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Synthesis and identification of an investigational essential precursor compound for the purpose of the development of a vaccine treatment against the peanut allergy

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SYNTHESIS AND IDENTIFICATION OF AN INVESTIGATIONAL ESSENTIAL PRECURSOR COMPOUND FOR THE PURPOSE OF THE DEVELOPMENT OF A VACCINE TREATMENT AGAINST THE PEANUT ALLERGY

by

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Dedication

I would like to dedicate this master's thesis to my mother and father for providing me with their unwavering support and love throughout this research program. Their commitment to my success was inspiring and kept me motivated to achieve my goals.

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First and foremost, I would like to thank my advisor, Dr. Catherine Yang. Training in her research lab has provided me with an excellent research experience that will benefit me for my lifetime. I feel grateful for her guidance and for the opportunity to have learned from such a successful and passionate role model. I also feel very grateful and humbled by the opportunity to have worked on such an important project, and to be part of a dedicated team of researchers. I am confident that the experiences and guidance I received here have provided me with the tools I'll need to be successful in my future endeavors.

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Abstract

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SYNTHESIS AND IDENTIFICATION OF AN INVESTIGATIONAL ESSENTIAL PRECURSOR COMPOUND FOR THE PURPOSE OF THE DEVELOPMENT OF A VACCINE TREATMENT AGAINST THE PEANUT ALLERGY
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Presented in this master's thesis are several studies carried out to determine the viability of several allergoid candidates utilizing the major peanut allergen Ara h 2. The Ara h 2 allergen protein appears naturally as a doublet when observed by gel electrophoresis, SDS-PAGE. Optimization of allergen purification methods successfully led to Ara h 2 purity, and the ability to standardize procedures to yield consistently pure samples. The purified allergen was chemically crosslinked with diketone derivatives selected for their abilities to react with specific amino acids accessible on the Ara h 2 protein. Chemically modified allergen samples were also evaluated using SDS-PAGE; successful protein modifications were identified by doublet band smearing or even oligomer formations such as dimers and trimers. Immunoassays were then applied to determine if epitope surface region is disrupted to indicate the diminished immune responsiveness to the modified allergen. Ara h 2 IgE specific antibodies, through western blot analysis, were used to determine antibody affinity between chemically modified and unmodified allergen proteins and to characterize any differences between the allergen samples. Results indicate that Ara h 2 may be chemically crosslinked using functional active reagents and that is possible to chemically shield IgE epitope surface regions after modification.

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Chapter 1

Introduction

Significance of Peanut Allergies

Food allergies are a major health concern for patients and for the healthcare providers who are tasked with the responsibility to treat them, because of the rapid onset and limited treatment options available in case of emergency. Currently, 8 foods are associated with food related allergies because of the immune reaction they have the potential to elicit in allergic individuals[1]. Food allergies are prevalent in the developed world, with more than 2% of the adult population (nearly 5.5 million Americans) estimated to suffer from them[2]. However, peanuts are especially disconcerting because of the low potential for patient to outgrow the condition. Common allergies to foods are likely to be outgrown or tolerated once the patient reaches adulthood, however an allergy to peanuts is not ameliorated with age and is virtually a life-long condition[3]. Peanut allergies are severe because they can sometime cause death almost immediately due to fatal anaphylaxis[4]. Widespread application and use of peanuts throughout the world pose an inescapable risk to patients, currently no preemptive treatments exist but to avoid exposure to peanuts altogether. Among all allergies, a peanut allergy is the most severe and widespread. It is believed that 1.3% of the adult American population and 0.5% of children under 4 years old currently suffer from allergies to peanuts[2].

IgE antibodies have an important role in allergic responses within the body. These antibodies are significant because, in the presence of an allergen, they promote mast cell degranulation. The observed symptoms of an allergic reaction are the results of mast cell

degranulation in the body. Degranulation is the term given when mast cells release mediator molecules such as leukotrienes, prostaglandins, and histamines[5, 6]. The medical condition used to define a severe allergic reaction is anaphylaxis. Anaphylaxis is characterized as an over exaggerated immune response as the body's chief reaction towards the presence of the absorption of food allergen proteins[6]. Food induced anaphylaxis can occur within only a few minutes after exposure. Anaphylaxis is a medical emergency that requires treatment usually with an epinephrine injector possibly followed by several doses of steroid medications - it is often necessary for the patient to visit the emergency room as well for evaluation[6]. Food-induced anaphylaxis is a life-threatening condition where exposed, allergic individuals experience symptoms targeted toward single or even multiple body systems and can lead the system to be severely compromised as a result[6]. Symptoms of anaphylaxis can be localized but eventually become pervasive and detrimental as time passes without treatment. Patients who are unlucky enough to come into contact with their allergen (complete ingestion not always necessary to provoke a response) experience a rapid onset of symptoms that lead to immediate health decline and, if untreated, sudden death. Localized symptoms include gastrointestinal pain as a result of inflammation of the GI tract, difficulty breathing or difficulty getting enough air into the lungs usually caused by airway organs becoming constricted and closed off - *this is usually identified by healthcare providers if wheezing is present*[6]. Other noticeable symptoms include rash; swelling of the face, extremities, or other areas of the body; or a severe drop in blood pressure resulting in dizziness eventually resulting in the patient losing consciousness[5].

Immunoglobulin E (IgE) and Immunoglobulin G (IgG) antibodies are specifically important in the discussion of peanut allergies because of their allergen binding affinities and for the roles they play in eliciting an over exaggerated immune response. Binding affinity of IgE antibody to a specific allergen is directly related to the occurrence of an allergic episode in the presence of the allergen protein. An allergen that is bound to IgE antibodies almost instantly produces an immune response and symptoms consistent with anaphylaxis and anaphylactic shock[5]. Allergen sensitivity, the body's overall sensitivity to an allergen and likelihood of producing an allergic episode, is understood to be directly related to the quantity of IgE specific antibodies currently within the body[7]. It is believed that IgG antibodies do not play a role in eliciting an allergic attack but are just as sensitive to the allergens compared with IgE antibodies. IgG antibodies act to safely and effectively remove the peanut allergen from the body without eliciting an allergic response. Other components and functions of the immune system exist that aid in desensitization, however the exact mechanism is not well understood[5, 6].

The following cascade comes from the current understanding of allergen immune reactions: upon exposure, an allergen enters the body and immediately comes into contact with the dendritic cell prior to promoting TH^0 cell conversion[8]. TH^0 cells are converted by the allergen activated dendritic cells. TH^0 cells are converted into either TH^1 or TH^2 cells, depending on which cell differentiates the TH^1 or TH^2 cells then travel to the B cells. The B cells then produce either IgG or IgE antibodies. TH^2 cells produce IgE antibodies, TH^1 cells produce IgG antibodies[8]. Mast cells are also important because they produce the usual immune response as well as the symptoms commonly associated with allergic reactions. The specific role mast cells have in producing an allergic response is

still not completely understood, but there is an evidence to support the presence of IgE receptors on the surfaces of mast cells, which when activated by allergen linked IgE allow the mast cells to produce histamines, prostaglandins, leukotrienes, and cytokines. In other words, when IgE antibodies are reacted with an allergen, antibodies can then bind to the membrane receptors on the surface of the mast cells which leads to a reaction cascade within mast cells leading to degranulation[5, 6, 8]. Degranulation is the process by which mast cells release mediator molecules into the extracellular space. When the mediator molecules come into contact with membranes of nearby cells and tissues the cells undergo self-destructive reactions usually local apoptosis directly resulting in irritation persistent in the region of the body where the reaction occurred[5, 6, 8]. There are a few other degradation pathways not discussed here that carry out immune responses to allergens, but the pathway described above is the most significant and potentially lethal to patients.

When the allergen enters the body the cross-linking of allergens to receptor bound IgE occurs and this is one of the leading causes for mast cell degranulation. Degranulation occurs when the mast cell releases prostaglandins, cytokines, histamines, and leukotrienes into the body; collectively these are known as mediator molecules. When nearby cells come into contact with these mediator molecules they can undergo apoptosis which leads to physiological symptoms of allergies *e.g.* difficulty breathing due to apoptosis of bronchial epithelial cells. The receptor FcyRIIB is known to crosslink receptor bound IgE with allergens, however this receptor is also known to crosslink allergen bound IgG as well Burton[9]. IgG is an antibody molecule that has the ability to

compete with IgE for binding and in cases where IgG is present there is less binding of IgE[5, 6, 8].

Unfortunately, it is possible for patients to suffer from polysensitization in which multiple cross reactions occur increasing the severity and even the occurrence of an allergic reaction. In other words, Ara h 2 sensitized antibodies are also known to cross react with different additional allergens therefore increasing the probability of a reaction if the patient is exposed to peanuts in general. Reactions to Ara h 1 and/or Ara h 3 antigens in addition to Ara h 2 are typically predictive of more severe allergic reactions[10, 11].

Aside from allergenicity, allergens also have antigenicity that can be disrupted, usually referred to as immunogenicity. Immunogenicity is the ability to recognize an allergen as a foreign substance, this is not the same as allergenicity because immunogenicity is not always associated with production of an allergic response[12]. If an allergen produces an immune response but loses the ability to produce an allergenic response then tolerance towards the allergen can be promoted without the threat of anaphylaxis. This can be achieved through chemical, mechanical, or even with the use of radiation (e.g. microwave)[13, 14, 15, 16]. By definition this is referred to as an allergoid, which is an antigen with reduced allergenicity and maintained immunogenicity[17]. An allergoid could be an essential component making up a potential vaccine designed to be exposed to patients. The synthesis and characterization of a viable allergoid is the first step in the development of an effective and safe vaccine to promote tolerance in patients with peanut allergies. Physical disruptions to allergen IgE epitopes can lead to decreased antigenicity[12].

IgE epitopes on the surface of peanut allergen proteins, like those present on the surface of Ara h 2, can become disrupted through chemical modification and could result in a lower affinity or complete loss of affinity to IgE sensitive antibodies[18]. Circumventing IgE binding could result in an increase in the degradation pathways responsible for digesting peanut allergens without harmful symptoms. This understanding is consistent with the findings of a clinical study where 21.5% of peanut allergic patients outgrew their condition after peanut challenges administered to patients with already low peanut IgE levels in the blood[19]. Peanut allergies are harmful to hypersensitive patients because of the major allergen protein, Ara h 2, and its resistance to chemical modification within the body. If the proper conditions are determined, chemically modifying the peanut allergen protein could be a simple and effective technique to develop a treatment for peanut allergies.

Currently, the only treatments available are post-occurrence treatments, which are usually given to patients after they are exposed to the allergen and the allergen is already ingested. Unless a pre-reaction treatment is developed, healthcare providers are limited to only emergency treatments given after allergen exposure and advising patients to abstain from peanuts altogether - and that's it. Proper prevention is the only definitive measure to lower the possibility of an allergic reaction to peanuts. Avoiding peanut products altogether is currently the only guaranteed way to prevent an allergic reaction. Patients need to develop an alertness against foods that could contain peanuts to avoid accidentally exposing themselves to the allergen. Patients are often burdened with this condition because of the required vigilance needed of them in questioning how everything they eat is prepared (*e.g.* when going out to dinner the allergic patron may

need to be reassured by the chef who prepared the meal of the absence of any remnants of peanut products). Care plans are often needed to be prepared in the event of an exposure to the allergen. The care plan is needed to be understood by anyone who is looking after an allergic child or even an allergic adult. Caring for a child who is allergic to peanuts is a large responsibility and requires guardians to ensure the child understands their own emergency plan and what to do in the event of an emergency, in most cases remaining calm is the best way to care for someone having an allergic reaction (if adrenaline is unavailable) before advanced help arrives. It is also usually recommended by healthcare providers that allergic individuals wear a medical emergency identification bracelet or necklace so that lay responders can be aware of their condition in the event of an exposure.

