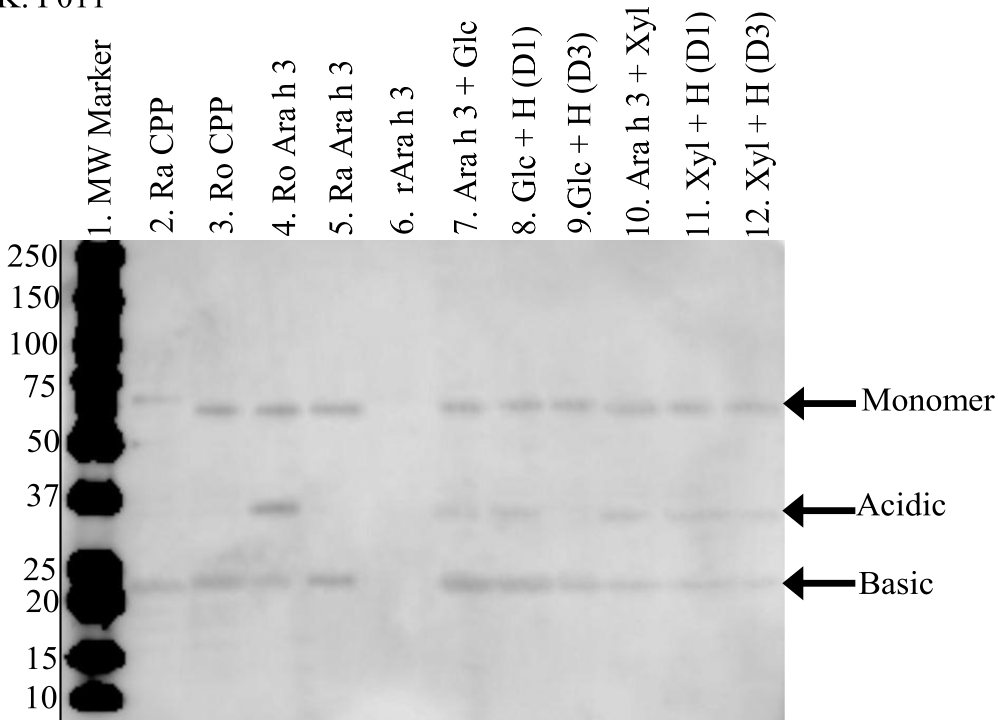


Figure 17 (A-K) Immunoblottings obtained after the transfer of samples obtained in Figure 15. Lane 4: native roasted Ara h 3. Lane 5: native raw Ara h 3. Lane 6: recombinant Ara h 3 acidic and basic subunits. Lane 7-12: simulated roasted Ara h 3. Lane 7: no heat. Lane 10: no heat. Lanes 8 and 11: D1: sample taken after 24 hours of heating. Lanes 9 and 12: D3: sample taken after 72 hours of heating.. Basic subunit, acidic subunit, formation of monomers, and trimers are indicated with an arrow and labeled. MW Marker: Precision Plus Protein WesternC.

IgE binding properties of Ara h 3 differed from patient to patient (Figure 17 (A-K)) particularly, when looking at recognition of the individual subunits. Serum IgE from P003, P006, P010, and P011 (Figure 17 (C, F, J, and K) respectively) all bound the native basic subunit with more intensity than the acidic subunit. P002, P004, P005, P007, and P008 (Figure 17 (B, D, E, G, and H) respectively) all bound the native acidic subunit with more intensity than the basic. P001, P009, and P010 (Figure 17 (A, I, and J) respectively) bound both native subunits with the same intensity.

