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Identification and Characterization of Putative Allergens in Pecan Species

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Identification and Characterization of Putative Allergens in Pecan Species

by

Jelena Spiric

A THESIS

Presented to the Faculty of
The Graduate College of the University of Nebraska
In Partial Fulfillment of Requirements
For the Degree of Master of Science

Major: Food Science and Technology

Under the Supervision of Professor Joseph L. Baumert

Lincoln, Nebraska

December, 2011
Identification and Characterization of Putative Allergens in Pecan Species

Jelena Spiric, M.S.
University of Nebraska, 2011

Advisor: Joseph L. Baumert

The identification and characterization of soluble pecan proteins was found to be qualitatively dependent on the chemical characteristics of the extraction buffer that also had a significant influence on the total protein content of an extract. The ionic strength of the extraction buffer had a greater impact on protein extractability than pH. A high salt phosphate buffer (0.01 M PBS, 1M NaCl, pH 7.2) was used as an optimal extraction solution that allowed remarkable detection of clinically relevant allergens.

The 2S albumin family of seed storage proteins has been shown to be one of the most stable allergens, thus potentially making these proteins clinically relevant for allergic sensitization. In this study, we sought to purify native 2S albumin from pecan to further characterize this putative allergen. The protocol for purification and isolation of 2S albumin protein from pecan, Car i 1, was developed employing two size-exclusion chromatography steps. Sequence identification of pecan proteins was done by means of LC-MS/MS ion trap mass spectrometry.

The soluble high molecular weight pecan proteins were rapidly digested by pepsin in simulated gastric fluid (SGF) whereas the low molecular weight proteins were more
stable or were reduced to digestion resistant proteins and polypeptides. The purified 2S albumin, Car i 1, from pecan was found to be resistant to digestion in SGF and comparatively stable to proteolysis by trypsin and pancreatin in simulated intestinal fluid (SIF).

Digestion of purified Car i 1 in SGF and SIF resulted in formation of different digestion-resistant peptides, which included important epitopes that were retained despite extensive in vitro digestion. These peptides were capable of binding IgE antibodies from allergic individuals. Digestion stability of Car i 1 and formation of stable, digestion-resistant antigenic polypeptides may contribute to allergic sensitization to pecans in susceptible individuals.
ACKNOWLEDGEMENT

I am very grateful to my adviser Dr. Joe Baumert for his guidance and support during the course of this project. Joe has been a wonderful mentor who has had an incredible impact on my life. He has helped me in becoming an independent researcher by providing me with indispensable knowledge and skills necessary for the development of effective experiments.

I also would like to recognize and express my gratitude to Dr. Steve Taylor, my committee member, for giving me the opportunity to continue my graduate education and to pursue research in my area of interest. I am thankful to him for believing in me and for sharing his great experience and expertise.

I would like to express my appreciation to the other members of my committee, Dr. Goodman, and Dr. Zeece for serving on my committee and providing valuable inputs throughout this project. Also, Dr. Elthon for providing his assistance and allowing me to use his instruments in my research. Similarly, I would like to recognize the contribution and help of Roger Powell from National Jewish Health Mass Spectrometry Facility.

I would like to thank all of the people from Food Allergy Research and Resource program who have made the completion of this thesis and my graduate program possible. Special thanks to Julie Nordlee for her patience, valuable criticism, and mentoring in laboratory techniques. I am also very grateful to all my fellow graduate students, especially Poi-Wah Lee, Rakhi Panda, and Ben Remington for giving me their assistance, advice, and encouragement.
Finally, I would like to thank my family and friends for all of their support and inspiration. They have always been my source of strength and comfort. This thesis would not be possible without their endless love. I am truly grateful for all of the amazing people in my life.
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CHAPTER 1: LITERATURE REVIEW

INTRODUCTION

Food allergy has a tremendous impact on the lives of millions of Americans. Strict avoidance diets are currently the only option for allergic individuals to remain symptom free as no treatment or cure for food allergies is currently available. Allergic consumers or their caregivers must diligently read ingredient labels on every packaged food product they purchase to ensure their safety. This can be a very challenging and time consuming task. Moreover, for individuals with food sensitivities, particularly food allergies, eating certain foods can be a life threatening experience; therefore, fear of adverse reactions diminishes the quality of life in many susceptible individuals.

Food allergens are a significant public health issue worldwide. Milk, eggs, wheat, peanuts, tree nuts, soybean, fish, and crustacean shellfish are considered the major allergenic foods. These foods, often mentioned as the “Big 8”, are responsible for vast majority of IgE-mediated food allergies on a worldwide basis (FAO, 1995). An estimated 5% of young children and 3% to 4% of adults in westernized countries are affected by food allergies (Sicherer and Sampson, 2010). More than 1% of the US population has allergic reactions to peanut and/or tree nuts, and some of these reactions can occasionally progress to life-threatening anaphylactic reactions (Sicherer et al., 2010). Additionally, peanut and tree nut allergies are rarely outgrown, resulting in lifelong afflictions (Fleischer et al., 2005; Roux et al., 2003).

The USDA, Agricultural Research Service (ARS) Western Human Nutrition Research Center, the International Tree Nut Council Nutrition Research & Education Foundation, and the Peanut Institute organized the “2007 Nuts and Health Symposium”
held in Davis, CA emphasized the emerging evidence of both positive and negative effects of nut (including both peanut and tree nuts) consumption on health. Nuts are abundant in polyunsaturated fatty acids, contain a variety of phytonutrients, and consumption of nuts in a diet has been linked to the reduction of the development of several cancers (Davis et al., 2008). However, the prevalence of tree nut and peanut allergies is believed to be increasing as well. The factors responsible for this apparent increase have not been identified (Sicherer et al., 2010).

Over the past two decades, knowledge of the clinical and biochemical aspects of the major tree nut allergens has grown substantially. Characterization of allergens from several commercially important tree nuts has been accomplished, but the identification of allergens in some tree nuts is still lacking. The stability of the prominent allergens of tree nuts to digestion may also play a key role in development of tree nut allergy where the exposure to undigested allergens may result in allergic sensitization. Studying IgE-reactive proteins of tree nuts is therefore critical to improve our knowledge of tree nut induced food allergies (Sathe et al., 2009).

Little information is currently available regarding the fate of digestion-resistant pecan allergens. These digestion-resistant proteins or peptides could have immunologically active epitopes that could sensitize individuals upon exposure. Moreover, a better understanding of allergen stability could be used to assess if these potentially stable proteins and peptides could be used as critical components in development of specific ELISAs for diagnosis of food allergies and potentially used as targets for development of immunotherapy treatment of food allergy.
This review will distinguish between different adverse reactions to foods and define true food allergies and their prevalence among children and adult populations, with a particular focus on tree nut allergy. The second part of the review will also focus on both gastric and intestinal digestion of dietary proteins and the potential role of stable proteins and fragments of proteins in sensitizing individuals via gastrointestinal exposure.

**FOOD SENSITIVITIES**

Food sensitivities are abnormal physiological responses to foods, which affect a small percent of the overall population (Taylor and Hefle, 2002). Food sensitivities encompass a wide range of adverse reactions to foods and they are further classified as food allergies and food intolerances (Figure 1.1). Some food sensitivities are manifested after preexisting health conditions such as various gastrointestinal illnesses or drug therapy (Taylor, 1987). The main distinction between food intolerances and food allergies is involvement of an individual’s immune system to in allergic reactions. True food allergies involve abnormal immunological response to a particular food. Food allergies can further be categorized as immediate hypersensitivity (IgE-mediated) reactions, or delayed hypersensitivity (cell-mediated) reactions (Taylor and Hefle, 2006a). The general population considers food sensitivities as a substantial health concern where often all adverse reactions to foods are mistakenly interpreted as food allergy (Taylor and Hefle, 2002). Therefore, it is important to emphasize that this review and research focuses on IgE-mediated allergic reactions.
Figure 1.1 Classification of the primary food sensitivity reactions (Modified from Taylor, 1987 and Taylor and Hefle, 2006a).

Food Intolerances

Food intolerances are individualistic adverse reactions to food that do not involve abnormal response of the immune system (Taylor and Hefle, 2006a). They are caused by the presence of certain genetic defects of the susceptible individual, or factors inherent in a food (Taylor, 1985). Individuals with food intolerances can typically tolerate small to modest quantities of the offending food or food component in their diets without experiencing adverse reactions. Lowering the intake amount of food causing the illness in the diet typically controls food intolerances, whereas strict avoidance diets are the only option for food allergic individuals. Moreover, food intolerances are further classified as anaphylactoid reactions, metabolic food disorders, or idiosyncratic reactions (Taylor and Hefle, 2006a).
**Anaphylactoid Reactions**

Anaphylactoid reactions to food are accompanied with the release of histamine and other mediators from mast cells and basophils without the involvement of the immune system. The mechanism behind the release of these mediators is still unknown (Taylor et al., 1992). Evidence for the existence of anaphylactoid reactions remains debatable since none of the endogenous substances in food causing the release of these mediators have been identified (Taylor and Hefle, 2002). Foods associated with causing anaphylactoid reactions include strawberries and chocolate, with symptoms similar to true food allergies (Hefle, 1996). Since diagnosis of anaphylactoid reactions is challenging, treatment for such reactions requires avoidance of the offending food (Taylor, 1987).

**Metabolic Food Disorders**

Metabolic food disorders are adverse reactions resulting from body’s inability to metabolize a particular food or its components (Taylor, 1987). This deficiency may be genetically inherited; however it can also be acquired as a result of transitory factors (Kocian, 1988). The best examples of metabolic food disorders are lactose intolerance and favism.