Even strict avoidance to peanuts is difficult, most of the time the ingestion is accidental. Allergic individuals always live in fear of accidental exposure and the possibility of a server allergic reaction. Patients with peanut allergies or any other food allergies are usually not limited to being allergic to one allergen *e.g.* peanuts, in fact they commonly have allergies to a whole host of tree nuts such almonds and walnuts (for peanut allergies). Therefore, the patient needs to avoid related foods as well as the specific allergy food.

Areas of Research

Peanut allergies are concerning because public health related research shows a significant increase in prevalence of these allergies within the U.S. over the last decade[20]. Unfortunately, the exact cause for an individual to become sensitized (allergic) to peanuts is not completely understood, however there are several literature supported theories

available. There is evidence supporting the proposition that patient sensitization occurs at a young age as a result of breastfeeding using milk containing trace amounts of allergen proteins[7, 21, 22]. As a rationale to this sensitization theory Jarrett E E.[21] hypothesized stimulation of helper T cells promotes IgE production, occurring in the presence of low initial antigen doses compared to high initial antigen doses in which case she hypothesized the antigen would actually stimulate suppressor T-cells inhibiting IgE production. Coupled with this theory, the research by Hourihane et al.[23] helps explain the increased prevalence of peanut allergies in westernized countries. Although their study did not control for recall bias, the mothers of children allergic to peanuts and age 5 years old or younger were significantly more likely than mothers of older children to have eaten peanuts regularly while pregnant or breastfeeding[23]. The increasing trend for mothers to consume peanuts while pregnant shouldn't be overlooked since it directly correlates with the outcome of having a child sensitized to peanuts (prior to their first peanut exposure) and also correlates with the overall increase in number of patients allergic to peanuts.

Currently, allergen-specific immunotherapy (SIT) is the only treatment able to cure allergic diseases and has been shown to be successful in treatment of insect venom allergy and in patients suffering from allergic rhinitis or rhinoconjunctivitis[24]. Immunotherapy treatments are well supported and understood, however desensitizing patients to a dangerous allergen poses a threat to the patient's safety that cannot be overlooked. Desensitizing patients to a dangerous allergen (*i.e.* no longer sensitized to the antigen) needs to be a careful process. Jarret's theory proposes that exposing patients to substantial allergen quantities stimulates suppressor T-cells within the body, and

promotes desensitization. This theory is consistent with the outcome of patients who were treated with allergen specific immunotherapy using insect venom allergens which ultimately lead to a cure of the allergy[24]. However, a method to safely expose patients to large quantities of otherwise harmful peanut allergens is not well understood and requires further exploration.

Among the research conducted around peanut allergies, a group of Australian researchers identified a type of oral immunotherapy that was effective in treating children with peanut allergies. Probiotic and peanut oral immunotherapy (PPOIT) was effective in inducing possible sustained unresponsiveness and immune changes that suggest modulation of the peanut-specific immune response in children with peanut allergy[25]. 62 children, as a part of a clinical study, were exposed over a duration of 18 months to a minimal amount of peanut protein sample daily that also contained the probiotic lactobacillus rhamnose, in extremely high quantities. Remarkably the research concluded that over 80% of the study participants achieved possible sustained unresponsiveness to peanuts[25]. The probiotic was believed to have aided the immune system in developing a protective response preventing the usual aggravated allergic response[25]. This altered immune response to the peanut protein presents potential investigational treatments of peanut allergies and could lead to the development of an overall cure. However, the modified allergen has not been evaluated yet for prolonged effectiveness so the return of usual symptoms still remains a possible concern to be studied[25].

Major peanut allergen protein Ara h 2 has been observed to produce severe allergic responses when exposed to endogenous antibodies[26, 27]. Life-threatening conditions such as anaphylaxis could be the result in allergic individuals who are not

treated immediately after exposure to this specific allergen[6]. Currently, there is little that can be done to preemptively treat allergic episodes resulting from peanut allergens. Identifying a safe, and preferably uncomplicated, method of exposing patients to the peanut allergen to increase the body's tolerance to the allergen is significant to increasing understanding and in the overall treatment of these allergies. More research is also necessary to increase understanding of the chemical properties of Ara h 2 and its role in eliciting an allergic response by the body.

Chapter 2

Major Peanut Allergen Ara h 2 Isolation and Identification

Ara h 2 is one of the known allergens in peanuts that causes the most severe reactions associated with peanut allergies. The peanut kernel contains 11 major allergen proteins that on their own elicit an allergic response[27]. Of the 11 peanut allergens, Ara h 2 has consistently shown to be the most significant. More than 90% of patients with peanut related allergies contain IgE antibodies with remarkably high binding affinity to Ara h 2[27]. Ara h 2 has also shown to be seemingly resistant to most protein degradative processes. For example, heating Ara h 2 actually increases its ability to elicit an allergic response within the body, and exposing Ara h 2 to conditions consistent with the stomach and GI tract showed to have little to no effect on reducing the protein's overall allergenicity[28, 29, 30, 31, 32].

Ara h 2 appears on SDS-PAGE as a doublet with a molecular weight between 17-19kDa. Ara h 2 is an allergen protein that is made up of 157 amino acids Figure 1[33]. Isolating Ara h 2 is the essential initial step in the synthesis of the potential allergoid. However, prior to immunoassays, experiments designed to observe the presence of structural changes to the allergen are needed. To perform experiments focused on the structure of Ara h 2, isolating Ara h 2 is important to avoid the presence of additional proteins in solution that could lead to variable results. A robust, reliable, and efficient method for purifying Ara h 2 is important for developing and screening crosslinking conditions.

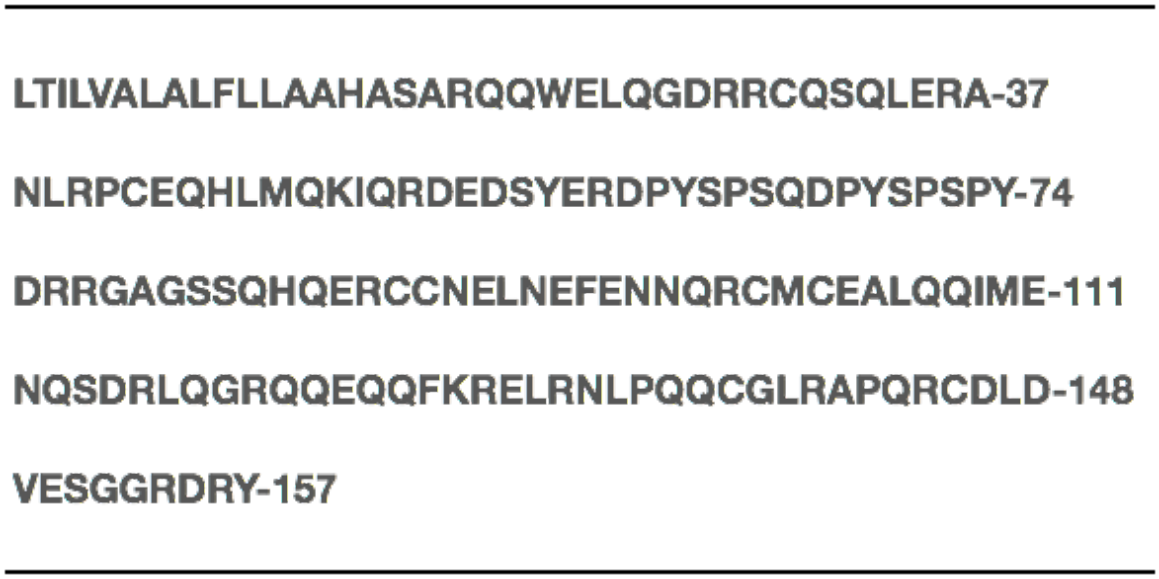


Figure 1. Ara h 2 amino acid sequence. Ara h 2 amino acid sequence represented as single letter amino acid code Burks[33].

Synthesizing an allergoid from Ara h 2 is the major objective of this research followed by characterizing its in-vitro immune affinity prior to in-vivo immunoassays. In addition to *in-vitro* experiments, reproducing the allergoid synthesis reaction is a strong factor in determining whether or not the product is ready to be exposed to biological subjects (small and large animals). Exposing animal subjects requires highly pure

allergoid samples. For example, if impurities do exist as an essential factor to the formation of the allergoid then reproducing the allergoid reaction would not be possible without the presence of the original impurity, even if impurities existed that do not benefit the overall allergoid reaction there is still potential for the impurity to alter the overall effectiveness of the product as a drug once administered to subjects leading to unpredictable and even dangerous outcomes. The most effective method for producing an allergoid consistently, that displays no uncertainty in usability and effectiveness, remains contingent on how well the synthesis reagents were isolated.

Materials and Methods

Sample Preparation

Ara h 2 was purified following the method by Sen[34] with additional modifications made to the procedure. Peanut kernels (*Arachis hypogaea* L., Florunner cultivar) purchased from Good Earth Peanut company were exposed to liquid nitrogen and the frozen kernels were then crushed by mortar and pestle prior to being defatted three times in a Soxhlet extractor starting with petroleum ether for the first two exchanges and then finally using chloroform for the last reflux. The defatted peanut kernels were pulverized to a fine flour. The defatted peanut flour was then added to a TBS buffer (pH 8.3, 65 mM Tris-base, 1 mM EDTA, 1 mM PMSF and 200 mM NaCl) and stirred in TBS for 1 hour on ice at 4° C. After stirring the peanut powder in the TBS buffer for about 5 minutes, the extract was sonicated (Sonics Vibra-Cell VCX-600) at 40% for 2 min over ice to increase the chances of extracting intracellular proteins into the sample solution, through non-ionizing vibration. The extract was then cleared by filtration through six layers of cheesecloth and then centrifugation at 30,000 × g for 30 min at 4° C. Ammonium sulfate

was used to fraction the supernatant. Ammonium sulfate was added to bring the solution to 40% saturation, the sample was then centrifuged at 30,000 × g for 30 min at 4° C and the supernatant was collected and the pellet was discarded. Ammonium sulfate was added again to bring the solution to 70% saturation and the sample was centrifuged at 30,000 × g for 30 min at 4° C and the pellets was collected and the supernatant was discarded. The pellets were resuspended in a total of 10 mL of TBS buffer (pH 8.3, 65 mM Tris-base, 1 mM EDTA, 1 mM PMSF) and sonicated on ice for 20 seconds at 39% power. Undissolved particles were removed by centrifugation (3,000 × g for 15 min).

Allergen purification was accomplished using a series of chromatography columns based on ionic charge and hydrophobicity. Q-sepharose column prepared with 100mL of resin to achieve the desired column bed volume of 2.5cm X 30cm. The protein sample was applied to the Q-sepharose anion exchange column which was equilibrated with pH 8.3 65 mM Tris buffer, 1 mM EDTA and1 mM PMSF prior to loading protein sample. After washing the column with 200 mL of Tris buffer containing 40 mM NaCl, bound proteins were eluted using a 400 mL linear salt gradient (40 mM - 500 mM NaCl, 65 mM Tris-HCl, 1 mM EDTA, 1 mM PMSF at pH 8.3) at 2 mL/min flow rate. 2.5 ml fractions were collected, analyzed with coomassie dye 50uL: 150uL (protein: dye) and then analyzed by SDS-PAGE. Pooled fractions containing Ara h 2 were dialyzed overnight at 4°C against 15 mM ammonium bicarbonate (pH 7.0) dialysis buffer exchanged 3-5 times. After dialysis, aliquots were lyophilized and stored at −70°C until needed.

SDS-PAGE Procedure

Samples solubilized in sample loading buffer (1.5 mL of pH 6.8, 1 M Tris-HCl, 1.5 mL of 1 M dithiothreitol, 0.3 g of sodium dodecyl sulfate, 0.015 g of bromophenol blue, 1.2 mL of glycerol - Final volume of 7.5mL). Samples solubilized by combining 10uL of 40% diluted sample (dilution with 25mM Tris buffer at pH 10.5) with 10uL of 2X sample buffer. Samples vortexed slowly, to avoid splashing, and then were heated in boiling water (100° C) for 5 minutes. Samples were vortexed on setting number 8 (after heating) and centrifuged at 3,000 × g for 5-10 seconds at room temperature (to accumulate sample toward the bottom of the centrifuge tube, and away from the container walls) prior to loading samples onto SDS-PAGE gel. Tris glycine (4-20%) SDS-PAGE gels (manufactured by *BioRad*) were used. A 10uL of sample volume was loaded into each well of the SDS-PAGE gel. A 10uL of molecular weight standard was loaded onto the gel only after protein samples were loaded. The gel was allowed to run at 120V until enough time passed for the proteins to run to the bottom of the gel. The gel was then stained in a coomassie dye and methanol based staining solution (1.25g Coomassie R-250, 30% methanol, and 10% acetic acid). After approximately 24 hours in the staining solution, the gel was then destained to remove excess stain using a water based destaining solution (30% methanol and 10% acetic acid).