Patients' sera that bound to acidic subunit in both native roasted and raw Ara h 3 with high intensity (Figure 17 lanes 4 and 5 respectively) also bound the recombinant Ara h 3 acidic subunit (lane 6). Those patients whose serum IgE bound the recombinant Ara h 3 acidic subunit were P001, P002, P003, P004, P005, P007, and P008 (Figure 17 (A, B, C, D, E, G, and H) lane 6 respectively). If patients weakly recognized the native Ara h 3 acidic subunit in either native roasted Ara h 3 (lane 4) or raw Ara h 3 (lane 5), they did not recognize the recombinant Ara h 3 (40 kDa) subunit (Figure 17 (C, F, I, J, and K) lane 6 respectively). Interestingly, patients whose serum IgE recognized and bound the native basic subunit preferably over the native acidic subunit in both roasted and raw Ara h 3 (lanes 4 and 5) did not recognize or bind the recombinant Ara h 3 basic subunit (lane 6) with one exception, P014 (Figure 14 (C) lane 4). This patient was the only patient who recognized and bound the recombinant Ara h 3 basic subunit.

Higher binding to roasted versus raw peanut proteins could be seen in all patients tested (Figure 17 (A-K)). Even when comparing raw CPP (lane 2) to roasted CPP (lane 3) and roasted Ara h 3 (lane 4) to raw Ara h 3 (lane 5).

Other experiments were performed to determine if the Maillard reaction was able to simulate the roasting process and to what efficiency patients' serum IgE bound the resultant Maillard reaction products that modified Ara h 3 (Figure 17 (A-K) lanes 7-12). Our Simulated Roasting Model (SRM) consists of an isolated reaction where on the protein and a reducing sugar are incubated together and heated to 55 °C. In Figure 15 and 17, the SDS-PAGE and anti-Ara h 3 (40 kDa) western blots are shown to determine the migration patterns of Ara h 3 proteins such as: smearing or the presence of higher order structures in raw and roasted CPP, native raw and roasted Ara h 3, and SR Ara h 3. Figure 16 showed the appearance of smearing and higher order structures better than Figure 15 and the immunoblots (Figure 17 (A-K) showed that IgE also bound to the higher order structures seen in Figure 16.

Immunoblot analysis (Figure 17 (A-K)) showed the appearance of smearing and higher order structures produced by protein crosslinking and chemical modifications. Patients who were tested had similar IgE binding reactivity to native Ara h 3 subjected to the SRM (lanes 7-12), roasted CPP (lane 3), and roasted Ara h 3 (lane 4). Binding of serum IgE to lower molecular weight proteins in SR Ara h 3 (lanes 7-12) decreases as incubation in the presence of sugar in heat increases, but the SRM enhanced

recognition of serum IgE to higher order structures. Patients' serum IgE recognized and bound the higher molecular weight proteins that appear as smears, as well as, truncated forms of Ara h 3 acidic subunit in SR Ara h 3 samples (lanes 7-12) (see Figure 15 for SDS-PAGE and truncated forms of acidic subunit). Table 3-2 summarizes each individual patient's serum IgE recognition and binding.

Table 3-2 Summary of Patient Serum IgE Binding to Ara h 3 Proteins

Patient ID	Trimer (180 kDa)	Monomer (60 kDa)	Acidic Subunit (40 kDa)	Basic Subunit (20 kDa)	Truncated Acidic Subunit (28/27 kDa)	Truncated Acidic Subunit (16/17 kDa)	Truncated Acidic Subunit (14 kDa)	rAcidic Subunit	rBasic Subunit
P001	Y	Y	Y	Y	Y	Y	Y	Y	N
P002	Y	Y	Y	Y[1, 2, 3]	Y[5]	Y[4, 5]	Y	Y	N
P003	Y	Y	Y	Y	Y	N	N	Y	N
P004	Y	Y	Y	Y[1, 2, 3]	Y	N	N	Y	N
P005	Y	Y	Y	Y	Y	Y	Y	Y	N
P006	Y	Y	Y	Y[3, 5]	Y[3, 5]	Y[3]	Y[4]	N	N
P007	Y	Y	Y	Y	Y	Y	Y[3, 4]	Y	N
P008	Y	Y	Y	N	Y	N	Y[3, 4]	Y	N
P009	N	Y	Y	Y	N	N	N	N	N
P010	N	Y	Y	Y	Y	Y	N	N	N
P011	N	Y	Y[3, 5]	Y	Y	Y	N	N	N
P012	N	N	N	Y	N	N	N	N	N
P013	N	N	Y	Y	Possible	N	N	N	N
P014	N	N	N	Y	Y	N	N	N	Y