Lactose intolerance results from a deficiency in the production of the enzyme lactase or β-galactosidase in the brush-border of the intestinal mucosa (Kocian, 1988). Therefore, lactose, the primary sugar in milk and other dairy products, cannot be hydrolyzed into the individual monosaccharides, galactose and glucose (Taylor and Hefle, 2002). The intact lactose cannot be absorbed by the epithelial cells of the small intestine and it reaches the colon, where colonic bacteria metabolize the lactose resulting
in the production of CO₂, H₂, and H₂O (Taylor and Hefle, 2006a; Kocian 1988). The prevalence of lactose intolerance varies between racial and ethnic groups, affecting estimated two thirds of the world adult population. The prevalence exceeds 50% in South America, Africa, and Asia. In the US, the prevalence among Caucasians is 15%, 53% among Mexican-Americans, and 80% among the Black population. Between six and nine percent of population is affected with lactose intolerance in Australia and New Zealand, respectively. Lactose intolerance affects only about 2% of Scandinavians, but the prevalence reaches 70% in Sicily (Vesa et al., 2000). Additionally, lactose intolerance seems to be more prevalent with advancing age as a result of the natural decrease in intestinal lactase activity (Taylor, 1985). The symptoms of lactose intolerance consist of bloating, abdominal cramping, flatulence, and frothy diarrhea (Taylor and Hefle, 2006a). The most frequent method used for diagnosis of lactose intolerance is a lactose tolerance test, where a fasting individual is given orally 50mg of lactose and blood glucose levels are monitored. Levels of glucose exceeding 25mg/dl are perceived as normal (Taylor, 1987). Avoidance of dairy products containing lactose is the most common treatment; however, the majority of lactose sensitive individuals can tolerate modest amounts of lactose. Additionally, sensitive individuals have widely used products with minimized amounts of lactose such as fermented dairy products and hydrolyzed lactose. Yogurt and acidophilus milk have also been shown to be tolerated presumably because of the presence of bacteria containing β-galactosidase (Vesa et al., 2000; Taylor and Hefle, 2002).

Favism is another example of a metabolic food disorder resulting in an acute hemolytic anemia caused by exposure to fava beans or the inhalation of pollen from the
Vicia faba plant (Taylor and Hefle, 2006a). With a rapid onset time, usually between 5 and 24 hours, the most common symptoms accompanied with favism include pallor, fatigue, dyspnea, nausea, abdominal and/or back pain, fever, and chills. More severe symptoms may include hemoglobinuria, jaundice, and renal failure (Taylor, 1987). Recovery is rapid and spontaneous due to the self-limiting characteristics of the disease (Taylor and Hefle, 2006a).

Favism affects individuals with an inherited genetic deficiency resulting in the inability to produce the enzyme, glucose-6-phosphosphate dehydrogenase (G6PDH), in the red blood cells (Taylor et al, 1992). This enzyme is responsible for preventing oxidative damage of the red blood cells by regulating levels of glutathione and nicotinamide adenine dinucleotide phosphate (Taylor, 1987). Vicine and convicine are naturally occurring oxidants in fava beans, which are capable for damaging the erythrocytes of G6PDH- deficient individuals (Taylor and Hefle, 2006a).

Favism is the most prevalent enzymatic defect in humans, affecting an estimated 100 million people worldwide. The highest frequency has been seen among Oriental Jewish communities, the American black population, and individuals in the Mediterranean region, the Middle East, China, and certain African populations. An assay for enzymatic activity on isolated red blood cells has been commonly used as a diagnostic tool for favism (Taylor, 1987).

**Idiosyncratic Reactions**

Idiosyncratic reactions are adverse reactions to foods affecting certain individuals for which the mechanism of action is unknown (Hefle, 1996). Some examples associated with these reactions caused by a food or food ingredients include sulfite-induced asthma,
tartrazine-induced asthma, and Chinese restaurant syndrome (Taylor, 1985). Symptoms associated with these reactions, due to many possible mechanisms and foods involved, can range from minor to severe and life-threatening reactions (Taylor et al., 1992). The prevalence of these reactions is also questionable and challenging to estimate, since the cause-effect relationship between a particular food and adverse reactions is hard to establish (Taylor and Hefle, 2002). Challenge tests are the only reliable diagnostic tool for this type of disease (Taylor, 1985). Sulfite-induced asthma is one of the well-documented idiosyncrasies known to occur in some individuals (Taylor, 1987).

Although sulfites can occur in food naturally, they are commonly used as additives in food processing industry to control enzymatic and non-enzymatic browning, to stop microbial growth, to condition dough, to prevent oxidation, and as a bleaching agent of certain products (Taylor and Hefle, 2002). Even though the mechanism of action still remains unknown, asthma induced by sulfite ingestion affects approximately 5% of steroid-dependent asthmatic individuals (Taylor and Hefle, 2006a). If the level of added sulfites in foods exceeds 10 ppm, it must be declared on a food label to protect sulfite-sensitive consumers who are maintaining strict avoidance diets (Taylor and Hefle, 2002).

**Food Hypersensitivities**

*Immune System Overview*

The immune system is a defensive structure with the main function to protect the host from harmful substances and pathogenic microorganisms by providing a variety of cells and molecules capable in recognizing and eliminating potential infectious agents. In order to effectively respond and provide protection against foreign invaders, the immune
system must complete four main tasks: immunological recognition, immune effector functions, immune regulation, and generation of immunological memory. This can only be accomplished by the involvement of both the innate and adaptive immune systems (Murphy et al., 2008).

The first line of defense against an infection is provided by the innate immunity. This system, however, lacks the ability to identify specific pathogens and is unable to provide the specific protective immunity (memory) that prevents reinfection. Cells and molecules employed by the innate immune system to combat an infection have broad specificity to pathogens. Cells involved in the function of innate immune responses include macrophages, granulocytes, mast cells, dendritic cells, and natural killer cells. Granulocytes are the collective name for the white blood cells called neutrophils, eosinophils, and basophils. On the other hand, an adaptive immune response is induced when an infection overcomes the innate defense mechanism and it is capable of selectively eliminating specific foreign agents. The primary cells involved in adaptive immune responses are B lymphocytes (B cells) that produce and secrete antigen specific antibodies and T lymphocytes (T cells) that provide help to differentiate B cells to switch to certain antibody or immunoglobulin types that have different effector functions. In addition, some T cells are cytolytic and have the ability to target and kill cells expressing certain surface markers (Murphy et al., 2008).

The responses generated by the innate immune system to pathogens and foreign substances are responsible for initiation of the adaptive immune responses. The main characteristics of the adaptive immune response include its antigenic specificity, diversity, immunologic memory, and ability to discriminate between self and non-self
molecules. The immunological memory is one of the most important outcomes of adaptive immunity that provides a heightened ability of the host to respond on subsequent exposure to the same antigen and eliminate it more effectively (Murphy et al., 2008).

The adaptive immune responses can be classified into T cell-mediated immune responses and B-cell humoral immune responses. Upon antigen recognition, a naïve T cell differentiates into several different classes of effector T cells that are responsible for cell-mediated responses by the host. Cytotoxic T cells have an ability to directly eliminate infected cells, while there are several different kinds of effector T cells that act on other cells with various outcomes. The extracellular spaces between cells are guarded by humoral immune responses, which involve activated B cells that are capable of differentiating into antibody secreting plasma cells. An antibody is a Y-shaped protein that can bind to a specific antigen. Antibodies can efficiently eliminate antigens by neutralization, facilitating the uptake of antigens by phagocytes, or by binding to a pathogen and activating the complement system (Murphy et al., 2008).

Under certain circumstances, the immune system can evoke an unsuitable response to a rather characteristically harmless antigen such as pollen, food, and drugs resulting in a hypersensitivity reaction. These reactions are sometimes denoted as allergic reactions, which can provoke mild to life-threatening responses (Murphy et al., 2008).

Abnormal immune responses to innocuous or harmless substances include several mechanisms that have been classified by Coombs and Gell (1975) into 4 main categories (Figure 2) based on the effector molecules that are produced during the reaction. Type I, type II, and type III hypersensitivity reactions consist of humoral immune responses, which are mediated by the interaction of antigen-antibody complex. The antigen-antibody
complexes are differentiated by the different types of antigens involved and the specific
classes of antibody engaged. On the other hand, type IV hypersensitivity reactions
employ the T cell-mediated responses that can be divided into three groups, and are
frequently denoted to as delayed-type hypersensitivity since symptoms may not develop
for several hours to days after the initial antigen exposure (Murphy et al., 2008).

**Figure 1.2** Adapted and modified from Murphy et al., 2008.

Food hypersensitivity is defined as “an abnormal immunological response to a
particular food or food component, usually a naturally occurring protein” and has been
viewed as an emerging public health problem (Bush and Hefle, 1996; Taylor and Hefle,
2002). The two most significant mechanisms involved in food hypersensitivities involve
cell-mediated hypersensitivity reactions (Type IV) and IgE-mediated, immediate
hypersensitivity reactions (Type I).

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<td>IgE</td>
<td>IgG</td>
<td>T\textsubscript{H1} cells</td>
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<td><strong>Effector mechanism</strong></td>
<td>Soluble antigen</td>
<td>Cell or matrix-associated antigens</td>
<td>Soluble antigen</td>
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<td></td>
<td>Mast cell activation</td>
<td>Cell-surface receptor</td>
<td>Complement, FcR⁺ cells (phagocytes, NK cells)</td>
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<tr>
<td><strong>Immune reactant</strong></td>
<td>Cell or matrix-associated antigens</td>
<td>Complement, FcR⁺ cells</td>
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| Complement, FcR⁺ cells (phagocytes, NK cells) | Cell-surface receptor | Complement, phagocytes | Macrophage activation | CTL |
| Soluble antigen | Soluble antigen | Soluble antigen | Cell-associated antigen | Cytotoxicity |
IgE-mediated hypersensitivity is the most important type of hypersensitivity associated with food allergies and involves the formation of IgE antibodies that specifically recognize certain allergenic proteins in foods, while cell-mediated hypersensitivity requires an interaction between a particular food antigen and sensitized T lymphocytes (Taylor, 2006a).