Results

Prior to running samples on SDS-PAGE, samples were evaluated for protein content by commassie dye analysis. Results using coomassie dye can be found in Figure 2 and Figure 3. Figure 2 and Figure 3 compare the results of different salt concentrations in preparing the protein elution gradients. Figure 2 shows the result of running a strong

elution gradient of 40mM – 1M NaCl (200mL) and resulted in eluted proteins being heavily concentrated in the middle of the gradient. Whereas Figure 3 displays elution results after running a gradient of 40mM – 140mM NaCl (200mL) it appears to suggest a larger dispersion of eluted proteins compared to Figure 2.

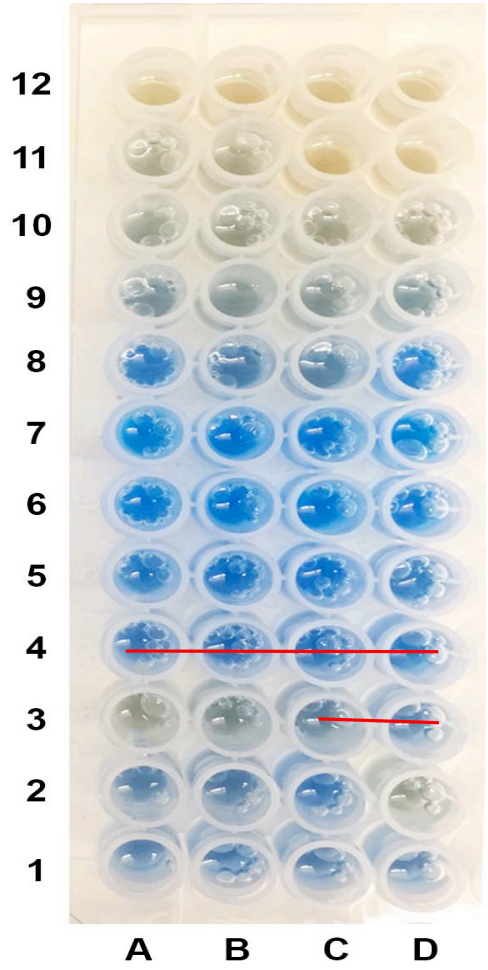
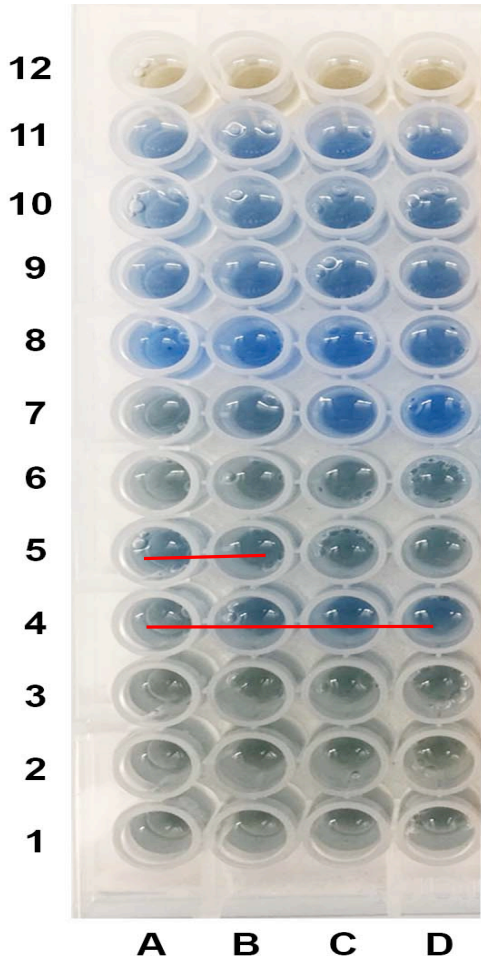


Figure 2. Coomassie dye analysis of fractions following the Q-column, eluted with 40 mM – 1 M NaCl gradient at 2mL/min 2mL fraction volume (65 mM Tris buffer, 1 mM EDTA, and 1 mM PMSF). Every 3rd sample out of 144 total collected samples was aliquoted starting in well 1A moving to the right. e.g. 2A corresponds to fraction # 9.



Indicates where Ara h 2 major allergen was found.

Figure 3. Coomassie dye analysis of fractions following the Q-column, eluted with 40 - 140mM NaCl gradient @ 2mL/min 2mL fraction volume (65 mM Tris buffer, 1 mM EDTA, and 1 mM PMSF). Every 3rd sample out of 144 total collected samples was aliquoted starting in well 1A moving to the right. e.g. 2A corresponds to fraction # 9.

Discussion of Allergen Purification

According to Sen[34] defatting peanut kernels can be achieved by refluxing petroleum ether over the sample. According to Packer[35] defatting samples not completely can result in poor extraction efficiency. Therefore, this step was modified, petroleum ether was used to start, which has a polarity index of 0.1, but ultimately the reflux was finished with chloroform that has a polarity index of 4.1 to extract less non-polar materials away from the peanut kernels. Following ammonium sulfate precipitation pellets should be dissolved completely to allow for complete separation. Our findings led us to determine that 40 mM – 500 mM NaCl was the optimal gradient to be used for Ara h 2 elution from the Q-sepharose column observed by commassie dye analysis (Figure 4) and SDS-PAGE analysis (Figure 5). This determination was made because of the inefficiency in eluting a mixture of unseparated proteins all at once (Figure 2) and in eluting the desired proteins too widely to be efficiently collected (Figure 3). Gradients that contained larger or smaller margins between concentrations of NaCl, than described above, were generally not well suited and caused inconclusive and inconsistent results. Also, to be noted, since Ara h 2 is only 17kDa in size, 4-20% Tris-glycine gradient gels (manufactured by BioRad) were used for precise separation of protein. Other gradient gels were not individually studied since additional gradient gels were not well documented by the literature for use in the separation of peanut allergen proteins.

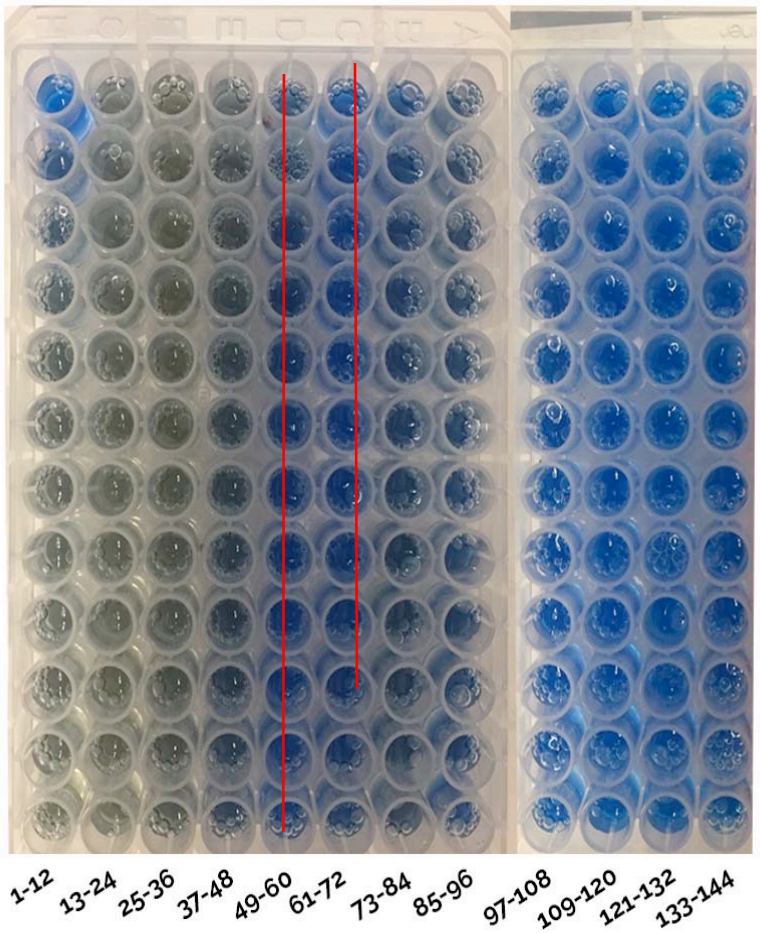


Figure 4. Coomassie dye analysis of all 144 fractions after running sample on Q-sepharose column eluted at 40 - 500 mM NaCl, 65 mM Tris buffer, 1 mM EDTA, and 1 mM PMSF. Well # 1 (1st fraction) located in top left well. Red lines are indicative Ara h 2 protein.

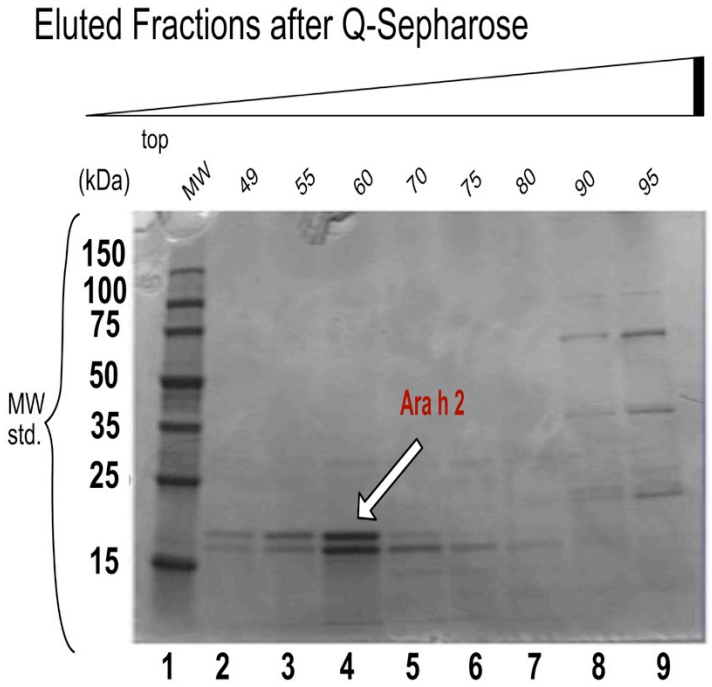


Figure 5. SDS-PAGE gel of specified fractions, which indicated protein after using coomassie dye, of sample after running sample on the Q-sepharose column eluted at 40 - 500 mM NaCl, 65 mM Tris buffer, 1 mM EDTA, and 1 mM PMSF.

Chapter 3

Protein Chemical Modification-Lead Compound Assessment

The major peanut allergens Ara h 2 and Ara h 6 were the focus of the research study completed by Koppelman[29] because of the apparent synergy of these allergens in eliciting an allergic response. Researchers confirmed Ara h 2 and Ara h 6 have an integral role in the biological mechanism leading to an allergic reaction, however, by reducing the allergens and subsequently chemically modifying [alkylating] them with glutaraldehyde the researchers determined that it was possible to alter the allergen's secondary and tertiary structures. Presuming allergen structure was necessary to allergen function, researchers designed a series of clinical studies to further evaluate the newly, chemically synthesized allergen products. Again, using children as participants, patients were injected with small doses of the chemically modified allergen sample. The participants exposed to the chemically modified samples displayed later time activated reactions and overall decreased response when exposed to the native allergens. The researchers concluded this to be a viable method in desensitizing patients to their allergen and because of the levels of effectiveness observed should serve as another potential candidate for peanut allergy therapy. *This finding (glutaraldehyde being a necessary protein modifier) was also identified by Park[36]; Silva[37]; and Stanić-Vučinić[38] however these groups of researchers experimented with proteins and allergens outside of Ara h 2.*

Proteins can crosslink through glycation reactions, and undergo physical structural changes[39, 40, 41]. Carbonyl containing reagents were previously identified as capable of eliciting protein cross-linking. According to Acharya[42] the protein RNase was successfully cross-linked using glyceraldehyde. The experiment called for 20 mM of

glyceraldehyde at 7.4 pH and 37° C. The formation of crosslinks with RNase using glyceraldehyde was concluded to be analogous to nonenzymic glycation reactions. In other words, by using (carbonyl containing) aldehydes it is possible to form advanced glycation end products i.e. cross-linked structural proteins Acharya[42].