Y: patient bound. N: patient did not bind. rAcidic Subunit: recombinant Ara h 3 (40 kDa) subunit. rBasic Subunit: recombinant Ara h 3 (20 kDa) subunit. Superscript number indicates the patient only bound the indicated protein in specific samples and the superscripts correspond to 1: Raw CPP 2: Roasted CPP 3: Roasted Ara h 3 4: Raw Ara h 3 5: SR Ara h 3.

Immunoblots Using Sera from P012 Taken at Two Different Time Points

In Figure 14, sera from three patients was randomly chosen to determine if IgE antibodies would detect the recombinant subunit inclusion bodies. Serum IgE from P012 was one of the three patients chosen and recognized the basic subunit in native raw Ara h 3. An immunoblot was performed with this patient's serum IgE and included all normalized samples shown in Figure 15. It was assumed that P012 would not only recognize the basic subunit in native raw Ara h 3 but also in CPP, native roasted, and SR Ara h 3. The immunoblots using P012 sera are shown in Figure 18 (A and B).

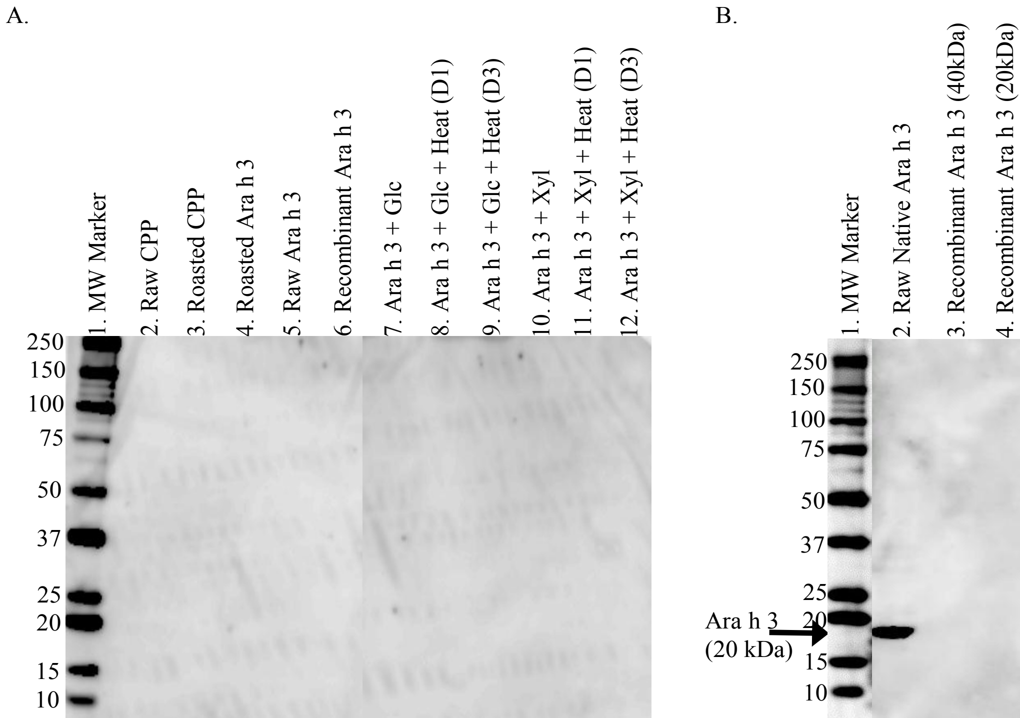


Figure 18 Serum IgE from P012 used in an anti-IgE immunoblot. (Figure A) Serum acquired from patient several years after last accidental peanut ingestion. (Figure B) Serum was acquired from patient after a recent accidental ingestion of peanut. Identity of the samples in each lane are indicated at the top of the figure. MW Marker: Precision Plus Protein WesternC.