**Cell-Mediated Hypersensitivity**

Non-IgE mediated immune responses to food components are generally thought to fall in the Type IV category or T-cell mediated reactions. These reactions are often referred as delayed hypersensitivity reactions with onset of symptoms generally occurring within 6 to 24 or more hours after ingestion of the offending food. Common types of cell-mediated hypersensitivity reactions include celiac disease, protein-induced enterocolitis, protein-induced enteropathy, food-protein induced proctitis, allergic eosinophilic gastroenteritis and esophagitis (Taylor and Hefle, 2006a). The immune responses attributed to this hypersensitivity are far less understood compared to IgE-mediated responses. Cell-mediated reactions and/or antigen-antibody complex may play a role in some of the diseases (Sampson 1991; Taylor and Hefle, 2006a). The primary example of a delayed hypersensitivity is celiac disease, also known as celiac sprue or gluten-sensitive enteropathy, a malabsorption syndrome triggered by a permanent intolerance to gluten in genetically predisposed individuals (Niewinski, 2008; Taylor and Hefle, 2002).

The prevalence of celiac disease according to the epidemiological studies is higher in women than men with 2.5:1 ratio. In the U.S. and Europe, the prevalence is estimated to be in range between 1:100 and 1:150 individuals. Moreover, the frequency
of the disease is higher (4-12%) in first degree relatives of patients with the celiac (Fasano et al., 2001; Gasbarrini et al., 2008).

The clinical presentation of celiac disease is characterized by localized inflammatory reactions in the intestinal tract causing infiltration of lymphocytes in the epithelium and the lamina propria, impaired absorptive function of the epithelium together with increased fluid secretion and enhanced permeability and increased production of IgM, IgG, and IgA antibodies causing crypt hyperplasia and villous atrophy (Niewinski, 2008; Taylor and Hefle, 2002; Gasbarrini et al., 2008).

Symptoms of celiac disease have a tendency to differ by age group and are typical of non-IgE mediated hypersensitivities described by chronic diarrhea, abdominal pain, malnutrition, vomiting, weight loss, failure to thrive, chronic constipation, and dermatologic or respiratory symptoms. Also, celiac disease is associated with various types of cancer, autoimmune disorders, IgA deficiency, or Down’s syndrome (Leffler et al., 2003; Green et al., 2007).

The inflammatory response is caused by wheat gluten and similar proteins of barley and rye that are resistant to endoluminal proteolytic digestion, due to a high amount of proline and glutamine in their amino acid structure. These Pro and Gln rich peptides can pass through the mucosal layer of the intestinal epithelial cells either by moving between tight junctions or by direct absorption, where they get deamidated by tissue transglutaminase 2, an enzyme secreted by the intestinal cells. Once inside the lamina propria, the deamidated gluten peptides can come into contact with antigen presenting cells (Schuppan et al., 2009; Niewinski, 2008; Green et al., 2007).
Genetically susceptible individuals usually express a subtype of the human leukocyte antigen (HLA) Class II molecules on their antigen presenting cells (APC), with more than 90% of patients expressing the HLA-DQ2 subtype while the HLA-DQ8 subtype is present in most of the remaining patients. The receptors formed by the DQ2 or DQ8 genes bind to deamidated gluten peptides more tightly than other forms of the antigen-presenting receptors. Consequently, these forms of the receptor are more likely to activate CD4+ T lymphocytes that produce proinflammatory cytokines and induce autoimmune responses, which are characterized by an increased density of activated T cells and plasma cells in the lamina propria, and an increase in IgA production. The IgA gets secreted into the gut lumen as secretory IgA or passes into the bloodstream. Plasma cells will also secrete antibodies reactive with transglutaminase and deamidated gluten (Schuppan et al., 2009; Niewinski, 2008; Green et al., 2007).

Even though genes encoding for HLA-DQ2 and DQ8 proteins are the single most important factor for development of the celiac disease, there are other environmental factors that have vital roles in triggering clinical symptoms of the disease since close to 40% of general population also carry these genes but do not experience any adverse response to gluten proteins. Among suggested triggers are the presence of particular gastrointestinal infections, the effect of breast-feeding, and the timing of the introduction of gluten into a child’s diet (Niewinski, 2008; Green et al., 2007). The exact trigger(s) that cause individuals to clinically react to gluten proteins while others with the same genetic predisposition for celiac disease do not experience adverse reactions upon consumption of gluten proteins remains unknown.
The gold standard for diagnosis of celiac disease requires both small bowel biopsy and a positive resolution of symptoms once the patient adheres to a gluten-free diet for an extended period (Green et al., 2007). Additionally, there are several non-invasive serologic tests also available for the diagnosis (Leffler et al., 2003).

Currently, there are no treatments for celiac disease and patients have to commit to a strict gluten-free diet accompanied with dietary supplementation and other immunosuppressant therapies (Leffler et al., 2003). However, significant progress has been made in the development of potential therapies for celiac disease with the most promising outcomes involving oral enzyme therapy that can degrade gluten into non-hazardous components (Leffler et al., 2003; Sollid and Khosla, 2005; Schuppan et al., 2009).

**IgE-Mediated Hypersensitivity and Food Allergy**

The primary biological role of IgE antibodies is in protective immunity, particularly in response to parasitic infection. However, in atopic individuals non-parasitic, innocuous, antigens are capable of stimulating inappropriate IgE responses triggering Type I hypersensitivity reactions (Goldsby, 2003). A true food allergy is an antibody (IgE) mediated, Type I immediate hypersensitivity reaction. It is defined as an adverse immunological reaction to an offending naturally occurring food component, most often a protein. IgE mediated symptoms develop shortly after the exposure to the offending food. They can range from mild to severe, and may include skin rash, hives, itching, swelling of the lips, throat, and other body parts, as well as, nasal congestion, trouble breathing, diarrhea, vomiting, dizziness, and fainting. A severe reaction to a food can also lead to anaphylactic shock (FDA, 2009). Multiple organ systems involved in
anaphylactic shock include respiratory, cardiovascular, gastrointestinal, and cutaneous systems, where complications in these systems combined with hypotension can result in death (Taylor and Hefle, 2006a).

**Mechanism and Pathogenicity of Food Allergy**

There are two fundamental phases involved in developing a food allergy and subsequently experiencing an allergic reaction, which include the sensitization phase and the elicitation phase (Figure 1. 3). The sensitization phase is symptomless and marks the first exposure where an allergen stimulates the production of IgE antibodies that attach to mast cells and basophils, thereby priming the immune system (Taylor and Hefle, 2006a). Moreover, the sensitization phase consists of a series of steps including antigen absorption, antigen processing and presentation, T cell and B cell activation, development of oral tolerance or allergic sensitivity, and synthesis of antigen specific IgE antibodies (Fraser et al., 2001). Although sensitization can occur upon the first encounter with the allergen, this does not always hold true as late onset of food allergies can occur in some individuals that have tolerated certain allergenic proteins in the past (Taylor and Hefle, 2001). An actual allergic reaction occurs upon subsequent exposure to the allergen where the allergen cross-links two IgE antibodies on the surface of mast cells or basophils causing degranulation and release of histamine and other inflammatory mediators (Fraser et al., 2001).
Figure 1.3 Mechanism of IgE-mediated food allergy (Adapted from Taylor and Hefle, 2002).

Antigen/allergen absorption occurs primarily in the small intestines of the gastrointestinal tract, which is also considered to be the largest immunologic organ in the human body (Chahine and Bahna, 2010). Mucosal tissue in the gastrointestinal tract has both immunological and non-immunological components that interact with antigens and preclude their intact absorption (Fraser et al., 2001). Increased intestinal permeability due to the dysfunctional epithelial barrier has been known to lead to inflammatory disorders of the human intestine, including food allergy (Helm and Burks, 2000; Chahine and Bahna, 2010). While lined by a single layer of epithelium, the gut-associated lymphoid tissue (GALT) is exposed continuously to enormous amounts of different antigens from the external environment (Chahine and Bahna, 2010; Chehade and Mayer, 2005). In addition, based on the specific antigen and gut maturity, there are several pathways that allow the uptake of macromolecules. They include enterocyte transcellular absorption, Microfold (M) cell uptake, or paracellular uptake (Fraser et al., 2001).
Moreover, intact antigens are still capable of passing through the mucosal epithelium, in spite the existing gut barriers. Antigen processing and presentation takes place in GALT, which is comprised of the appendix, Peyer’s patches, lymphocytes and plasma cells, and interepithelial lymphocytes (IELs). Before lymphocyte activation, antigens are processed by specialized antigen presenting cells (APCs) and presented on their surface in conjunction with HLA Class II molecules that are recognized by specific receptors on T cells. APCs are located above Peyer’s patches, and they include dendritic cells, macrophages and enterocytes. Antigen presentation generally activates IgA B cell secreting precursors, while suppressing IgM, IgG, and IgE (Fraser et al., 2001).

T cell and B cell activation involves two steps, resulting in cell proliferation and cytokine production. The initial step involves the recognition of the HLA:antigen complex on APC by the T cell receptor (TCR) located on the surface of the T cell followed by binding of CD28 on the T cell surface to the B7 ligand on the APC surface. Moreover, the T cell expresses CD3 molecules that allow transduction of signals as a result of TCR binding to APC (Fraser et al., 2001).

In physiologically normal individuals, oral tolerance is developed upon exposure to a food protein in the gastrointestinal tract either through synthesis of protein-specific IgG, IgM, or IgA antibodies or without any immunological response at all (Taylor and Hefle, 2006a). It has been hypothesized that a consequence in failure to develop oral tolerance or the breakdown in existing tolerance leads to food hypersensitivity (Chehade and Mayer, 2005; Chahine and Bahna, 2010). The breakdown in the oral tolerance mechanism promotes excessive generation of IgE antibodies (Sampson 1991). Therefore, exposure to antigen can lead to development of oral tolerance or allergic sensitivity
where oral tolerance is induced and sustained by CD4+ T cells (Fraser et al., 2001). Oral tolerance denotes a state of active inhibition of immune responses to an antigen by means of prior exposure to that antigen by the oral route (Chase, 1946). Oral tolerance may be overcome resulting in allergic sensitization during ingestion of proteins, or may be circumvented completely by presentation of proteins by different routes, such as via respiratory tract or skin (Sicherer and Sampson, 2009).