There are several studies in the literature that can be referred to for support where several reagents were evaluated for success in modifying Ara h 2 and in eliminating or at least significantly reducing allergenicity. Experiments conducted by Chung[43] displayed significant decrease in allergenicity of peanut allergens using phenolic compounds. According to Chung[44] tannic acid is successful at significantly reducing allergenicity of peanut allergens. One study actually concluded that using ferulic acid actually increases the likelihood of Ara h 2 antibody binding[45].

According to Chung[46] caffeic acid and chlorogenic acid are examples of chemical reagents that can be oxidized to form quinone derivatives susceptible to bonding to free amine groups, sulfhydryl groups, and tryptophan groups and capable of catalyzing a crosslink between two separate complete proteins. Caffeic acid and chlorogenic acid have been studied for their potential to modify the allergen protein and have successfully formed Ara h 2 allergoids. From the experiments carried out by Chung[46] oxidizing caffeic acid require a basic environment above pH 10 to form the reactive quinone necessary to potentiate the crosslinking reaction. Caffeic acid and chlorogenic acid are just two examples of chemical modification reagents, where it is important the proteins being crosslinked are examined for the appropriate amino acid content needed to catalyze the reaction. Since these chemical reagents were found to be successful by Chung[46] we

proposed the possibility of reagents closely related to these molecules also containing potential reactivity with Ara h 2 as well.

Proteins capable of crosslinking reactions, catalyzed by arginine targeting chemical reagents, contain roughly 5% arginine residues[47]. The quantity of arginine residues in Ara h 2 is approximately 12%[33]. The ketone aldehyde group of the compound Phenylglyoxal acts as an oxidizing agent and targets arginine residues in proteins[48]. The guanidine group of arginine interacts with phenylglyoxal and forms heterocyclic condensation products which could potentially promote a structural change to occur in Ara h 2 which results in the crosslinking between two reactive Ara h 2 proteins[48]. Diketone reacting groups of aromatic molecules also react well with arginine amino acids and have been studied to promote crosslinking in proteins. Based on this understanding, the compounds phenylglyoxal monohydrate and its derivatives4-fluorophenylglyoxal hydrate, 4-methoxyphenylgyoxal hydrate, 2,3-butandione, 2,3-hexandione, 3,4-hexanedione, and 2,3-heptanedione were examined to determine their ability and efficiency in crosslinking Ara h 2. Figure 11 shows a highly simplified proposed reaction mechanism that utilizes arginine residues of Ara h 2 to promote a crosslinking reaction to occur.

Materials and Methods

Chemically Crosslinking Ara h 2

Allergoid synthesis reactions were prepared in sealed 1.5 mL centrifuge tubes. The cone shaped centrifuge tubes allowed reaction mixture to pool near the base of the container for simple extraction by micropipette. Allergoid synthesis reagents were

dissolved in 25 mM Tris-base buffer at pH 10.5, using additional solvents when needed (Figure 9) and serial diluted to desired concentrations.

Lyophilized protein was dissolved into 25 mM Tris buffer at pH 10.5 at a working protein concentration of approximately 1-2 mg/mL. Ara h 2 protein concentration was quantified against a BSA (bovine serum albumin) standard curve. To prepare the BSA standard curve the following BSA dilutions were prepared (according to the manufacturer *ThermoFisher Scientific*): 2000, 1500, 1000, 750, 500, 250, 125, and 25ug/mL, and 0ng/mL for blank control. The BSA dilutions were prepared using 25 mM Tris-base buffer at 10.5 pH (same buffer used to dissolve Ara h 2). 5uL of each BSA stock dilution was added to the appropriate well on a 96-well microplate. BSA stock dilutions were added to the microplate, three trial replicates evaluated. A 5uL of the unknown concentration of Ara h 2 sample was added to 3 clean wells on the microplate (three trial replicates evaluated). 250uL of the coomassie dye reagent was then added to each well containing unknown or stock samples and the microplate was allowed to mix on shaker for 30 seconds prior to measuring absorbance. Stock and unknown absorbance was read with a microplate reader using UV-visible light at 595 nm wavelength. The average absorbance at 595 nm for each BSA standard dilution vs. its concentration in ug/mL was plotted after correcting for the blank (0ng/mL concentration) for each concentration. The BSA standard curve was then used to determine the protein concentration of the unknown Ara h 2 sample.

Stock solutions of the modifying reagents were prepared using buffer and any additional solvent(s) necessary to dissolve the specific reagent. The desired concentrations for the stock solutions were prepared by serial dilution making sure to

keep the overall solvent concentration consistent for each reagent concentration. Once stock reagent solutions were prepared, stock Ara h 2 solutions were prepared using 2mg/mL protein concentration (1mg/mL final protein concentration after reaction). Finally, we combined the stock reagents with the Ara h 2 solution by adding an aliquot of each stock concentration into the tube already containing Ara h 2 protein (equal volume to volume ratio). The centrifuge tubes were then sealed off and incubated at the conditions described above. A graphic of the modification procedure can be found in Figure 6.

Several reagents were evaluated for allergoid synthesis potential (Table 2 and Table 3). The evaluated reagents were reacted with Ara h 2 under various conditions to determine reaction viability. The evaluated conditions included variations in reaction buffer, reaction buffer pH, exposing the reaction mixture to agitation vs. non-agitation, reaction temperature, concentration of reagent exposed to protein, and time exposed to protein evaluated to understand which conditions were ultimately most effective in forming an allergoid (Table 1).

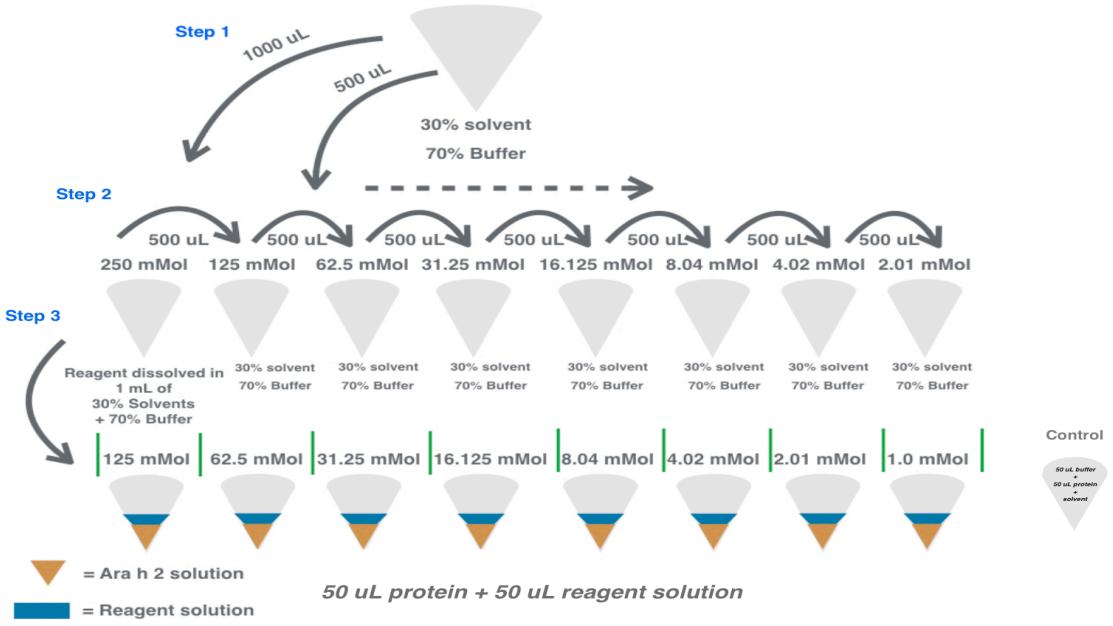


Figure 6. 3-step infographic procedure used to chemically modify the allergen. Depending on the reagent used specific solvents were required to fully dissolve the reagent into solution. Following the solvent concentration guidelines listed above for the specific reagent to be dissolved. It is necessary to keep the solvent concentration consistent for all reagent serial dilutions. The highest reagent concentration to be evaluated was determined and then multiplied by two because the prepared stock concentrations are actually 2X that of the prepared working concentrations. 1mL of solvent/buffer solution was used to prepare the first reagent concentration within a 1.5 mL centrifuge tube then vortexed until reagent dissolved completely. Serial dilutions of first reagent concentration prepared by adding 500 uL of solvent/buffer to 5-10 empty 1.5 mL centrifuge tubes. 500 uL of original reagent solution was combined with solvent/buffer within one of the prepared centrifuge tubes and mixed until homogeneous. Samples were prepared by a 2-fold serial dilution to create several stock reagent solutions.

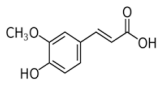
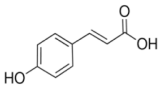
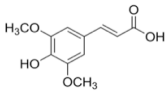
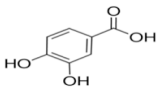
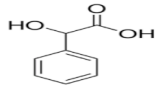
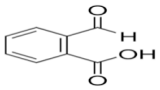
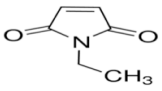
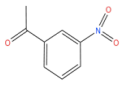
Table 1

Evaluated Ara h 2 allergoid synthesis reaction conditions

	Conditions						
Reaction Temperature	4° C	20° C	37° C	37° C	37° C	37° C	37° C
Reaction pH	10.5 & 7.4	10.5	10.5	7.4	10.5	10.5	9.5
Reaction Buffer	Tris-Base	Tris-Base	Tris-Base	Tris-Base	Tris-Base	HEPES	Borate
Sample Agitation	NO	NO	NO	5 RPM Rotation	5 RPM Rotation	5 RPM Rotation	5 RPM Rotation

Table 2

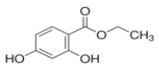
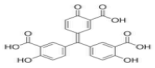
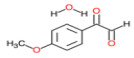
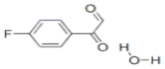
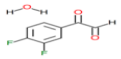
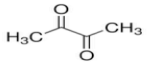
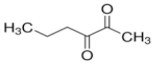
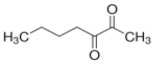
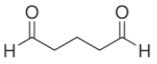
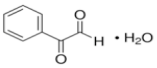
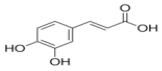
Allergoid synthesis reagent candidates not effective in promoting a reaction with Ara h 2.

Reagent	Structure	Hit (smear)	1st oligomer	2nd oligomer
Ferulic acid		-	-	-
Trans-P-Coumaric		-	-	-
Sinapinic acid		-	-	-
3,4-dihydroxybenzoic acid		-	-	-
Mandelic acid		-	-	-
2-Carboxybenzaldehyde		-	-	-
N-ethyl-Maleimide		-	-	-
Isonitroacetaphenone		-	-	-

(+) indicates positive reaction and (–) indicates no reaction.

Table 3

Allergoid synthesis reagent candidates effective promoting a reaction with Ara h 2.

Reagent	Structure	Hit (smear)	1st oligomer	2nd oligomer
ethyl-2,3-dihydroxybenzoate		+	-	-
Aurinticarboxylic acid		+	-	-
4-methoxyphenylglyoxal hydrate		+	+	+
4-Fluorophenylglyoxal		+	+	-
3,4-difluorophenylglyoxal hydrate		+	+	+
2,3-Butandione		+	+	-
2,3-Hexandione		+	+	+
2,3-Heptandione		+	+	-
3,4-Hexandione		+	+	-
Phenylglyoxal monohydrate		+	+	+
Caffeic acid ***(already in literature)***		+	+	-

(+) indicates positive reaction and (–) indicates no reaction.