Referring to the Patient ID table (Table 3-1), P012 suffers from relatively mild symptoms to peanut, such as, gastrointestinal upset and only recognized the Ara h 3 basic subunit (Figure 18 (B)). In Figure 18 (A), IgE antibodies from this patient's serum recognized none of the peanut proteins, including Ara h 3 (20 kDa) subunit or Ara h 2 proteins which most patients recognize. In Figure 18 (B), serum IgE from P012 recognized the 20 kDa subunit very specifically in native raw Ara h 3 but not the acidic subunit or either recombinant subunits. Serum from P012 used in Figure 18 (B) was acquired after an accidental ingestion of peanut and serum used in Figure 18 (A) where the patient had avoided peanut and had not ingested peanut in years. This accounts for the discrepancy in the two immunoblots.

Discussion

This study was carried out to further characterize a major immunodominant peanut allergen, Ara h 3. Many studies have focused on the other immunodominant allergens, Ara h 1 and Ara h 2 and their recombinants forms, however, this study focused on Ara h 3, both in its native and recombinant form. It has been documented that roasted peanuts bind serum IgE from individuals who are allergic peanuts approximately 90-fold higher than raw peanuts[9] and the Maillard reaction is the main contributor to this finding.[9] An examination of roasting and the chemical modifications that occur was assessed by an in vitro simulated roasting model to determine for the first time if the Maillard reaction in vitro could mimic roasting on Ara h 3 and if serum IgE would bind simulated roasted Ara h 3 proteins similarly to roasted peanuts. Moreover, analysis of serum IgE binding to the acidic subunit and basic subunit was compared between purified raw and roasted Ara h 3. Finally, the recombinant acidic and basic subunits were compared to native raw and roasted Ara h 3 through analysis with serum IgE to determine if patients bind the basic subunit or the acidic subunit preferably in the recombinant form and also to determine if patients' serum IgE recognize the recombinant subunits with as much efficiency as the purified native raw and roasted Ara h 3.

Recombinant Ara h 3 Acidic and Basic Subunit Expression and Basic Subunit Solubility and Purification

High level expression of recombinant Ara h 3 (40 kDa) and (20 kDa) subunits can be achieved when induced using a low-copy expression vector, pET-9a and expressed at a low temperature (20 °C) overnight (Figure 4-2 lane 6 and 4-3 lane 3). The two recombinant subunits differed in solubility where recombinant Ara h 3 (20 kDa) was found to be insoluble in every buffer used. Under denaturing conditions using high concentrations of urea, a majority of recombinant Ara h 3 (20 kDa) was soluble but a portion of the protein remained in the insoluble pellet. High concentrations of a denaturant being unable to completely solubilize a protein, the recombinant basic subunit is characteristic of improper protein folding and inclusion body formation. Consequently, recombinant Ara h 3 basic subunit was purified using an inclusion body isolation and gel purification method. Highly specific custom made chicken antibodies against the basic subunit were produced using gel slices.

Recombinant Ara h 3 Acidic Subunit Solubility and Purification

Contrary to the basic subunit, recombinant Ara h3 (40 kDa) becomes soluble in a high Tris, high NaCl, and high pH buffer and precipitated at 50 % ammonium sulfate saturation. Several 50 % ammonium sulfate pellets containing Ara h 3 (40 kDa) were used in multiple attempts to purify Ara h 3 (40 kDa) using ion exchange chromatography. The 50 % ammonium sulfate pellets were subjected to various conditions using both a strong anion-exchange resin and a strong cation-exchange resin.

Recombinant Ara h 3 (40 kDa) was found to bind the High-Prep Q resin at a pH value of 9.0 and eluted off the column at approximately 275 mM – 375 mM NaCl. Ara h 3 was present in fractions selected for SDS-PAGE but had a substantial amount of contaminating proteins. In conclusion, this purification was successful in terms of Ara h 3 binding and eluting from the column in fractions during the NaCl gradient.