Three basic mechanisms for induction of oral tolerance are clonal anergy, active suppression, and clonal deletion. Clonal anergy is a state of unresponsiveness, where cells fail to proliferate or produce proinflammatory IL-2. Active suppression is a state induced by the inhibitory role of Treg cells that secrete inhibitory factors such as TGF-β and IL-10. TGF-β is efficient in suppressing cell-mediated immune responses and promotes immunoglobulin class switching to IgA, which have non-inflammatory effects (Chehade and Mayer, 2005; van Wijk and Knippels, 2007). Clonal deletion involves induced cell death through apoptosis (Fraser et al., 2001). A few non-host factors such as physical properties of the antigen and the dose and frequency of exposure can affect the development of oral tolerance (Sicherer and Sampson, 2010). The antigen dose is an important factor in determining which state of the oral tolerance is activated, low doses of antigen favor active suppression, whereas high doses of antigen are believed to induce clonal anergy and deletion (Fraser et al., 2001; Chehade and Mayer, 2005; Faria and Weiner, 2005; van Wijk and Knippels, 2007).

IgE plays a pivotal role in the pathogenesis of allergic diseases. Its synthesis begins with the interaction of B cells and T cells, and is regulated by surface molecules on activated T cells (Prussin and Metcalfe, 2006). B cells differentiate into IgE secreting
plasma cells where their synthesis is directed by IL-4 and IL-13 cytokines. Other important cell interaction molecules for the antibody response are CD40 on B cells and CD40L on CD+4 T cells as well as TNF-α and CD2 on T cells. Th2 cells are involved in stimulating IgE antibody production and induction of allergic reactions. Th2 cells secrete various cytokines including IL-4, IL-5, IL-6, IL-10, and IL-13 that promote IgE mediated allergic inflammation and promote sensitization by food antigens and the induction of food allergic reactions (Fraser et al., 2001; Groschwitz and Hogan, 2009).

Allergic reactions occur upon subsequent antigen exposure to the antigen specific IgE which is bound to the high-affinity receptors (FceRI) on the surface of mast cells and basophils at the constant domain (Fc epsilon) of IgE (Presta et al., 1994). Crosslinking of adjacent FceRI Receptors occurs when their associated IgE antibodies bind to epitopes on a single antigen. Normally that requires at least two specific epitopes on the antigen, or co-valent linking of antigens (e.g. through disulfide bonds). Crosslinking leads to the release of preformed mediators such as histamine, proteases, heparin, proteoglycan, eosinophil chemotactic factors, neutrophil chemotactic factors, leukotrienes, prostaglandins, and platelet-activating factors that increase vascular permeability, bronchial smooth-muscle contraction, along with an increase in mucus production, and increase in chemotaxis of eosinophils and neutrophils (Fraser et al., 2001).

Currently, the available information concerning the mechanisms for development of oral tolerance or allergic sensitization is still incomplete. Several risk factors have been identified that may contribute to IgE-mediated food hypersensitivity. These include the genetic predisposition of the host, the dose of the antigen, the nature of the antigen,
inadequate intestinal microflora of the host, gastrointestinal infections, immature mucosal
immune system and increased permeability, IgA deficiency and other immunologic
defects, formula feeding, and the age of the host (Chehade and Mayer, 2005; Chahine and
Bahna, 2010). It is important to note that many of these risk factors are associated with
infants and young children that naturally have an immature gastrointestinal epithelium,
immune system and aberrant gut flora. Neonates have a sterile gastrointestinal tract and
do not begin to acquire their gut microbiota until birth (during birth for vaginal
deliveries). It has been hypothesized that the lack of early exposure to microorganisms in
infancy may be one of the factors influencing increased allergic responses in westernized
populations. Several theories exist ranging from the belief that microorganisms could
either be lacking during infancy, perhaps the presence of certain microorganisms in
excess could be an issue, or an overall diversity in the microbiota could be responsible for
the failure in development of tolerance (Wang et al., 2008). Lack of lactic acid
fermenting bacteria (bifidobacteria and lactobacilli) in non-breast fed infants has been
associated with higher rates of atopic sensitization (Bjorksten, 2005). Additionally,
murine studies have shown that absence of constant stimuli by toll-like receptor 4 (TLR-4) in germ-free mice leads to a high susceptibility to food allergy (Bashir et al., 2004).
Moreover, the intestinal epithelial barrier is regulated by a complex of protein-protein
networks including desmosomes, adherens junctions (AJs), and tight junctions (TJs).
Disruption of the TJs leads to dysfunction of the intestinal epithelial barrier and increased
intestinal permeability that has been associated with several intestinal diseases, including
food allergies, celiac disease, and type I diabetes (Groeschitz and Hogan, 2009).
Probiotics have been shown to be effective in restoration of TJs function, enhanced
mucus production, inducing the polarization of Th1 cells responses, and suppression of inflammatory responses (Strobel and Mowat, 2006). The gut microbiota, the immature gut epithelium, and dysfunction of TJ s seem to be especially important factors that influence the possible exposure to intact food allergens and could lead to sensitization of infants.

**PREVALENCE OF IgE-MEDIATED FOOD ALLERGY**

Age, geographic location, exposure to the offending allergen, and possibly race/ethnicity in addition to many other factors influence the varying prevalence of food allergies throughout the world (Sicherer, 2011). The prevalence of IgE-mediated allergic reactions in the overall United States population is estimated to be around 4%, while 6-8% of children under the age of 3 years are affected by food allergies (Sampson, 2005). The perceived prevalence of food allergies by general population is much higher (1 in 5 Americans believe they have a food allergy) due to the self-diagnosis, parental diagnosis, and the lack of differentiation between IgE-mediated food allergy and other food sensitivities (Sampson, 2004; Taylor et al, 1999). Comparable overestimation of food allergy has also been reported throughout the world (Altman and Chiaramonte, 1996).

Even though any food has a potential to elicit an IgE-mediated reaction, milk, eggs, and peanuts are considered as the most frequent allergenic foods groups among U.S. children, while in adults crustacean shellfish, peanuts, and tree nuts are more prevalent causative agents of adverse reactions (Sampson, 2004). Additionally, many children tend to outgrow their food allergies and develop tolerance especially to milk, eggs, wheat or soy by their teenage years (Sicherer and Sampson, 2010). However, allergies to peanuts, tree nuts, fish or shellfish are more persistent and rarely outgrown (Samartin et al., 2001). In
addition to the “Big 8” group, over 160 other foods have been recognized to provoke allergic reactions (Hefle et. al., 1996). Peanuts and tree nuts are responsible for the majority of fatal allergic reactions in the US, whose incidence of occurrence also seems to be on the rise (Sicherer, 2010). Peanut and tree nut allergy affects approximately 1.15% of the total United States population or more than 3 million people (Sicherer, 2010). Allergy to peanuts and tree nuts are especially burdensome as only an estimated 20 and 10% of peanut and tree nut allergic individuals, respectively, outgrow these allergies by adulthood resulting in life-long afflictions (Kulis et al., 2009). Table 1.1 illustrates prevalence estimates of specific food allergy in North America. Moreover, allergic reactions to specific foods may vary among population groups based on the cultural and dietary habits, along with food preparation methods (Taylor et al., 1999).

While allergy to cow’s milk and eggs is common throughout the world, hazelnut allergy for example, is rather prevalent in primarily European countries, and soybeans and seafood are more common allergenic foods in Japan (Taylor, 2002).

<table>
<thead>
<tr>
<th>Prevalence</th>
<th>Infant/child</th>
<th>Adult</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk</td>
<td>2.5%</td>
<td>0.3%</td>
</tr>
<tr>
<td>Egg</td>
<td>1.5%</td>
<td>0.2%</td>
</tr>
<tr>
<td>Peanut</td>
<td>1%</td>
<td>0.6%</td>
</tr>
<tr>
<td>Tree nuts</td>
<td>0.5%</td>
<td>0.6%</td>
</tr>
<tr>
<td>Fish</td>
<td>0.1%</td>
<td>0.4%</td>
</tr>
<tr>
<td>Shellfish</td>
<td>0.1%</td>
<td>2%</td>
</tr>
<tr>
<td>Wheat, soy</td>
<td>0.4%</td>
<td>0.3%</td>
</tr>
<tr>
<td>Sesame</td>
<td>0.1%</td>
<td>0.1%</td>
</tr>
<tr>
<td>Overall</td>
<td>5%</td>
<td>3% to 4%</td>
</tr>
</tbody>
</table>

**Table 1.1** Estimated food allergy rates in North America (Adapted from Sicherer and Sampson, 2010).
There are multiple theories as to why the prevalence of food allergy may be increasing: the hygiene hypothesis, changes in dietary components, use of antacids, food processing, and delay of oral exposure to a particular food (Sicherer and Sampson, 2010). The originally proposed hygiene hypothesis indicates that the westernized countries have reduced the incidence of infections in early life, and that these infections could possibly have a protective effect on the development of allergies. The growing availability of products and practices that promote a sterile environment, especially for children, could have decreased our exposure to the numerous microbes influencing the maturation of immune responses (Martinez, 2001). For example, a naturally occurring exposure to bacterial endotoxin in childhood has been associated with a decreased incidence of allergy and asthma (Liu and Murphy, 2003). Thus, limited immune responses to infections could lead to a state where perhaps the immune system is looking at food proteins that are otherwise harmless as harmful foreign proteins. The hygiene hypothesis suggests that exposure to pathogens may prevent the onset of allergies, possibly by skewing the immune system towards a Th1 rather than Th2 response. Differences in fat, nutrient/vitamin D, and antioxidant intake have also been proposed as a mechanism for increasing allergy prevalence (Lack, 2008). Generally, proteins in food are the causative antigens in food allergies. Strong antacids may reduce proteolysis and result in an increased exposure of the immune system to intact or immunologically reactive food proteins (Pali-Scholl et al., 2010). The way that food is processed can also affect whether proteins become denatured and, in some instances, food emulsions may act like an adjuvant of a vaccine and perhaps up-regulate the immune cells associated with the gut (Sicherer and Sampson, 2010). Moreover, many of the clinical pediatric associations in
westernized countries recommended delayed introduction of major allergenic foods up to the first three years of the life in infants who are at high risk of developing allergies. More recent studies suggest that exposure to high doses of food allergens early in infancy may drive oral tolerance rather than sensitization (Du Toit et al., 2008). As a result of this emerging research and a lack of evidence to suggest that the delayed introduction of allergenic foods prevents the onset of food allergies, the universal recommendation for restricting the introduction of food allergens has been revised and is only recommended on a case-by-case basis if needed. Additional research is needed to determine if either early high dose exposure or avoidance early in life helps to prevent the development of food allergies. In the healthy gastrointestinal tract, the dendritic cells located between the Peyer’s Patches and M cells seem to elicit a more tolerogenic response when presented with antigens by M cells. So, when the infant gastrointestinal tract and immune system have matured to some degree, the introduction of foods may help prevent allergy compared to having the allergen first encountered topically, which is considered a potential route of sensitization as well (Sicherer and Sampson, 2010). Further research is needed to gain more insight on the prevalence of the specific types of food allergy in the general population and the populations of various countries, regions, and groups.