Centrifuge tubes were used to prepare the protein modification reactions. The desired volume of reagent was added to an equal volume of protein to start the allergoid reaction. The reaction solutions were incubated and agitated for 30 days at 37° C. Reactions were carried out in a rotating incubator rotating 360 degrees at a speed of 5 RPM. After the desired time had passed, samples were evaluated by SDS-PAGE.

Excess modification reagent was removed by dialysis. Samples were dialyzed against 3L of 15mM ammonium bicarbonate buffer at pH 7.4 in a 0.1 - 0.5 mL dialysis cassette (MWCO 20kDa). Using a 1 mL syringe, separate dialysis cassettes were individually loaded with modifications samples at different reagent concentrations until all concentrations had been effectively dialyzed. Dialysis buffer exchanges were carried out until samples appeared clear in color and no longer exhibited the original reagent influenced color. Once modifying reagents were removed from the sample, modified protein samples were lyophilized and stored at -70° C.

Results

According to Lundblad[47] borate buffer at "basic pH" should facilitate protein crosslinking reactions when solubilized proteins are exposed to diketone crosslinking reagents. However, this is not reflected in our data. Figure 8 displays several SDS-PAGE gels of unsuccessful allergoid reactions using borate buffer at different concentrations of reagent. This is significant because literature has cited borate buffer as being an effective buffer that should be used in protein modification reactions when using the reagents that were used here.

Ara h 2 was exposed to varying concentrations of Phenylglyoxal monohydrate, 4-Fluorophenylglyoxal hydrate, 4-methoxyphenylgyoxal hydrate, 2,3-Butandione, 2,3-Hexandione, 3,4-Hexanedione, and 2,3-Heptanedione. Allergoids were reproducibly produced by using Tris-base buffer at pH 10.5 and sample agitation at 5 RPM by rotisserie at 37° C. Instead of the usual doublet band observation around 17kDa and 19kDa (in the case of native un-modified Ara h 2) a smearing pattern occurred between and around the doublet bands which indicated some degree of protein modification to the allergen monomer. In addition to the simple allergen protein modifications, where the monomer was still the only oligomer present, the results of higher intensity modification reactions were also observed. These high intensity reactions resulted in an observed possible crosslink between allergen proteins by the formation of multiple oligomers, appearing on the SDS-PAGE as dimers and even trimers as shown in Figure 9.

Discussions

The potential allergoid, the new product formed between Ara h 2 and the modification reagent, is prepared in aqueous buffers. The reaction solution is a mixture of the newly formed allergoid, un-reacted Ara h 2, reaction buffer, and reagent dissolving solvent. Ara h 2 is an aqueous soluble protein, however most of the modification reagents used were not soluble in water, so a range of solvents were needed to dissolved and expose these reagents. Some of the reagents evaluated did not react well with Ara h 2, this failure in reactivity may be attributed to reagent solubility or an incompatibility between the solvents used and the reagent.

To determine which reaction conditions consistently produced a positive potential allergoid formation, from natural Ara h 2 allergen, variations of these conditions were

examined. However, for consistent results we found that using a simple 25 mM Tris-base buffer worked well.

Most of the reagents used were completely insoluble in water based buffers, so organic solvents were necessary. However, to avoid damaging or altering the Ara h 2 protein through the use of organic solvents alone it was also necessary to use the least amount of solvent needed to fully dissolve the reagent in the buffer solution. To do this, several trials were prepared where a fixed amount of crosslinking/modification reagent was weighed out and then dissolved in the lowest possible volume of a solvent, finally buffer was added to the reagent-solvent mixture to determine if the reagent would remain soluble. Frequently the reagent used would precipitate of the solution after being mixed with the buffer. After several attempts to determine the solubility of the desired reagent quantity, varying the concentrations of reagent and organic solvents used (for each reagent evaluated), the minimum amount of organic solvent required to solubilize the reagent was determined. After this was determined we could then add the reagent to the protein and begin the modification procedure.

Previously it was determined that agitation during incubation with the crosslinking reagent was important for efficient reactivity. The reactor used in the lab was constructed from a rotisserie style incubator device, allowing control of reaction temperature. High temperatures are well known to increase the likelihood of collisions between reacting molecules in solution, we found that incubating at 37 degrees Celsius on a constant rotation speed of 5 RPM produced consistent reaction outcomes. The rotating reactor allowed for the modification reaction to occur in a much shorter duration

of time, which proved to be much more efficient and even more effective than the stand alone stationary incubator.

Several reagents were attempted for allergoid synthesis potential and the complete list can be found in Table 2 and Table 3. However, phenylglyoxal monohydrate derivatives and simple diketone containing molecules were ultimately identified to generate strong chemical crosslinking of Ara h 2. Based on these results, Ara h 2 modified with these reagents were further investigated for immunoreactivity with IgE and IgG.

Positive Hit Reagents	Method to dissolve in polar buffers
Caffeic acid -	add 10% MeOH & 10% EtOH
Phenylglyoxal monohydrate -	add 10% MeOH
2,3-dihydroxybenzoate -	add 10% MeOH & 10% EtOH
Aurinticarboxylic acid -	add 10% EtOH
4-Fluorophenylglyoxal -	add 10% MeOH & 10% EtOH
2,3-Hexandione -	add 5% MeOH & 5% EtOH & 7.5% DMSO
4-methoxyphenylglyoxal hydrate -	add 12% MeOH & 22% EtOH & 0.1% NaOH
3,4-difluorophenylglyoxal hydrate -	add 15% MeOH & 15% EtOH
2,3-Heptandione -	add 10% DMF, 7.5% DMSO, 5% MeOH & 12% EtOH
3,4-Hexandione -	add 12% DMF, 7.5% MeOH & 17% EtOH
2,3-Butandione -	NO SOLVENT NEEDED

Figure 7. Methods for dissolving allergoid synthesis reagent candidate compounds in polar buffers. Only candidates effective at promoting reactions with Ara h 2 are displayed.

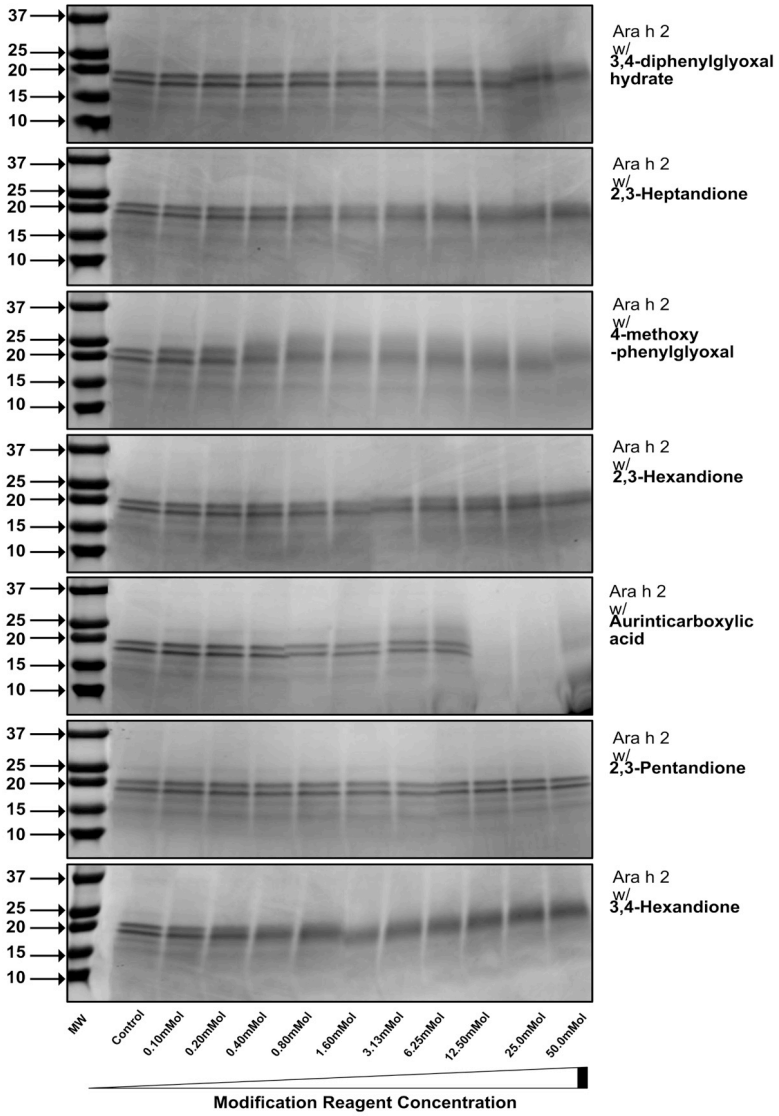


Figure 8. SDS-PAGE of allergoid synthesis reagent candidates using borate buffer under basic pH conditions.

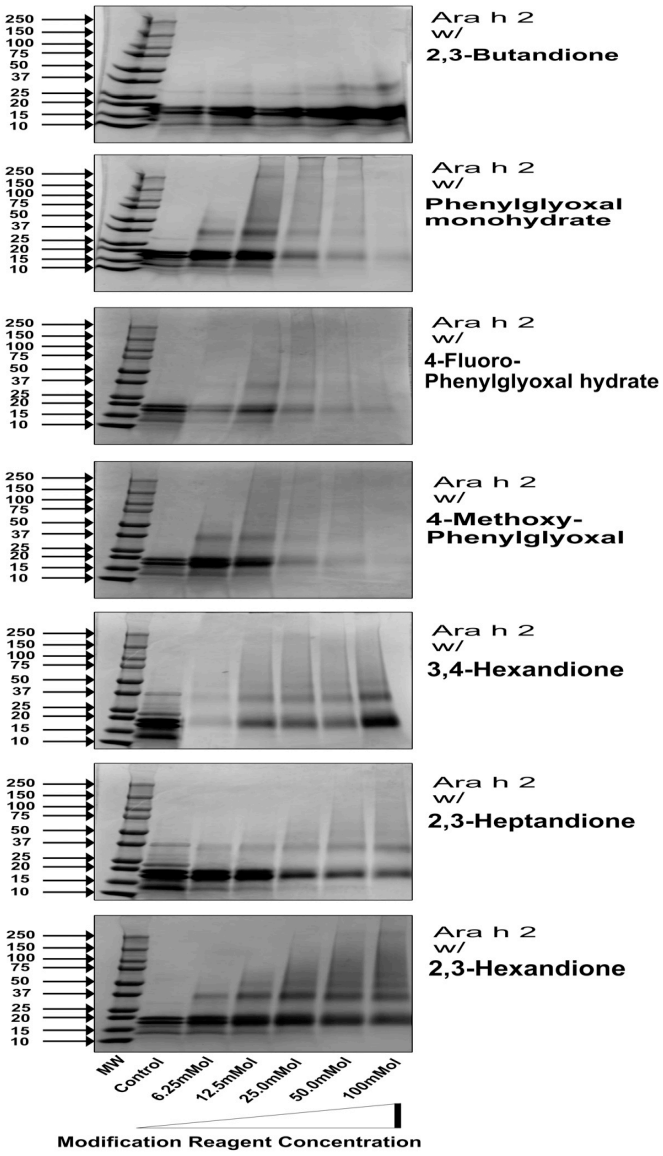


Figure 9. SDS-PAGE of successful allergoid synthesis reactions, reacted under basic conditions in 25mM tris buffer.

Chapter 4

Allergoid Viability Determination by *In-Vitro* Assay

A modified protein sample that displays a shift in mobility observed by SDS-PAGE is a necessary initial confirmation that the protein sample deserves further evaluation for its immune affinity. Unfortunately, however, a shift in mobility is not sufficient to identify a modified allergen protein as a potential and useful allergoid. Further experiments are required before any assumptions can be made about the sample. Using specialized experiments founded in immunology, it may be possible to further interpret if a potential allergoid product was actually formed. Immunoassays specific to the native allergen are necessary to provide additional characterization details for the allergoid. These details could include chemical and biological properties.