Caveats to these first two purification attempts are two variables were changed (pH value and salt concentration). This rendered incomplete purifications from both of these attempts. Although useful information was obtained by performing a purification changing two variables including:
Ara h 3 (40 kDa) bound to High-Prep Q resin more efficiently at a pH value of 9.0 versus 8.4 yet, a pH value of 8.4 resulted in a significant increase in the amount of protein that went into solution prior to loading onto the column and resulted in minimal precipitation of the protein prior to loading. Both purification attempts provided data that could be applied in subsequent trials.

A final anion-exchange purification was attempted and buffers were used at a pH value of 9.0. Approximately 410 mg of protein was loaded onto the column and Ara h 3 bound tightly to this resin. Total amount of protein in solution, loaded onto the column, and strength of Ara h 3 binding to High-Prep Q resin was consistent with the previous attempt. Recombinant Ara h 3 appeared in fraction numbers 73 and 75 and SJM - 500 wash which was inconsistent with the previous attempt. Fraction numbers 59 - 80 and SJM - 500 wash were pooled and diluted to approximately 250 mM NaCl and re-loaded onto a High-Prep Q column at pH value of 9.0 to maintain Ara h 3 in solution and bound to the resin. Absorbances on the chromatogram from the linear NaCl gradient (250 mM – 520 mM) of fractions collected were low. The majority of absorbance readings from Figure 9 (A) were at or below 0.02, except fraction number 49. SDS-PAGE in Figure 9 (C) lane 6 contained a sample of fraction 49 and there was a faint band at the molecular weight of Ara h 3 (40 kDa). Anti-Ara h 3 (40 kDa) western blot confirmed the presence of Ara h 3 (40 kDa) in the load onto the column and in fraction number 49.

A final attempt at purifying Ara h 3 (40 kDa) before proceeding to other conditions or options used a strong cation-exchange resin, High-Prep S. Approximately 380 mg of protein was loaded onto the column at a pH value of 6.5 and it appeared that Ara h 3 bound to the column. Linear NaCl gradient of SJM - 50 – SJM - 500 buffer was applied and absorbance readings of fractions collected were low and below 0.02 (Figure 10 (A)). Fractions selected for SDS-PAGE confirmed the absence of Ara h 3 in wash fractions, fractions collected from the NaCl gradient, and in 1 M NaCl wash. Ara h 3 (40 kDa) bound to High-Prep S column at pH value of 6.5 but never eluted off. It was concluded that even though recombinant Ara h 3 (40 kDa) becomes soluble in a high Tris, high NaCl, high pH buffer and precipitated at 50 % ammonium sulfate saturation, recombinant Ara h 3 acidic subunit was expressed as unfolded, aggregated, insoluble inclusion bodies, and the high concentration of Tris in the solubilization buffer

could possibly denature the protein leading to solubility. Therefore, under low Tris concentrations, certain NaCl concentrations and pH conditions, Ara h 3 (40 kDa), which was soluble, actually precipitated on the columns or degraded.

Other methods of purification were explored for the acidic subunit and an inclusion body isolation followed by gel purification was chosen to proceed with. The inclusion body purification of the acidic subunit produced a pure and concentrated protein.

Immunoblot Analyses Using Serum IgE from Peanut Allergic Patients

Purified inclusion bodies of recombinant Ara h 3 (40 kDa) and (20 kDa) subunits were used in immunoblot analyses with samples of raw and roasted crude peanut proteins, purified from peanut native raw and roasted Ara h 3, and native raw Ara h 3 subjected to the Maillard reaction. The detection of IgE binding to higher molecular weight proteins produced by the isolated in vitro Maillard reaction, to the acidic versus basic subunits in raw and roasted Ara h 3, and the recombinant protein recognition was performed using serum IgE from peanut allergic patients.