**DIAGNOSIS AND TREATMENT OF FOOD ALLERGIES**

An accurate diagnosis of food allergies is very important for food-allergic individuals in order to receive proper treatment, as dietary management cannot be effective until a firm diagnosis is obtained (Taylor et al, 1999). The first step in diagnosing a food allergy usually involves a detailed review of the patient’s medical history by an allergist, along with a physical examination of a patient to determine causal
foods and to rule out other possible diseases and conditions (Taylor and Hefle, 2006a; Hamilton, 2010). Once a specific food is identified as a probable cause of the allergic reaction, *in vitro* and *in vivo* laboratory tests are usually performed to confirm the diagnosis. There are several *in vivo* and *in vitro* tests used in diagnosing food allergy, however, no single test is preferred over another due to differences in the sensitivity, specificity, and ease of use in a clinical setting (Chafen et al., 2010). More importantly, a patient’s clinical reactivity cannot be consistently predicted by using any of the tests available, since they only detect sensitization and the presence of specific IgE antibodies (Asero et al., 2007). Additionally, given the variety of foods and possible symptoms involved, the diagnosis of food allergy becomes a challenging task.

The skin-prick test (SPT) is the most frequently used test for *in vivo* detection of IgE antibodies to food allergens, and is the simplest procedure to perform (Taylor and Hefle, 2006a). During a skin prick test, a small amount of food extract is applied to the skin and the site is assessed for wheal formation (Taylor and Hefle, 2006a). IgE is measured for its ability to induce the release of inflammatory cell mediators from mast cells. Generally, SPT have a great sensitivity and negative predictive accuracy (>90%), but poor specificity and positive predictive accuracy (50-85%) (Asero et al., 2007). Therefore, negative SPT results generally confirm the absence of IgE mediated allergic reactivity, while positive tests do not prove clinical reactivity (Sicherer and Sampson, 2010).

*In vitro* diagnostic tests for allergen-specific IgE detection include the traditional radioallergosorbent test (RAST), the ImmunoCAP® test (Thermo Fisher Scientific), and the enzyme-linked immunosorbent assay (ELISA) (Samartin et al, 2001; Ebo and
Stevens, 2001). The RAST method uses a sample of patient’s blood serum where serum is allowed to react with allergen bound to a solid matrix, and the degree of binding of allergen-specific IgE in the serum to the solid-phase allergen is measured with radiolabeled antihuman IgE (Taylor and Hefle, 2006a). These tests are viewed as less sensitive compared to in vivo skin tests, and can give both false negative and positive results (Samartin et al, 2001; Ebo and Stevens, 2001). However, because of the safety reasons in patients with extreme sensitivity these tests may be preferred methods (Taylor and Hefle, 2006a). RAST for the most part have been replaced by the ImmunoCAP® tests (or similar methods) for routine diagnostic analysis of blood sera for specific IgE. The ImmunoCAP® method is set up much like the RAST test; however, antihuman IgE antibodies labeled with a fluorescent enzyme are utilized in place of radiolabeled antihuman IgE.

Oral food challenge tests give strong evidence of a food allergy, and can be conducted openly, single-blind, or double-blind with blinded challenges controlled by placebo (Asero et al., 2007). The most reliable and consistent diagnostic procedure, considered the gold standard for diagnosis of food allergy, is the double-blind, placebo-controlled food challenge (DBPCFC). The DBPCFC indisputably associates ingestion of a specific food to elicitation of a certain set of allergic symptoms (Taylor and Hefle, 2006a). DBPCFC should only be performed in a clinical setting that has access to emergency care in the event that a severe allergic reaction should occur. For this reason, it is recommended that the DBPCFC should not be used in situations where there is a high possibility of life-threatening anaphylaxis to a suspected food even though it is recognized as the most reliable method in determining the casual role of specific foods in
adverse reactions (Taylor et al., 1992; Daul et al., 1994). Exquisitely sensitive individuals can undergo a DBPCFC if the starting dose of the challenge is in the low mg of the food rather than 200-500 mg of the allergenic food. In the U.S., many diagnostic food challenges have started with these high doses resulting in an estimated 20-25% of allergic individuals reacting with moderate to severe allergic reactions (Sicherer and Sampson, 2000).

The basic approach for treating any type of true food allergy is practicing a specific avoidance diet. Food(s) that can provoke the adverse reactions in affected individuals must be avoided. The tolerance or threshold for the offending food can be particularly low in the case of IgE-mediated allergic reactions (Taylor et al., 1992). Several cases have been documented where exposure to very small amounts of the offending foods provoked adverse reactions in allergic individuals. Taylor et al. (2002) indicated that doses in the range of 1.25 to 2.5 mg protein are capable in causing symptoms in peanut allergic individuals. Mild reactions are also noted in adults at 0.1 mg peanut protein using partially defatted, roasted peanut meal (Wensing et al., 2002). However, Sicherer and Sampson (2007) imply that for many individuals objective symptoms occur from ingestion of about 1 to 3 peanut kernels. Therefore, the most successful treatments known to date include identifying and avoiding the offending food(s) (IFT Expert Panel, 1985). In addition to being nutritionally and socially limiting, avoidance diets can be quite challenging as undeclared allergens can on occasion be found in packaged food products and cross-contact of allergens commonly occurs in food service (restaurant) settings so avoidance diets do not completely prevent the risk of an adverse reaction occurring (Sicherer and Sampson, 2009). Therefore, assistance from an
An expert allergist and dietician is recommended when developing a proper avoidance diet to ensure that an adequate diet can be maintained while ensuring that the offending food(s) are avoided. Several factors need to be carefully considered including the degree of tolerance (if known) or exquisite sensitivity, the selectivity of safe foods that will maintain a balanced diet, the possibility of cross-reacting foods, and the adequacy of labeling (IFT Expert Panel, 1985). It is essential that affected individuals diligently read food labels, take care in getting foods from restaurants, and avoid cross-contact of foods containing an allergen during meal preparation (Sicherer and Sampson, 2010). In addition to the specific avoidance diet, there are other approaches that can help manage food allergies since accidental exposures to the offending food still occur, including overall elimination diet, pharmacologic treatments, and prophylactic treatments (Taylor, 1985).

Pharmacologic methods are useful in some cases in management of allergic symptoms, but typically offer only brief, symptomatic relief (IFT Expert Panel, 1985). Antihistamines may alleviate mild to moderate symptoms such as mild itching and inflammation associated with oral allergy syndrome (OAS) and IgE-mediated skin disorders (Sicherer and Sampson, 2010). Antihistamines block histamine receptors in the tissue and preclude a number of the symptoms associated with allergic reactions (Taylor and Hefle, 2006a). Since antihistamines only block the cell receptors of one of the many inflammatory mediators that trigger the symptoms associated with allergic reactions, they are often not effective alone in resolving moderate to severe allergic reactions. Individuals suffering from severe reactions are advised to carry an epinephrine (adrenaline) kit for immediate self-treatment followed by seeking medical treatment at a
hospital on those occasional instances when reactions occur in spite of efforts to avoid the adverse foods.

The food industry must meet the needs of these consumers by providing accurate and complete ingredient lists on packaged food products. It is also necessary for those in the foodservice industry to be knowledgeable about ingredients in the foods they serve and avoid cross-contact of allergens in the food service kitchen. Ultimately, further research is required in product development to produce more specific products for individuals with different types of food allergy (IFT Expert Panel, 1985). Currently, few hypoallergenic foods exist for use by food allergic individuals. This is especially important for allergic populations such as infants that may not have many foods that can be substituted in their diet to maintain adequate nutrition. Several substitute formulas are available for infants with cow’s milk allergy, where protein hydrolysates (that have undergone extensive acid hydrolysis) are present in most of these formulas that are on the market (Samaritin et al, 2001). For example, soybean-based formula is a viable substitute for milk-allergic infants and is generally well tolerated in many cases. Casein hydrolysate formula (hypoallergenic infant formula) has also been shown to be safe for the vast majority of milk-allergic infants and can be used effectively in cases where an infant is sensitive to both milk and soy (Taylor and Hefle, 2006a). Rare acute reactions to various hypoallergenic formulas have been documented, in which case newborns are nurtured with elemental formula, for example amino acid based formula (Samaratin et al., 2001).