An appropriate immunoassay to determine the viability of the formed allergoid is a western blot analysis. Western blot exposes the modified allergen to peanut specific IgE antibodies to evaluate binding affinity. This immunoassay is based on the understanding that IgE antibodies bind to regional protein epitopes that are intact and unshielded. Variations to protein solubility can result in alterations to these epitope surfaces, possibly due to exchanges between non-polar amino acids and polar amino acids. Surface IgE epitopes that are disrupted or shielded, through chemical modification, could result in decreased antibody binding affinity. In other words, if there is an observed difference in binding affinity between the native allergen and that which was modified then antibodies failed to bind to the modified allergen, and therefore resulted in the successful formation of a potential allergoid.

Materials and Methods

Preparing pAb for Western Blot analysis

Our research lab prepared the allergen sample provided to *Neo Scientific* to raise and purify Ara h 2 specific IgE antibodies. IgE antibodies were raised against antigen protein Ara h 2 in rabbits to be used in immunoassays. Antigen samples need to be approximately 88% pure when raising IgE antibodies to avoid the potential of raising antibodies against unknown impurities which could weaken overall affinity to Ara h 2. Once the antigen is prepared and the purity is verified, using SDS-PAGE, the antigen can then be exposed to rabbit organisms. Approximately 3-4 mL of antigen at a concentration of 1-2 mg/mL is needed to prepare the antibodies. This is the process known as sensitizing, since the antibodies are being sensitized to the antigen. The antigen is exposed to the endogenous immune system of rabbits to form sensitized IgE antibodies. After antibody sensitization, the animal is stimulated to produce IgE antibodies in the presence of the allergen protein. Antibodies can then be removed simply by extracting the animal's blood and following up with a series of centrifuge purifications. The impure antibody solution is then run on an affinity column to purify and collect only the desired antibodies. Finally, a standard curve is used to determine antibody affinity toward the antigen protein.

Western Blot Procedure

Prior to running the western blot analysis, an SDS-PAGE was completed where replicates of the evaluated samples were prepared and run on the same SDS-PAGE gel. The first half of the SDS-PAGE gel (lanes 1-6) contained replicate samples of the second half of the gel (lanes 7-12). The SDS-PAGE gel was prepared in this way for the purpose

of (after electrophoresis) exposing half of the gel to coomassie dye stain and half of the gel to western blot analysis to eliminate the possibility of any variations between the two samples. After successfully running the SDS-PAGE, the proteins were transferred from gel to a cellulose membrane using the semi-dry western blotting technique. To start, 2 extra thick blotting pads and the cellulose membrane were exposed to transfer buffer (25 mM Tris buffer, 190 mM glycine, 20% methanol, dH_2O to 1000 mL) for 15 minutes. The gel was secured on top of the cellulose membrane between two blotting pads. Sandwiching the samples to be transferred, the cathode plate assembly was secured to the anode plate. The trans-blot semidry apparatus was run for 60 minutes at 15V. The cellulose membrane was removed first washed with distilled water prior to being immediately exposed to 5% stopping buffer (5% low fat milk in PBST) agitated at room temperature for 60 minutes. The cellulose membrane was then washed 5 times with PBS-T for about 5 minutes each. The membrane was exposed to 10 mL of 1:250 primary IgE antibody in 5% stopping buffer and agitated overnight at 4^{o} C. Membrane was then washed 5 times with PBS-T for about 5 minutes each. The membrane was then exposed to 10 mL of 1:1000 secondary antibody in 5% stopping buffer and agitated at room temperature for 60 minutes. Membrane then was washed 5 times with PBS-T for about 5 minutes each. The membrane was then immediately exposed to 5 mL of chemiluminescence substrate. *Substrate components were pre-prepared by the manufacturer (ThermoFisher). Substrate components consisted of "Substrate A" and "Substrate B" which needed to be mixed together prior to exposing to the membrane. Substrate A and B were mixed equally, totaling 10 mL.* Exposed membrane was then incubated for 1 minute at room temperature (exposed to substrate). Photo of western blot

was captured using a CD chemi-blot UV camera at normal intensity settings and 30 seconds exposure.

Results

Experiments were completed that exposed IgE antibodies to modified and unmodified Ara h 2 to observe if there were any variations in antibody affinity. Figure 10 displays SDS-PAGE and western blot results of reagent exposed Ara h 2 samples. There appears to be visible indication of protein chemical modification of Ara h 2 through mobility shift observed by SDS-PAGE analysis. This figure appears to display visible smearing and even polymerization dependent on the reagent used. There also appears to be a direct correlation between higher reagent concentration exposure and an increased susceptibility for the protein to undergo chemical modification. Higher concentrations of exposed reagents modified Ara h 2 at higher intensities compared to lower reagent concentrations. Western blot analysis of the samples appeared to display decreased affinity of IgE antibodies toward the modified Ara h 2 allergen samples. Antibody affinity also appeared to be dependent on the concentration of reagent exposed to the Ara h 2 protein.

Discussions

As we experimented with chemical modifications to Ara h 2 and compared the results of formed potential allergoids and unmodified allergens we noticed a visible mobility shift by SDS-PAGE that directly correlated with a change in IgE antibody binding affinity (Figure 10). A control using bovine serum albumin (BSA) was used to identify any imprecise antibody binding and showed to be conclusive in that the antibodies were actually specific to Ara h 2. Unmodified Ara h 2 was also used as a

control to show the effectiveness of the antibody binding. Figure 10 displays a mobility shift of modified Ara h 2, the modified allergen is still visible on the SDS-PAGE even after being modified by the specific reagent. However, the western blot analysis shows a stark difference between the antibody affinity towards the unmodified control and the affinity towards the modified samples. This is evidence that the allergen protein is still present in the sample solution but is failed to be detected by the highly specific antibodies.

As Ara h 2 is modified there is a chance of losing the original protein solubility character [49]. SDS-PAGE cannot determine protein solubility specifically, but a lack of visible protein on the SDS-PAGE, especially after protein modification, could be the consequence of poorly dissolved protein as a result of the chemical modification. The protein allergoid could potentially become so insoluble (compared to the native un-modified protein) that it could form an aggregate pellet and precipitate out of the sample solution [49]. If the protein does become so insoluble that it is no longer observable by SDS-PAGE, and therefore western blot, attempts to solubilize the protein may be necessary so it can be observed by SDS-PAGE and therefore able to be analyzed by western blot. The idea that modified Ara h 2 could be less soluble in solution than the native Ara h 2 was a reasonable consideration. However, due to time limitations and extreme limitations on resources, specifically scarce quantities of allergen protein it was not possible for us to perform experiments to fully test this argument.

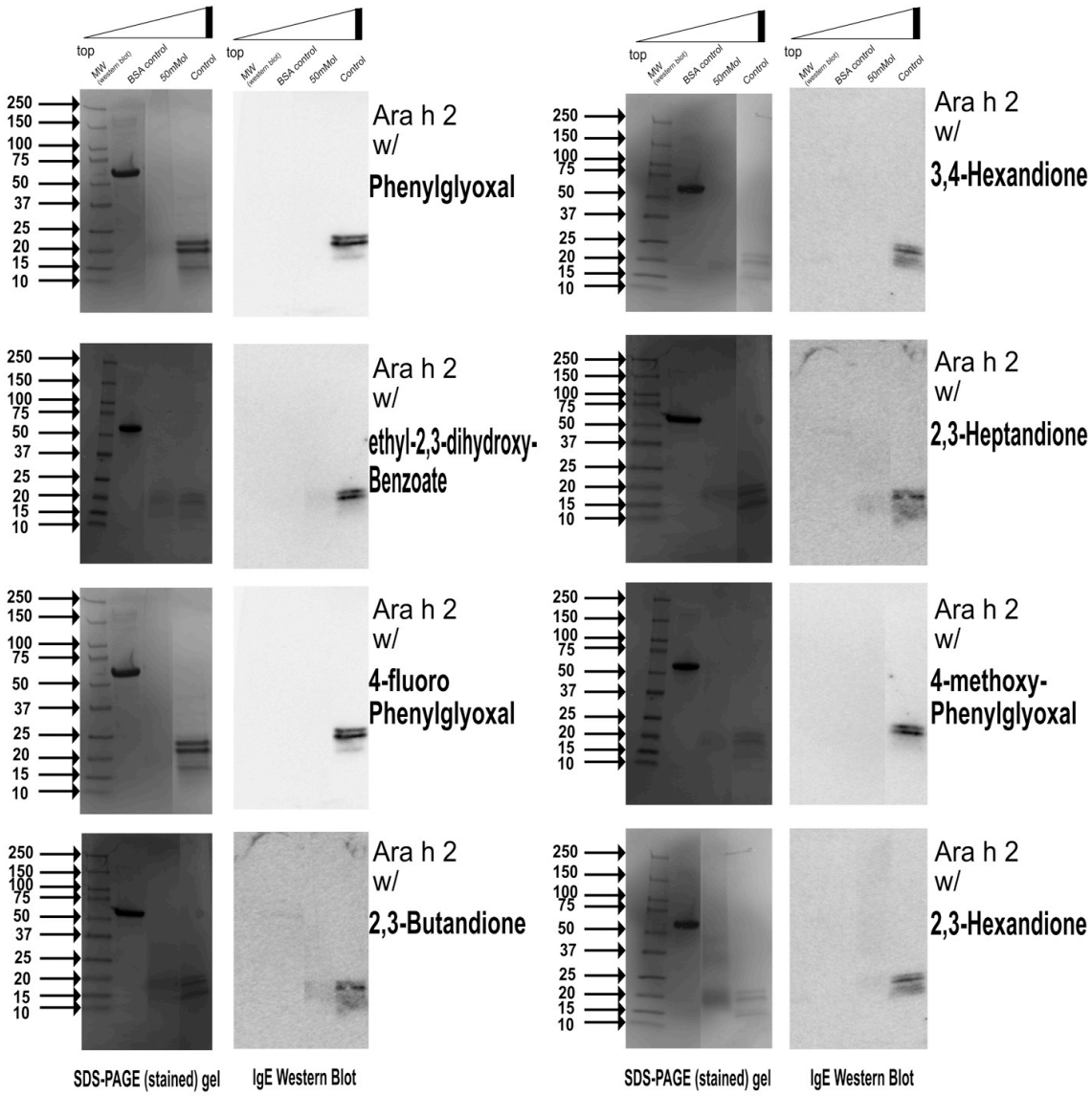


Figure 10. SDS-PAGE and ant-Ara h 2 IgE western blot of successful allergoid synthesis reactions, reacted under basic conditions in 25mM Tris buffer.

Chapter 5

Overall Allergen Crosslinking Discussion

Peanut protein Ara h 2 has been observed to produce severe allergic responses when exposed to endogenous antibodies. Life-threatening conditions such as anaphylaxis could be the result in allergic individuals who are not treated immediately after allergen exposure. Currently, there is little that can be done to preemptively treat allergic episodes resulting from peanut allergens. More research is necessary to increase understanding of the complete role of the peanut allergen and its effects in the body. Identifying a safe method of allergen exposure to patients could result in an endogenous immune system tolerated to peanuts, and specifically the major peanut allergen Ara h 2.

The major allergen Ara h 2 was isolated successfully following the procedure described in chapter 2. Several reaction conditions were attempted, listed in chapter 3. The variables of these conditions that were mostly responsible for generating a positive chemical modification reaction with Ara h 2 were determined. Tris-base buffer at 10.5 pH and at temperature of 37° C on constant rotation facilitated the reaction well. The allergoid reaction procedure was then standardized to maintain consistency in reagent target screening which allowed for reproducibility and resulted in the identification of several vaccine candidate lead compounds. Several novel Ara h 2 allergoid synthesis reaction mechanisms were proposed. Since the presence of epitope surfaces are the result of the native protein structure, a significant disruption to this structure could lead inaccessible or even eliminated epitope surface regions. Protein chemical modification was the chief method used to alter the protein structure. According to Feeney[50] protein chemical modification is an effective method for altering protein structure. Reagents

known to react with specific amino acids were used to promote the protein chemical modification. SDS-PAGE was used to determine if the chemical modification was successful.