Recognition by serum IgE varies between Ara h 3 subunits in the raw form, but roasting appears to increase binding to both subunits. Patient binding patterns to simulated roasted Ara h 3 are very similar to how each individual patient binds roasted Ara h 3. By day 3 of the SRM, recognition of the individual subunits appears to decrease in simulated roasted Ara h 3, although, the presence of higher order structures and smearing by day 3 increases significantly. This increase in smearing and recognition of higher order proteins by serum IgE is indicative of protein/peptide crosslinking and chemical crosslinking that is normally seen following roasting.

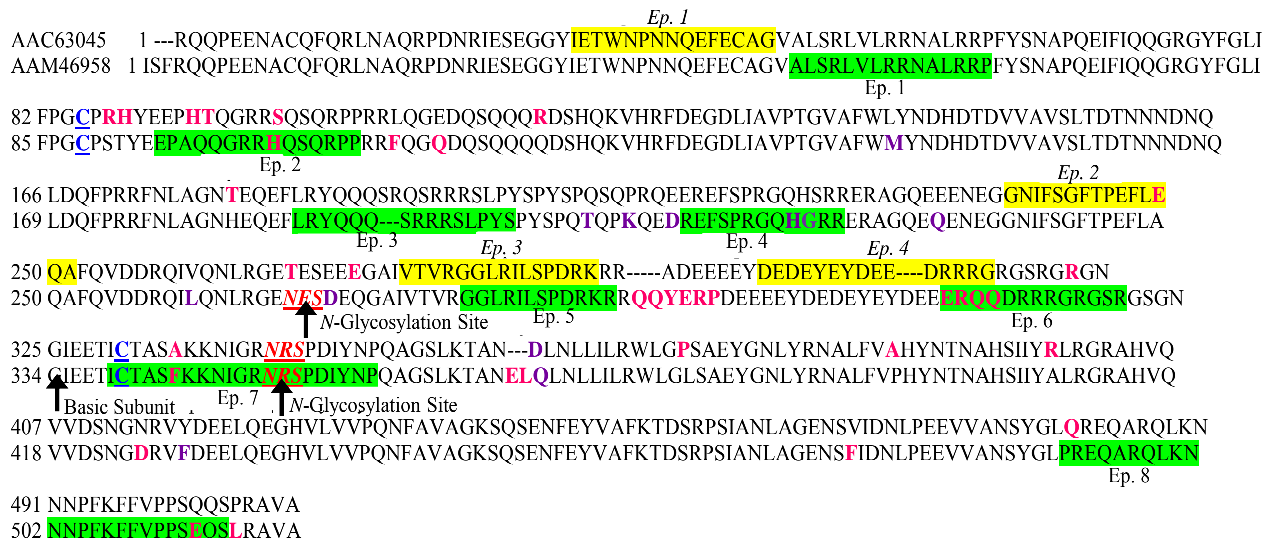


Figure 19 Sequence Alignments used by Rabjohn et.al (top sequence, accession number AAC63045) and Rougé et.al. (bottom sequence, accession number AAM46958). Epitopes (Ep.) are identified by Rabjohn et.al. labeled in italics above the sequence and highlighted in yellow. Epitopes identified by Rougé et.al. are labeled in non-italics under the sequence and highlighted in green. Conserved cysteine residue that is involved in the disulfide bond between the acidic and basic subunits is bolded, underlined, and in blue. The *N*-glycosylation sites are bolded,

underlined, italicized, red, and labeled. The start of the basic subunit is indicated. Non-conserved residues are in magenta and similar residues are in purple.

In regards to serum IgE recognition of the recombinant acidic subunit, 50 % of patients tested recognized the recombinant acidic subunit. This appeared to follow a trend of the patient binding to the acidic subunit with high intensity in both the raw and roasted native forms of Ara h 3. It appeared that patients whose serum IgE bound the acidic subunit with high intensity in the native raw and roasted or simulated roasted samples recognized the recombinant subunit. According to Rabjohn et.al, all epitopes are located within acidic subunit.[32] This could account for our patients sera IgE recognizing the recombinant acidic subunit more frequently. Only 7.1 % (1/14) of our patients bound the recombinant basic subunit, P014. A few of our patients exhibited serum IgE binding and recognition to the basic subunit only in crude peanut protein samples, native Ara h 3 samples, and simulated roasted Ara h 3 samples. Possible explanation is even though the basic subunit contains two IgE binding epitopes only one, epitope 7, is considered immunodominant.[37] In addition, the recombinant basic subunit is only recognized by one patient which can be explained by the absence of an *N*-glycosylation site which is present within epitope 7 of native proteins.[37] Recognition by serum IgE of the recombinant basic subunit may be specific to this post-translational addition of an *N*-glycan chain within epitope 7.