The prophylactic treatment of food allergies is a somewhat debatable subject (Taylor, 1985). Breastfeeding and delayed introduction of solids studies have shown
some correlation to delay the onset of atopic symptoms, but have not been consistently shown as effective in preventing the development of food allergy so the conclusions should be reasoned with caution (Chafen et al., 2010). Conflicting findings in various studies have also been observed when examining the effectiveness of a maternal allergen avoidance diet during pregnancy, lactation, or both. Kramer and Kakuma (2006) conveyed no evidence to encourage a protective effect of maternal diet while studies by Hattevig et al. (1989) and Sigurs et al. (1992) found considerably reduced occurrence of atopic dermatitis in children whose mothers had diets free from cow’s milk, hen’s egg, and fish during lactation. There is also inconsistent evidence on the prevention of atopic disease by exclusive breastfeeding; however, it is recommended when possible to breastfeed for the first few months after birth since breast milk has been shown to provide the infant with best balance of nutrition (Chafen et al., 2010). Hydrolyzed formulas may reduce cow’s milk allergy in infants that may be at high risk of developing allergies compared with cow’s milk formula (Chafen et al., 2010). Finally, the independent role of probiotics is still uncertain, but it has been suggested that introduction of beneficial bacteria could prevent food allergy in combination with breastfeeding, hypoallergenic formula, or both (Chafen et al., 2010).

There is a lot of research interest in the prevention of food allergies. Areas of active research, as already mentioned, involve breastfeeding and delayed introduction to solids, exclusive breastfeeding for the first year, maternal diet during pregnancy and breastfeeding, special diets for infants and young children, and probiotics for mothers during breastfeeding. This is a relatively new area of research and none of these prevention strategies show strong or conclusive results yet.
Clinically proven treatment of food allergies currently does not exist, however various approaches are being considered including anti-IgE injections, Chinese herbal remedies, probiotics, the use of mutated epitopes to retrain the immune system, and oral immunotherapy (Davis et al., 2008). Even though subcutaneous immunotherapy with birch pollen has been shown to improve allergies associated with birch pollen-associated food allergies and oral allergy syndrome, subcutaneous immunotherapy cannot be used widely for tolerance induction of all food allergies as unacceptably high rates of systemic allergic reactions using peanut extract have been documented (Fleischer, 2007; Sicherer and Sampson, 2009). Therefore, current research efforts are targeted toward changing the route of administration of allergens, or modifying the epitopes of the major allergenic proteins, as well as developing non antigen-specific immunomodulatory therapies (Sicherer and Sampson, 2009). At present, the method undergoing the most clinical research is oral immunotherapy, in which doses of the allergenic food protein are given in gradually increasing amounts over a period of weeks to months in an effort to reach a maintenance dose that will help drive immune desensitization or tolerance to the particular allergenic food. In the effort treat or perhaps cure the food allergy, the maintenance dose of food allergen is then administered daily for months to years. Based on the results from preliminary studies examining the efficacy of oral immunotherapy with peanut, egg or milk, the data indicates that oral immunotherapy could be a beneficial therapy for a number of affected individuals (Sicherer and Sampson, 2010). One of the key questions that remain unresolved at this point is whether oral immunotherapy will help the individual eventually to develop oral tolerance to the offending allergenic food or only desensitization can be achieved. The desensitization is only temporary and
requires consistant, maybe daily dosing while tolerance, which is a lack of reactivity likely, takes years to achieve. The desentization results in a higher threshold for the allergenic food that may allow allergic individuals some increased level of safety but does not provide complete oral tolerance. The other major question that remains to be answered is whether or not desensitization or oral tolerance will continue if the maintenance dose is missed for a period of time or if discontinued all together.

**TREE NUT ALLERGY**

Many individuals consume tree nuts in their diets because of their pleasing taste and potential health benefits. Among the most popular tree nuts are almond, Brazil nut, cashew, hazelnut, macadamia nut, pecan, pine nut, pistachio, and walnut. Approximately one-third of Americans consume nuts (tree nuts or peanuts) on daily basis. Seven percent of the European population eats nuts; nonetheless the amount eaten by Europeans (31 g/d) is greater than that of Americans (21 g/d) (King et al., 2008). Based on the association of tree nut consumption with reduced risk of certain diseases, the U.S. Food and Drug Administration (FDA) in 2003 issued a health claim stating “Scientific evidence suggests but does not prove that eating 1.5 ounces per day of most nuts as part of a diet low in saturated fat and cholesterol may reduce the risk of heart disease” (FDA, 2003). Consumption of tree nuts has been on a rise, where per capita consumption has reached 3.5 lb annually (USDA, 2010). Almond (25%), pecan (22%) and walnut (17%) account for approximately 64% of the total tree nut consumption in the US (FAO, 2009; USDA, 2000). The United States ranks first in global tree nut production and is the largest producer of almonds and pecans, accounting for more than 80% of pecan’s world
production. The US is also the main exporter of walnuts, with China being the leading producer of the walnuts (AMRC, 2010, FAO, 2009).

Typically, many of the tree nuts are eaten raw or roasted as snack foods. Moreover, they are also being used in various food products as ingredients, including toppings in salads, cereals dessert toppings, confectionery (e.g. ice cream and candy), and a variety of baked goods. While tree nuts are high in calories (271kcal/1.5oz (42g)), they are also a good source of beneficial mono- and polyunsaturated fatty acids (King et al., 2008). The major monounsaturated fatty acid in tree nuts is oleic acid (C18: 1). However, macadamia nuts also contain a significantly higher amount of plamitoleic acid (C16: 1) compared to the rest of the tree nuts. The most common polyunsaturated fatty acid in tree nuts is linoleic acid (C18: 2). In addition to linoleic acid (59.79%), a substantial amount of the total content of polyunsaturated lipids in walnut is linolenic acid (C18: 3) (Venkatachalam and Sathe, 2006). Tree nuts also provide a good source of protein, fiber, magnesium, vitamin E, and antioxidants (King et al., 2008, Lin et al., 2001). Walnuts, pecans and chestnuts are among the tree nuts that have the highest content of antioxidants. Walnuts have a range of polyphenols and tocopherols (King et al., 2008). In a review of the clinical literature conducted by Blomhoff et al. (2006), they suggest that the high nutritional value of tree nuts in conjunction with the complex and rich combination of the fatty acids and antioxidants might have a potential role in reducing the risk of heart diseases by lowering cholesterol levels.

Coates and Howe (2007) have indicated several beneficial effects of tree nuts on metabolic health. It has been suggested that inclusion of nuts in a diet helps in prevention and management of cardiovascular diseases by maintaining the function of endothelial
cells (the interior lining of blood vessels) responsible for the control of blood pressure. Moreover, the improvements in regulation of glucose and insulin levels have been seen in patients with type II diabetes. Incorporation of nuts in diet regimens also has a positive effect on weight management and reduction of low-density lipoprotein (LDL) cholesterol. Even though they are safely consumed by a vast majority of the population, tree nuts can also elicit allergic reactions in allergic individuals. An allergic reaction to tree nuts and peanuts is an IgE-mediated, immediate hypersensitivity reaction. The severity of the allergic reactions can occasionally be life threatening. Also, tree nuts and peanuts are present to a great extent in various foods making avoidance diets very difficult, and reactions due to cross-contamination and accidental ingestion can be common, especially in food service settings (Sicherer and Sampson, 2000). The study conducted by Le et al. (2008) indicates that severe symptoms were more frequently seen in patients with a tree nut and peanut allergy than in patients with a fruit allergy, where the impact of these allergies on daily life is high; it does not differ from fruit allergic patients. There are no clear reasons to explain this observation, but it may suggest that many patients with fruit allergy show clinical symptoms to more than one fruit, and the quality of life is correlated to the number of food allergies. Furthermore, nutritional deficiency of vitamin C, minerals, and dietary fibers could possibly lead to impaired health by eliminating fruits from the diets (Le et al., 2008).

In the United States fatal anaphylactic reactions to foods are predominantly caused by either peanuts or tree nuts (Fleischer, 2007). Tree nut allergies, similar to peanut allergies, are rarely outgrown so they become lifelong afflictions that must be carefully managed through proper avoidance diets (Roux et al., 2003). More than 3
million people in the U.S. population are estimated to be allergic to peanut and tree nuts, which typically begin in childhood (Roux et al., 2003; Sicherer et al., 2010). Fleischer (2007) reported that the median age for onset of tree nut allergy in one study was 36 months, and in another study the onset was 62 months, compared to an onset time of 14 to 24 months for peanut allergy. This may be due to the differences in the timing of the initial exposure to these allergens as peanut is typically introduced earlier in the child’s diet than tree nuts. Around 9% of affected individuals tend to outgrow tree nut allergies, including those who had experienced severe reactions. These results indicate that the resolution of tree nut allergy is less than in peanut where an estimated 20% of peanut-allergic children are believed to outgrow their peanut allergy (Skolnick et al., 2001), however; over time it is still possible for a subset of tree nut allergic children to outgrow their allergy. Approximately, 68% of patients involved in the Fleischer’s study were also allergic to peanut, and patients allergic to multiple tree nuts were less likely to outgrow their allergy (Fleischer et al., 2005).

The most common tree nuts capable of inducing an allergic reaction include walnut, cashew, almond, pecan, Brazil nut, hazelnut, macadamia nut, pine nut, and pistachio. In addition, IgE-mediated allergy to walnut is thought to be the most common among tree nuts, followed by cashew, almond, pecan, and pistachio (Fleischer, 2007). Fleischer (2007) found that walnut was the most prevalent tree nut allergen, affecting 34% of respondents, followed by cashew (20%), almond (15%), pecan (9%), pistachio (7%), and the other nuts (<5% each).

The prevalence of tree nut and peanut allergies is believed to be increasing among children in the U.S. The documented rates obtained using a random digital dial telephone
survey for self-reported peanut and tree nut allergies conducted in the 3 years 1997, 2002, and 2008 present no statistically significant differences in prevalence among adults. A statistically significant increase in prevalence rates was observed in children with peanut allergy (increase from 1.2% to 2.1%) and tree nut allergies (from 0.5% to 1.1%) from 2002 to 2008. Additionally, a significant increase in self-reported allergies among children is reported in this study, while the total population prevalence of self-reported peanut allergy, tree nut allergy, or both in did not change among adults since 1997. Self-reported tree nut allergies rose from 0.2% in 1997 to 1.1% in 2008, and peanut allergy increased from 0.4% to 1.4% in the children younger than 18 years of age. The present estimates are comparable to reports obtained from Canada, the United Kingdom, and Australia (Sicherer et al., 2010).