As for protein chemical modification experiments, specifically when using SDS-PAGE to observe results, the evaluated sample protein may appear at a molecular weight different from the native protein, if there is a shift in gel mobility of the protein from the native control sample then this could be the first observation of polymerization of the protein. Even though it may be too soon to make any type of determination having only molecular weight data, the data is still important because of the provided opportunity to secure the polymerized samples to be further evaluated using additional experiments that could lead to an enhanced understanding of the chemical properties of Ara h 2.

There were a few significant factors to keep in mind in our attempts to modify the allergen. There was always a possibility of altering the allergen's solubility when synthesizing the allergoid thus causing the potentially formed allergoid to be insoluble. Solubility changes appeared to occur more quickly at higher reagent concentrations. Protein concentration, modification reagent concentration, and reaction conditions with each concentration were evaluated thoroughly and in duplicates to determine the most effective and direct route to a viable allergoid synthesis. There appeared to be relationship between the quantity of reagents used and the overall strength of the chemical modification to protein. Ultimately, protein concentrations around 1-2mg/mL and chemical reagent concentrations around 50mM appeared to be the most effective in modifying Ara h 2. In addition to reagent concentrations, higher pH and warmer temperatures appeared to increase the likelihood of a potentially successful reaction.

SDS-PAGE was the most effective technique available to us to identify protein modification and the viability of the modification at the conditions being tested. There always existed the possibility of only partially modifying Ara h 2, resulting in a modified allergen protein that still retained IgE excitability potential. Therefore, the attempted reaction conditions needed to be well refined to successfully facilitate the desired allergoid forming reaction.

Another important consideration was the solvent (or solvents) used to dissolve reagents within the buffer which were limited to a minimum so not to interfere with the crosslinking reaction and to avoid any negative and destructive effects to the allergen as a whole. The buffer used to maintain the reaction pH was based on the conditions essential to the reagent being tested and if Ara h 2 could be safely stored in the specific buffer over the duration of time needed to carry out the reaction.

The validity of the allergen modification reaction was first confirmed through mobility shifts that suggested a change in protein molecular weight between the native allergen and the allergen exposed to the evaluated compound. The change in molecular weight is the initial indicator of a potential change to the epitope surface regions. A positive change to the epitope surface regions is presumed to be indicative of a change in immune-reactivity as well. A change in immunoreactivity could potentially result in a change in how the allergen functions during *in-vitro* and *in-vivo* testing. Several modification reagents were attempted, the complete list can be found in Table 2 and Table 3, however the reagents capable of generating an actual allergoid are discussed in further detail here. According to *Packer*[35] proteins capable of crosslinking by chemical modification to arginine residues contain roughly 5% arginine. The quantity of arginine

residues in Ara h 2 is approximately 12%. The ketone aldehyde group of the compound Phenylglyoxal acts as an oxidizing agent and targets arginine residues in proteins. The guanidine group of arginine interacts with phenylglyoxal and forms heterocyclic condensation products promoting a structural change to occur in Ara h 2 which results in the crosslinking between two reactive Ara h 2 proteins. Diketone reacting groups of simple organic molecules also react well with arginine amino acids and have been studied to promote crosslinking in proteins [35].

Since phenylglyoxal monohydrate was previously determined to chemically crosslink proteins at arginine residues when prepared at pH 10.5, and phenylglyoxal monohydrate displayed crosslinking capabilities when exposed to Ara h 2, 4-Fluorophenylglyoxal hydrate, *3,4-difluorophenylglyoxal hydrate*, 4-methoxyphenylgyoxal hydrate appeared to be good candidates as well for crosslinking peanut allergen. Our successful attempts led us to our current understanding that 4-Fluorophenylglyoxal hydrate and 3,4-difluorophenylglyoxal hydrate could be crosslinking Ara h 2 proteins in the same fashion as phenylglyoxal monohydrate where the Fluorine(s) group on the aromatic ring could be acting as an electron withdrawing group. It could be essential to pull electrons away from the diketone to react effectively to Ara h 2. Another explanation could be how effectively the reagents were dissolved in the reaction buffer. Each of these reagents was different in terms of solubility which could have played a role in overall reactivity as well. There is currently no prior works using 4-Flurophenylglyoxal hydrate in modifying the peanut allergen protein Ara h 2. No associated IgE data could be obtained for *3,4-difluorophenylglyoxal hydrate*, due to the sample limit in the end. There was no 45 day SDS-PAGE data or IgE data to evaluate,

however this modification reagent was effective in modifying Ara h 2 after observing the results of resulting sample batches that were completed at another time using a different native Ara h 2 sample.

To expand our understanding of the importance of a diketone reacting group in performing allergoid synthesis reactions with Ara h 2, 2,3-Butandione, 2,3-Hexandione, 3,4-Hexanedione, and 2,3-Heptanedione were evaluated. Currently there are no prior works identifying the ability of these reagents to modify the peanut allergen protein Ara h 2. Each reagent displayed strong evidence of crosslinking, however 2,3-Hexandione was observed to be the most effective reagent to crosslink Ara h 2. Our successful attempts led us to the understanding that 2,3-Butandione, 2,3-Hexandione, 3,4-Hexanedione, 2,3-Heptanedione and therefore the diketone group in phenylglyoxal could be essential in crosslinking Ara h 2 through glycol and glycosylation reactions. It should also be noted that O'Hehir[48] claimed in their patent potential success in crosslinking Ara h 2 using Phenylglyoxal, 2,3-Butandione, and 2,3-Hexandione, however no scientific studies could be located to support this claim until now.

It is also worth noting that aside from the 2nd derivative series evaluated there were 2 additional reagents evaluated that displayed evidence of crosslinking as well. The reagents were Aurintricarboxylic acid and ethyl 2,3-dihydroxybenzoate. Further research into the reason for modification with Aurintricarboxylic acid and ethyl 2,3-dihydroxybenzoate is suggested since time prevented any further derivatives of these reagents from being evaluated. However, 2,3-dihydroxybenzoate contains a quinone group and successful reaction with Ara h 2 was observed in above 10.5 pH which is consistent with the reaction conditions and mechanism proposed by Chung[46].

In-vitro immunoassays were also successfully performed and yielded results consistent with the degree of observable allergen structural change. A direct correlation was observed between the protein shift in mobility (observed by SDS-PAGE) and the concentration of the reaction compound used. According to Figure 10 all reagents tested appeared to successfully chemically modify Ara h 2 and physically disrupt epitope regions as identified by western blot.

Several conditions were also evaluated to increase effectiveness during the initial compound screening experiments and proved invaluable in increasing the rate of identified hit compounds. The evaluated reaction conditions included variations in reaction buffer, reaction buffer pH, exposing the reaction mixture to agitation vs. non-agitation, reaction temperature, concentration of reagent exposed to protein, and time exposed to protein we all evaluated to understand which conditions were ultimately most effective in forming an allergoid. To determine which reagent concentrations to use with the protein a large range of concentrations was evaluated to identify where the extremes were and to identify the potential working concentration. The apparent working reagent concentration was determined to be between 25mM - 50mM. A standard range of 250mM - 1mM reagent concentration was frequently used to obtain the optimal working range for each reagent examined. Upon identifying which reagents react with Ara h 2, it is then necessary to make the fine-tune adjustments to optimize the allergoid where these adjustments can even as extensive as acquiring the chemical derivatives for the molecules being tested to determine the potential for stronger reactivity when compared to the original parent molecule structure.

Protein concentration was also important to remain as consistent as possible under the circumstances so to only evaluate one variable at a given time. The desired working protein concentration was around 1 – 2mg/mL, which was the seemingly effective working concentration identified by past researchers who determined that a 5mg/mL mixture of Ara h 2 and Ara h 6 was appropriate to modify Ara h 2 with phytic acid [49].

Conclusions

Moving forward we hope to increase our understanding of the allergoid chemical structure as well as identifying if any therapeutic benefits exist. Further characterization experiments are needed to better understand the allergoid structure, and to possibly make further modification enhancements that could benefit the overall product. In addition to characterization, higher order biological testing is also necessary to identify product stability and to determine what possible therapeutic benefits this product is capable of. It is also essential to provide further understanding for the allergoid theory in general, which could ultimately yield more public support for research projects like this one.

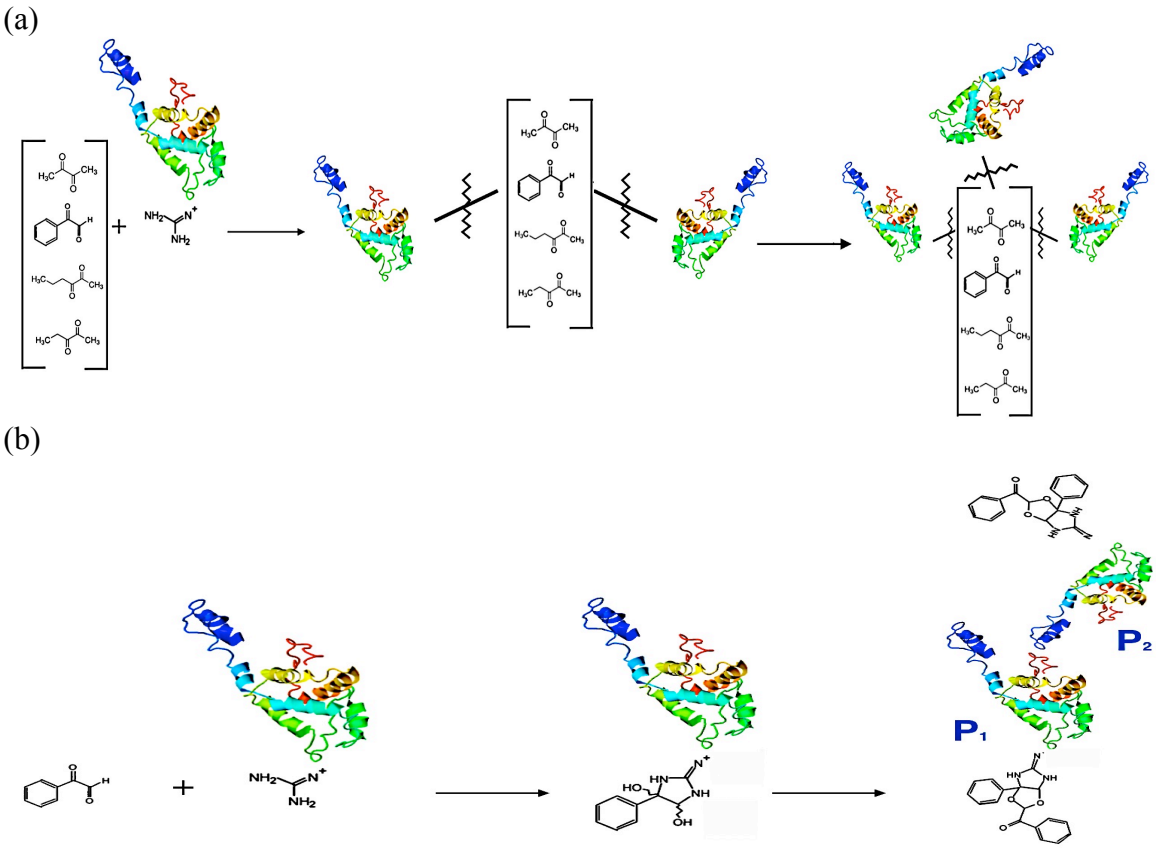


Figure 11. Ara h 2 crosslinking reaction mechanism. (a) Abbreviated Ara h 2 dimer and trimer formation reaction using diketone reagents and phenylglyoxal derivative compounds (only phenylglyoxal parent structure displayed here). (b) Phenylglyoxal mediated Ara h 2 crosslinking reaction through accessible arginine residues producing a crosslinking event between protein 1 and 2 (P1 and P2). In this schematic, reactants are not to scale.