Patient IgE binding to the recombinant subunits was less intense than to their native counterparts. This is most likely due to the fact that the recombinant subunits are not glycosylated and indicated that glycosylation may be important in IgE recognition of Ara h 3 by peanut allergic individuals. If any structure is maintained by the native allergens following denaturing SDS-PAGE followed by IgE binding, the perhaps these maintained structures contribute to IgE binding.

The ability of proteins to aggregate to form oligomers, form disulfide bonds, cross-link between peptides and proteins, bind sugar moieties, and undergo glycosylation has been shown to stabilize food allergens by enhancing their properties, such as, resistance to heat, proteolytic degradation in the gastrointestinal tract, and possibly enhance their recognition by immune cells.[11, 23] The cupin motif seems to promote the allergenic potency of food allergen because the secondary structure is highly packed together, perhaps even following SDS-PAGE.[36] Since recombinant Ara h 3 basic subunit is expressed as inclusion bodies, the cupin fold is most likely lost. Unfortunately, studies into the secondary or tertiary structures of the recombinant protein were unable to be performed and remain to be studied more extensively. It can be assumed that the stability of the recombinant basic subunit was affected since the protein was unable to undergo glycosylation since it was expressed in *E. coli* cells and the ability to form the native disulfide bond was affected due to the acidic subunit absence.

An additional important characteristic to consider is the second amino acid residue in epitope 7 is the conserved cysteine residue that is important for the intermolecular disulfide bond that links the acidic and basic subunits together and allows the protein to fold into its native conformations of trimers and hexamers.[36, 37] This cysteine residue in the recombinant basic form is unable to covalently associate with the acidic subunit, therefore, recombinant Ara h 3 basic subunit is not able to fold correctly. Improper folding of the protein can lead to alterations in the secondary and tertiary structure.[36, 37]

An alternative theory would be that the sera used in this study only contained IgE antibodies that bind to the conformational epitopes to the basic subunit[36] and it is possible that some structural motifs remain intact in the native roasted and SR Ara h 3 subunits even after denaturing SDS-PAGE. Since the protein was subjected to SDS-PAGE, it can be assumed that the recombinant subunits of Ara h 3 are most likely denatured, but there is a possibility that roasting and the SR Ara h 3 proteins maintain some structure due to chemical crosslinking and oligomerization even after SDS-PAGE. This indicates that there are structural components of Ara h 3 that contribute to an increase in IgE binding.

Separating the subunits and studying them in recombinant forms demonstrated that the acidic and basic subunits rely on each other to fold properly in order to assemble into the native structure.[36] The appearance of several bands corresponding to truncated versions of the acidic subunit visible on SDS-PAGE (Figure 15) supported the conclusions from Piersma et.al. that the acidic subunit in native Ara h 3 undergoes extensive proteolytic processing.[33] *In planta*, Ara h 3 acidic subunit is proteolytically truncated and several peptides arise corresponding to these truncations. C-terminally truncated peptides of Ara h 3 acidic subunit that contain conserved cysteine residue number 88 (C88) are able to form intermolecular disulfide bonds with the basic subunit.[33] Truncated peptides corresponding to approximate molecular weights at 16 kDa and 17 kDa are N-terminally truncated versions of the acidic subunit and are incapable of bonding with the basic subunit because C88 is absent.[33] It is interesting that all the truncated versions of the acidic subunit in native Ara h 3 contain IgE epitopes, but the number of epitopes contained varies by the length of each truncated product.[33] The immunoblots shown in this study correspond with these conclusions. A majority of patients tested recognized some form of the truncated acidic subunit in both native raw and roasted Ara h 3 and simulated roasted Ara h 3. The variation of which truncated form patients recognized is characterized by which epitopes are present in the peptide and brings up questions of which subunit and epitopes are immunodominant.