**Pecan Allergens**

Most food allergens are naturally occurring abundant proteins, and only a small percentage of all types of naturally occurring proteins in food are characterized as allergens (Taylor and Hefle, 2006b). The most common allergenic foods have relatively high protein content, particularly foods of plant or marine origin (Taylor et al., 1987). Some common allergenic foods contain multiple allergenic proteins (e.g. peanut, eggs, cow’s milk, and walnuts), while only one allergen has been identified in other foods (e.g. Brazil nuts, codfish, and shrimp) (Taylor and Hefle, 2006b). Proteins are considered to be major allergens if serum IgE from more than 50% of patients with a specific food allergy binds to them by immunoassay (Taylor and Hefle, 2006b). Allergenic food proteins tend to have several common features: many have molecular weights ranging from 10 to 70kDa, are water-soluble glycoproteins, and they are often resistant to food processing.
and gastrointestinal digestion (Taylor et al., 1987; Ebo and Stevens, 2001). Also, immunologically active proteins or their peptide fragments must sustain digestion and come into the contact with IgE-producing cells of the gut wall (Taylor et al., 1992). These characteristics tend to allow proteins to retain their immunologic form and reach the intestinal mucosa; however, factors that govern which proteins drive allergic sensitization and become the major allergens are still unknown (Taylor et al., 1987).

Proteins having sequence identities of 30% or greater, or based of their functional and structural properties, can also be classified into families and superfamilies (Breiteneder and Radauer, 2004). Almost 60% of all plant food allergens belong to one of four protein superfamilies. These families include the prolamine superfamily, the cupin superfamily, the profilins, and the Bet v1 (major birch pollen allergen) superfamily (Radauer and Breiteneder, 2007). The two allergenic proteins described to date in pecan include a 2S albumin, Car i 1 (10kD), and an 11 S legumin, Car i 4 (Sathe et al., 2005, Sharma et al., 2010a, Sharma et al., 2010b). The close family lineage of walnut and pecan could be used to explain a possible high degree of sequence homology between some of the proteins, particularly 2S albumins that share 88% sequence identity and 92% sequence similarity (Sharma, 2010). Clinical cross-reactivity of walnut with pecan has been reported but the identity of the offending allergens has not been thoroughly studied (Teuber et al., 2000).

**DIGESTION OF DIETARY FOOD PROTEINS**

It has been hypothesized that the major route of food allergen sensitization occurs via gastrointestinal tract; therefore digestion and gut permeability are main factors to take into account when assessing an individual’s susceptibility to food allergies (Moreno,
As suggested by Wickham et al. (2009), knowing the outcome of protein digestion is of great significance in understanding the basis of food allergies. Digestion usually facilitates the development of tolerance by altering the protein structure or hydrolyzing the intact protein into small peptide fragments that are not immunologically reactive. Generation of soluble antigens may play a key role in driving tolerance to food proteins primarily by altering of the immunogenic epitopes (Chahine and Bahna, 2010). The breakdown of food starts in the mouth where fluid comprising primarily of water, electrolytes, mucus and enzymes enables easy transport of food through esophagus to gastrointestinal track. In certain cases, this is considered the first site of antigen uptake where food proteins come into contact with the oral mucosa. The lumen of stomach is acidic in nature comprising mostly of mucus, hydrochloric acid and proteases. The mucous cells produce the bicarbonate rich fluid that serves as a protective covering to buffer the gastric mucosa from the acidic environment of the stomach. Hydrochloric acid is the secretory product in the stomach serving as a principal bactericidal and parasitic barrier due to its acidic nature. Hydrochloric acid also aids in the denaturation of proteins. Additionally, low pH is a prerequisite for the activation of pepsin, the foremost gastric protease present in the gut. The optimum pH range for pepsin activity is from 1.8 to 3.2. The gastric empting usually lasts 2 hours on average at which time the remaining peptides present in the chyme are further digested with a variety of pancreatic proteases including trypsin, chymotrypsin, and carboxypeptidase in the small intestine at neutral pH (Untersmayr and Jensen-Jarolim, 2006).

The gastrointestinal (GI) tract aids food antigen diffusion through its gut epithelial barrier, where epithelial cells are linked with neighboring cells via tight junctions and
mucus. In the upper bowel, the vast majority of antigen exposure originates from foods, while in the lower bowel; the antigenic exposure comes from the microorganisms living in the GI tract. Besides its physical barrier function, the mucosal system of the small intestines has two adaptive immune mechanisms to preclude antigen circulation: (i) antigen exclusion mediated through the secretion of IgA and IgM antibodies to regulate the colonization of microorganisms and diminish penetration of soluble agents, and (ii) suppressive mechanisms that prevent hypersensitivity to harmless foreign substances such as food proteins. Moreover, it has been acknowledged that these suppressive immune mechanisms are responsible for induction of oral tolerance to food proteins in the diet. Even with these defense mechanisms, antigens are still absorbed and circulated in the body. Intact food proteins and gut bacteria have been detected in plasma and in mesenteric lymph nodes. Protein persistence is increased with impaired digestion, theoretically triggering sensitization or allergic symptoms. Currently, it has been recognized that the resistance to *in vivo* digestion of allergenic food proteins heightens their potential for causing allergic sensitization and subsequent allergic reactions in susceptible individuals (Schnell and Herman, 2009).

As indicated earlier, most but not necessarily all food allergens tend to share a number of biochemical characteristics. These consist of glycosylation patterns of the allergens, abundance in the food, and resistance to proteases, heat, and denaturants. Stability to the proteolytic and acidic conditions of the digestive tract is considered as one of the more important characteristics of food allergens, which contributes to an increased probability of reaching the intestinal mucosa, where absorption and interaction with the immune system can occur (Moreno, 2007). Another important characteristic that is
common among food allergens is their great stability and resistance to denaturation. Structural stability is a significant characteristic because the immune responses are more likely to be triggered by intact proteins. Nevertheless, this property is not a major characteristic of aeroallergens since the route of exposure for aeroallergens is through the respiratory tract. The protein structure could be a significant factor in an allergen’s ability to resist denaturation since many allergenic food proteins contain intramolecular disulfide bonds. Also, in vitro digestion assays are used to identify regions of the proteins that are more resistant to proteases than other portions of allergen or other non-allergens (Sen et al., 2002). Observations made from studying Ara h 2, the major peanut allergen recognized by serum IgE from 90% of peanut-allergic individuals, show that disulfide bonds are likely a contributing factor in the resistance of this allergen to digestion with proteases commonly encountered in the GI tract (Koppleman et al., 2010).

In 1996, Astwood et al. pointed out that stability of proteins in an in vitro digestion assay in simulated gastric fluid (SGF) with pepsin may be used to help predict whether a new dietary protein might become an allergen. The potential of food proteins to bind IgE is decreased considerably with gastric digestion, potentially increasing the threshold dose of allergens required to elicit symptoms in food allergy individuals (Untersmayr and Jensen-Jarolim, 2008). A number of food allergens have been evaluated for their stability in SGF, and when compared with non-allergenic food proteins, they were able to survive for extended periods of time. An additional factor to be considered in conjunction with allergen’s structural stability is the relative abundance of the allergen in the food since abundance may influence the dose of allergen that endures gastrointestinal digestion. In order to sensitize naïve individuals and/or elicit an allergic
reaction, proteins should have the capability to stimulate the immune system in addition to having digestion stability (Moreno, 2007).

The digestion stability of various food allergens can be affected differently with different digestion enzymes. A good example of this involves the major allergen in crustacean shellfish, tropomyosin, where the digestion stability of this allergen is affected by different cleavage sites for peptide bonds of the proteases (Liu et al., 2011). Pepsin preferentially cleaves peptide bonds following Phe or Tyr residues. Trypsin shows a different specificity, cleaving next to the hydrophilic amino acid residues of Lys and Arg while chymotrypsin has a broader specificity, cleaving next to aromatic amino acids (Tyr, Trp, Phe) and Leu. Liu et al (2011) also suggests that the enzyme to substrate ratio was a significant parameter in the stability of tropomyosin. The protein was extremely unstable when the ratio (weight to weight) of pepsin to tropomyosin was increased from 0.02:1 to 1:1. Human digestion is variable from person to person; therefore, accurately reflecting the physiological conditions is a challenging task when choosing an optimal protease to substrate ratio in in vitro digestion assays. For digestion studies, it is more practical that the amount of proteases used is based on proteolytic activity rather than on weight basis (Liu et al., 2011).

Changes in protease concentration, pH, protein purity, and method of detection could have considerable effects on the interpretation of in vitro digestion assays. Studies conducted on Ara h 2 show that protein structure plays a significant role in the stability in in vitro digestion assays and provide a link between food allergen structure, stability to digestion, and the immune-dominant IgE-binding epitopes in food allergic individuals (Bannon et al., 2003). Moreover, the observations made by Fu (2002), imply that food
allergens may be more, equally, or less liable to digestion in simulated gastric and intestinal fluid than non-allergenic proteins, suggesting that digestibility alone is not the only factor to consider when evaluating the allergenic potential of a protein (Bannon et al., 2003). *In vitro* digestion assays should be used to estimate of the relative integrity of a protein and thus the probability of eliciting the allergic reactions (Fu, 2002). The allergenic status of purified proteins should not be assessed only by *in vitro* digestion assays as the food matrix may have a significant effect *in vivo*. SGF assays could be used for estimating protein persistence *in vitro* and isolating peptide fragments with potential allergenic epitopes. Consequently, the assessment of the allergenic potential of food allergens demands the use of both digestion and immunologic assays as methods to evaluate consumer safety to novel food proteins or proteins introduced through means of genetic engineering (Schnell and Herman, 2009).