References

1. FDA. (2006). Food allergens labeling and consumer protection act of 2004. In *Public law 108-282*, vol. Report to the committee on health, education, labor, and pensions (pp. p.1-33).

2. Sampson, H. A. (1999). Food allergy. Part 1- Immunopathogenesis and clinical disorders. Journal of Allergy and Clinical Immunology. 103(**5**), 717-728.

3. Burks, A. W., Tang, M., Sicherer, S., Muraro, A., Eigenmann, P. A., Ebisawa, M., Fiocchi, A., Chiang, W., Beyer, K., Wood, R., Hourihane, J., Jones, S. M., Lack, G., & Sampson, H. A. (2012). ICON: food allergy. Journal of Allergy and Clinical Immunology. 129(4), 906-920.

4. Burks, W., Bannon, G. A., Sicherer, S., & Sampson, H. A. (1999). Peanut-Induced Anaphylactic Reactions. International Archives of Allergy and Immunology. 119(**3**), 165-172.

5. Sampson, H. A. (1991). Immunologic mechanisms in adverse reactions to foods. Immunology and Allergy Clinics of North America. 11701-716.

6. Taylor, S. L., & Hefle, S. L. (2001). Food Allergies and Other Food Sensitivities. Food Technology. 55(**9**), 68-83.

7. Van Asperen PP, K. A., Mellis CM. (1983). Immediate food hypersensitivity reactions on the first known exposure to the food. Archives of Disease in Childhood. 58253–256.

8. Akdis, M., & Akdis, C. A. (2007). Mechanisms of allergen-specific immunotherapy. Journal of Allergy and Clinical Immunology. 119(4), 780-791.

9. Burton, O. T., Logsdon, S. L., Zhou, J. S., Medina-Tamayo, J., Abdel-Gadir, A., Noval Rivas, M., Koleoglou, K. J., Chatila, T. A., Schneider, L. C., Rachid, R., Umetsu, D. T., & Oettgen, H. C. (2014). Oral immunotherapy induces IgG antibodies that act through FcgammaRIIb to suppress IgE-mediated hypersensitivity. Journal of Allergy and Clinical Immunology. 134(6), 1310-1317 e1316.

10. Asarnoj A, M. R., Ostblom E, Poorafshar M, Lilja G, Hedlin G, van Hage M, Ahlstedt S, Wickman M. (2010). IgE to peanut allergen components: relation to peanut symptoms and pollen sensitization in 8-year-olds. Allergy. 65(9), 1189–1195.

11. Merima Bublin, & Breiteneder, H. (2014). Cross-Reactivity of Peanut Allergens. Current Allergy and Asthma Reports. 14(4), 426.

12. Achouri, A., & Boye, J. I. (2013). Thermal processing, salt and high pressure treatment effects on molecular structure and antigenicity of sesame protein isolate. Food Research International. 53(1), 240-251.

13. Jedrychowski, L., & Wroblewska, B. (1999). Reduction of the Antigenicity of Whey Proteins by Lactic Acid Fermentation. Food and Agricultural Immunology. 11(1), 91-99.

14. Hanane, K., Kamel Eddine, E. M., Omar, K., & Djamel, S. (2006). Microwave treatment modify antigenicity properties of bovine milk proteins. African Journal of Biotechnology. 5(13), 1267-1270.

15. Kim, S. B., Ki, S. K., Khan, M. A., Lee, W. S., Lee, H. J., Ahn, B. S., & Kim, H. S. (2007). Peptic and Tryptic Hydrolysis of Native and Heated Whey Protein to Reduce Its Antigenicity. Journal of Dairy Science. 90(9), 4043-4050.

16. Hu, C. Q., Chen, H. B., Gao, J. Y., Luo, C. P., Ma, X. J., & Tong, P. (2011). High-pressure microfluidisation-induced changes in the antigenicity and conformation of allergen Ara h 2 purified from Chinese peanut. Journal of the Science of Food and Agriculture. 91(7), 1304-1309.

17. MARSH, D. G., LICHTENSTEIN, L. M., & CAMPBELL, D. M. (1970). Studies on 'Allergoids' Prepared from Naturally Occurring Allergens. Immunology. 18705-722.

18. Bencharitiwong, R. P. D., van der Kleij, H. P. M. P. D., Koppelman, S. J. P. D., & Nowak-We ̨grzyn, A. M. D. (2015). Effect of chemical modifications on allergenic potency of peanut proteins. Allergy and Asthma Proceedings. 36185-191.

19. Helen S. Skolnick, M., Mary Kay Conover-Walker, M., RN, CRNPa, , Celide Barnes Koerner, M., RDa, , Hugh A. Sampson, M., Wesley Burks, M., & Robert A. Wood, M. (2001). The natural history of peanut allergy. Journal of Allergy and Clinical Immunology. 102(2), 367-374.

20. Grundy, J., Matthews, S., Bateman, B., Dean, T., & Arshad, S. H. (2002). Rising prevalence of allergy to peanut in children: Data from 2 sequential cohorts. Journal of Allergy and Clinical Immunology. 110(5), 784-789.

21. E., J. E. (1977). Activation of IgE regulatory mechanisms by transmucosal absorption of antigen. Lancet. Lancet223-225.

22. SA, B. (1982). The Natural History of Food Sensitivity. Journal of Clinical Immunology. 69(2), 173-177.

23. O'BHourihane, J., Dean, T., & Warner, J. (1996). Peanut allergy in relation to heredity, maternal diet, and other atopic diseases: results of a questionnaire survey, skinprick testing, and food challenges. British Medical Journal. 313513-521.

24. Crameri, R., & Rhyner, C. (2006). Novel vaccines and adjuvants for allergen-specific immunotherapy. Current Opinion in Immunology. 18(6), 761-768.

25. Tang, M. L., Ponsonby, A. L., Orsini, F., Tey, D., Robinson, M., Su, E. L., Licciardi, P., Burks, W., & Donath, S. (2015). Administration of a probiotic with peanut oral immunotherapy: A randomized trial. Journal of Allergy and Clinical Immunology. 135(3), 737-744 e738.

26. Burks, W. A., Williams, L. W., Connaughton, C., Cockrell, G., O'Brien, T. J., & Helm, R. M. (1992). Identification and characterization of a second major peant allergen, Ara h II, with use of the sera of patients with atopic dermatitis and positive peanut challange. Journal of Allergy and Clinical Immunology. 90(6, Part 1), 962-969.

27. Chatel, J.-M., Bernard, H., & Orson, F. M. (2003). Isolation and Characterization of Two Complete Ara h 2 Isoforms cDNA. International Archives of Allergy and Immunology. 13114-18.

28. Baumert, J. L. (2008). *IMMUNOCHEMICAL STUDIES OF DIGESTION RESISTANT ARACHIS HYPOGAEA 2 (Ara h 2) PEPTIDE.* Unpublished A Dissertation, University of Nebraska, The Graduate College of the University of Nebraska.

29. Koppelman, S. J., Hefle, S. L., Taylor, S. L., & de Jong, G. A. (2010). Digestion of peanut allergens Ara h 1, Ara h 2, Ara h 3, and Ara h 6: a comparative in vitro study and partial characterization of digestion-resistant peptides. Molecular Nutrition & Food Research. 54(12), 1711-1721.

30. P.E., J., I., V. d. P., A., B., F.A., H., T., G., M., H., D., K., E.N., M., & A.R., M. (2010). High pressure, thermal and pulsed electric-field-induced structural changes in selected food allergens. Molecular Nutrition & Food Research. 54(12), 1701-1710.

31. Verhoeckx, K. C., Vissers, Y. M., Baumert, J. L., Faludi, R., Feys, M., Flanagan, S., Herouet-Guicheney, C., Holzhauser, T., Shimojo, R., van der Bolt, N., Wichers, H., & Kimber, I. (2015). Food processing and allergenicity. Food and Chemical Toxicology. 80223-240.

32. Wu, Z., Lian, J., Han, Y., Zhou, N., Li, X., Yang, A., Tong, P., & Chen, H. (2016). Crosslinking of peanut allergen Ara h 2 by polyphenol oxidase: digestibility and potential allergenicity assessment. Journal of the Science of Food and Agriculture. 96(10), 3567-3574.

33. Burks, W., Sampson, H. A., & G.A., B. (1998). Peanut allergens. Allergy. 53(**2**), 725-730.

34. Sen, M., Kopper, R., Pons, L., Abraham, E. C., Burks, W. A., & Bannon, G. A. (2002). Protein Structure Plays a Critical Role in Peanut Allergen Stability and May Determine Immunodominant IgE-Binding Epitopes. Journal of Immunology. 169882-887.

35. Packer, L. (2001). *Flavonoids and other polyphenols* (Vol. 335): Academic Press.

36. Park, S. K., Bae, D. H., & Rhee, K. C. (2000). Soy protein biopolymers cross-linked with glutaraldehyde. Journal of the American Oil Chemists' Society. 77(8), 879-884.

37. Silva, C. J. S. M., Sousa, F., Gübitz, G., & Cavaco-Paulo, A. (2004). Chemical Modifications on Proteins Using Glutaraldehyde. Food Technology and Biotechnology. 42(1), 51-56.

38. Stanić-Vučinić, D., & Veličković, T. C. i. (2012). Enzymatic and Chemcial Modifications of Food Allergens. In P. C. P. (Ed). (Ed)., *Allergic Diseases - Highlights in the Clinic, Mechanisms and Treatment*, (pp. 83-106). Hardcover: InTech.

39. Ashraf, J. M., Rabbani, G., Ahmad, S., Hasan, Q., Khan, R. H., Alam, K., & Choi, I. (2015). Glycation of H1 Histone by 3-Deoxyglucosone: Effects on Protein Structure and Generation of Different Advanced Glycation End Products. PLoS One. 10(6), e0130630.

40. Schmitt, A., Schmitt, J., Munch, G., & Gasic-Milencovic, J. (2005). Characterization of advanced glycation end products for biochemical studies: side chain modifications and fluorescence characteristics. Analytical Biochemistry. 338(2), 201-215.

41. Jumnongpon, R., Chaiseri, S., Hongsprabhas, P., Healy, J. P., Meade, S. J., & Gerrard, J. A. (2012). Cocoa protein crosslinking using Maillard chemistry. Food Chemistry. 134(1), 375-380.

42. A. Seetharama Acharya, S. A., Cho, Y. J., & Manjula, B. N. (1988). Cross-Linking of Proteins by Aldotriose: Reaction of the Carbonyl Function of the Keto Amines Generated in Situ with Amino Groups. Biochemistry. 274522-4529.

43. Chung, S.-Y., & Champagne, E. T. (2009). Reducing the allergenic capacity of peanut extracts and liquid peanut butter by phenolic compounds. Food Chemistry. 115(4), 1345-1349.

44. Chung, S. Y., & Reed, S. (2012). Removing peanut allergens by tannic acid. Food Chemistry. 134(3), 1468-1473.

45. Chung, S.-Y., & Champagne, E. T. (2011). Ferulic acid enhances IgE binding to peanut allergens in Western blots. Food Chemistry. 124(4), 1639-1642.

46. Chung, S.-Y., Kato, Y., & Champagne, E. T. (2005). Polyphenol oxidase/caffeic acid may reduce the allergenic properties of peanut allergens. Journal of the Science of Food and Agriculture. 85(15), 2631-2637.

47. Lundblad, R. L. (2014). *Chemical Reagents for Protein Modification* (4 ed).: CRC Press.

48. O'hehir, R., Rolland, J., & PRICKETT, S. (2014). Patent - Novel immunotherapeutic molecules and uses thereof. In, vol. WO2014066939 A1): Monash University.

49. Chung, S.-Y., & Champagne, E. T. (2007). Effects of Phytic Acid on Peanut Allergens and Allergenic Properties of Extracts. Journal of Agricultural and Food Chemistry. 55(**22**), 9054-9058.

50. Feeney, R. E., Yamasaki, R. B., & Geoghegan, K. F. (1982). Chemical Modification of Proteins: An Overview. 1983-55.