There was some variation between patients in regards to the recognition of the basic and acidic subunits in native Ara h 3. Seventy-one percent of patients tested bound both the acidic and basic subunits in roasted Ara h 3, which is up from 36 % that recognized both subunits in raw Ara h 3. A specific example is in Figure 17, P007 recognized the 14 kDa truncated version of the acidic subunit very intensely in raw Ara h 3, however, in roasted Ara h 3, serum IgE from this patient still recognized the

14 kDa protein, but bound the 20 kDa more significantly and appears to have bound a protein at the molecular weight of approximately 32 kDa. The protein observed at 32 kDa is the 14 kDa peptide covalently linked to the basic subunit.[33] This is just one example of how roasting can stabilize the structure of a protein through disulfide linkages and other covalent interactions to form higher order structures but also enhancing the recognition of the protein by the immune system. Another example is shown in Figure 18 using serum IgE from P012. In Figure 18 (A), serum IgE used was acquired several years after the last accidental ingestion of peanut, whereas in Figure 18 (B), the patient had recently been exposed to peanut. The differences between the two immunoblots from this patient indicated that the immune system may not necessarily stimulate a high production of IgE antibodies toward an allergen to the degree of being undetectable using a high concentration of protein and low dilution of serum. Nevertheless, upon exposure to the antigen several years later, an immune reaction can occur and stimulate the production of IgE antibodies.

It has been postulated that the basic subunit might be more significant in other geographical areas other than in the United States.[31] Western blots in this study using serum IgE from peanut allergic patients living in America demonstrated that 71.4 % (10/14) patients recognized the basic subunit in roasted Ara h 3, 71.4 % (10/14) patients recognized the basic subunit in raw Ara h 3, and 57.1 % (8/14) patients recognized the basic subunit in simulated roasted Ara h 3. These percentages are approximately the same for patient recognition to the acidic subunit. It is more probable that other allergens found in peanut are more significant geographically which has been shown by several studies.[29, 30, 31] Recognition to the basic subunit of Ara h 3 by children in Italy described by Restani et.al. is presumably the cause of unidentified epitopes in the basic subunit.[12, 31]

To better understand Ara h 3, it needs to be transcribed as one mRNA and translated as a whole proprotein then cleaved into an acidic and basic subunit post-translationally that remain covalently linked by their intermolecular disulfide bond as it is naturally done in the plant.[33] It is known that there are five genes that encode Ara h 3 in a single peanut cultivar, which allows for sequence heterogeneities and multiple isoforms of Ara h 3 to arise.[36] This can produce multiple peptides and various IgE binding sites that are not able to be obtained by a single gene. Allowing for two or more Ara h 3 genes to be recombinantly transcribed and translated in one organism that allow for post-translational processing might have potential for multiple polypeptides that contain various IgE binding sites and could assist in proper protein folding. Recognition of the basic subunit by serum IgE might be elucidated for the reason that if patients are sensitized to conformational epitopes versus linearized; this would allow conformational epitope formation and expression.

Possibly many differences that have been observed among different laboratories could be resolved if a recombinant source was developed that allowed 11S globulins to be studied recombinantly as close to their native form but still allowed the possibility of genetic alterations in the DNA encoding the IgE epitopes to look at altering allergenicity. Thus far, a recombinant source has not been developed that would allow for this extremely complex process to occur as it does in the native plant. Through the SRM and western blot analyses, it can be determined that more research needs to done on Ara h 3 to gain a better understanding of this highly complex, multi-subunit, and extensive proteolytically processed protein in order to address improving immunotherapies for peanut allergies.

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Vita

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