**SUMMARY**

The analysis of the stability of proteins within the gastrointestinal tract may provide potential testing for allergenicity and could be an important and effective parameter that characterizes food allergens from non-allergens (Liu et al., 2011). The digestion resistance studies of proteins should be deduced in combination with other factors such as sequence homology to known allergens or specific serum screening. No single criterion can be used to predict the allergenic potential that food proteins may have on the human immune system (Moreno, 2007). The frequency of food allergies continues to rise in part due to increased awareness and knowledge of food allergies by consumers, better access to clinical allergists for proper diagnosis of food allergies, as well as some indication of a true increase in the prevalence of food allergies. With the increase in the
prevalence of food allergies around the world comes a need to continue research in identification and characterization of allergenic food proteins, examination of what makes a certain food protein allergenic (i.e. stability to heat, digestion, etc.), development of new methods to detect allergens, and the development of potential methods to reduce protein allergenicity through biotechnology or processing.

The prevalence of the tree nut allergies seems to be increasing along with its increased consumption. The majority of reported tree nut allergies in the U.S. occur to walnuts, almonds, cashews, and pecans. As indicated earlier, efforts have been made in characterizing major allergens in walnuts, almonds, and cashews, however; little information is currently available on characterizing pecan allergens. Therefore, the main objective of this study was to further characterize pecan allergens, evaluate their digestive stability, and evaluate the allergic potential of the pecan 2S albumin, which may be one of the most important allergens from pecan.
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CHAPTER 2: EXTRACTION AND CHARACTERIZATION OF SOLUBLE PROTEINS IN PECANS

Abstract

Rationale: An increase in the prevalence of allergic reactions to tree nuts may be due to their increased consumption. Pecans are one of the major allergenic nuts and little is known about their allergens. Characterization of soluble proteins in pecans is important and a major concern for clinicians and the food industry in order to assure safety of its consumers. The presence of clinically relevant allergens in extracts used for assessment is dependent on the solvent used for extraction of protein residues from whole foods. The objective of this study is to examine the effects of various buffers in the preparation of protein extracts that could be applied in clinical diagnostic tests and development of methods for detection of pecan residues in foods.

Methods: A range of extraction buffers was chosen to determine the optimum buffer and conditions for extraction of soluble proteins in raw and defatted pecans. Protein yields and size distribution profiles were monitored from extracts obtained using different time/temperature extraction combinations. The influence of pH and ionic strength was evaluated using extraction buffers having various levels of NaCl. Protein profiles were visualized under reducing conditions by SDS-PAGE.

Results: Insignificant differences in protein content and profiles were obtained for raw and defatted pecan samples extracted at 1 h at room temperature or overnight at 4°C using deionized reverse osmosis water (ROD) at various pH values (pH 2 – 12). Protein solubility and polypeptide patterns were quantitatively and qualitatively dependent on the buffer used for the extraction. Pecan proteins are not easily solubilized in ROD water at
neutral and acidic pH; solubility increases at pH>9. Protein solubility and its yield greatly increase as the NaCl concentration increases from 0.0 to 4.0M.

**Conclusion:** The allergen content in solution is dependent on the buffer used for the extraction. Improvements in extraction procedure of soluble pecan proteins observed in this study could be used in developing rapid and sufficiently sensitive assays that can be used in clinical diagnosis as well as in food processing settings for detection of pecan proteins in various foods or residue on equipment surfaces.

**INTRODUCTION**

Used by pre-colonial inhabitants, pecans (Carya illinoinensis) are the sole native tree nuts grown in North America. Pecans belong to Juglandaceae family, which also includes a variety of tree nuts such as walnuts, butternuts, hickory nuts, and heartnuts. The U.S. is the leading global producer of pecans, accounting for more than 80% of its total production (USDA, 2005).

Pecan seeds have high nutritional value. They are a good source of protein, poly and mono-unsaturated fatty acids, fiber, and antioxidants that are believed to play a role in reducing the risk of heart disease by lowering cholesterol levels. Raw, roasted, or salted, pecans are commonly eaten as snack food. Moreover, pecans are being used in various food products as ingredients including pecan pie, salads, dessert toppings, candy and confections, and variety of baked goods (NPSA, 2010).

The high lipid content of edible nut seeds and polyunsaturated fatty acid profiles rich in oleic, linoleic, and linolenic acids, which increase a satiety factor, have led to increased consumption of nuts to aid in weight management diets due to the ability of
tree nuts to subdue appetite and fat absorption (Venkatachalam and Sathe, 2006; Coates and Howe, 2007). Additionally, a low fat diet enriched with pecans has been shown to significantly lower total cholesterol and LDL cholesterol levels in men and women with normal to slightly elevated blood serum cholesterol levels (Rajaram et al., 2001). Moreover, nut seeds and nut seed derived ingredients are increasingly being used to produce and/or improve value-added products to help increase economic revenues for the food industry and also increase food choices for consumers (Sathe et al., 2009).

Despite their potential health benefits, tree nuts are one of the most frequent allergenic foods. Allergy to tree nuts is responsible for severe anaphylactic reactions in both children and adults. The significance of peanut and tree nut allergies is highlighted by the potential risk of severe or even fatal allergic reactions as these foods have been implicated in the majority of severe or fatal allergic reactions. Additionally, 1.4% of the U.S. population is estimated to have IgE-mediated food allergies to peanut, tree nuts, or both allergies. The onset of this type of allergy is usually observed in children, where approximately only 10% of the effected individuals tend to outgrow their allergy to tree nuts (Fleischer, 2007; Polenta et al., 2009). The majority of food-induced allergic reactions in American adults are caused by peanut, tree nuts, fish, and crustacean shellfish (Sampson, 2004). Tree nuts in general are particularly challenging food allergens for allergic consumers to manage through avoidance diets due to their widespread consumption either directly from the shell or as ingredients in a variety of foods. In addition, tree nuts are often harvested with shared equipment and processed in on shared equipment, which increases the chance of comingling and cross-contact of various tree nuts.
Pecans are included in the group of the 8 most common foods capable of inducing an allergic reaction, often referred to as the “Big 8” allergens. Milk, eggs, wheat, peanuts, fish, crustacean shellfish, soybean, and other three nuts also belong to this group. The prevention of allergic reactions is accomplished only by strict avoidance of pecans in the diet of pecan-allergic individuals. Inadvertent contamination or mislabeling of food products can lead to unexpected exposure to foods containing undeclared pecan residues that can induce allergic reactions in sensitized individuals. Hence, it is vital to develop a specific and sufficiently sensitive assay to detect the presence of pecans in foods.

Most of the time food proteins are causative agents of Type I-IgE mediated food allergies (Taylor and Hefle, 2002). Therefore, studying of IgE-reactive proteins in nut seeds is important in understanding of true food allergies. Protein type, functional properties, protein content, and the allergenic properties of peanuts have been well documented and characterized (Breiteneder and Radauer, 2004; Chassaigne et al., 2007; King et al., 2005; Maleki et al., 2000; Mills et al., 2002; Sathe et al., 2009; Shin et al., 1998; Yan et al., 2005; Yu et al., 2007). Research on the characterization of the allergenic proteins of tree nuts and their properties remains limited.

Sathe et al. (2005) suggested a strategy to systematically study allegens in food. First, one should identify a protein(s) from the food source of concern, which includes characterizing the protein profile, and identify particular proteins potentially responsible for causing allergic reactions in humans using IgE immunoblotting experiments. The protein(s) of interest should then be isolated/purified and further characterized from a biochemical, molecular, and allergenic perspective. Gaining an understanding of the biochemical, molecular and allergenic properties of food allergens can aid in further
development of approaches to moderate the allergenicity of these proteins and perhaps develop immunotherapy treatments to reduce a patient’s clinical reactivity to the proteins.

Detection of clinically relevant allergens intended for use in various bioassays is depended on the solvent used for extraction of particular proteins. Aqueous solutions are commonly used in immunoassays assessing the immunoreactivity of such extracts. The use of various solvents among different laboratories may explain variations observed in the polypeptide pattern when studying a particular food (Venkatachalam et al., 2008). Wallowitz et al. (2006) and Venkatachalam et al. (2008) have focused on the importance of using appropriate extraction buffers for preparation of extracts planned for use in clinically relevant diagnostic testing for tree nut allergies. Optimization and evaluation of extraction buffers is necessary in order to maximize the extraction of soluble proteins, which in turn increases a probability of detecting IgE developed against relevant allergens. Therefore, misleading diagnostic test results could be avoided with optimized extraction and preparation of diagnostic extracts containing the majority of soluble proteins.

Furthermore, knowledge about naturally occurring proteins becomes important when their recombinant counterparts do not show similar molecular behavior. The differences between the naturally occurring and recombinant form of the proteins may become apparent from several factors such as glycosylation, formation of multiple native isoforms due to either multiple genes coding for the same protein, post-translational protein modification, or a combination thereof (Sathe et al., 2009). Kang and Gallo (2007) have demonstrated the importance of identifying native isoforms in their study where they recognized that a natural isoform of Ara h 3 from peanut had a lower
immunoreactivity than the originally identified Ara h 3 allergen. Moreover, tissue maturity, tissue components, and the solvent employed for protein extraction, including storage conditions, are all variables that influence the quality of protein extracts being used for diagnostic, clinical, or investigative reasons (Sathe et al., 2009). Extracts from natural sources are recognized to be heterogeneous in nature, containing both non-allergenic and allergenic proteins. Additionally, the composition and potency of allergenic proteins in natural extracts can vary from extract to extract. Contamination by other allergenic sources is quite common due to cross-contact of various foods so care must be taken to ensure that the diagnostic extracts are prepared from pure sourced foods of interest (Hamilton, 2010).

Of similar importance to proper extraction of proteins for diagnostic purposes, detection of proteins from the allergenic food source serves as the basis for commercially available immunoassays used by the food industry to monitor the removal of allergenic residue from equipment surfaces or to ensure that undeclared allergenic residue is not present in finished food products. A critical step in food analysis is the effective extraction of the analyte/proteins from composite foods (Besler, 2001). Optimization of the sample preparation was illustrated in a study conducted by Keck-Gassenheimer et al. (1999) who have shown a remarkable increase in protein detection and recoveries of peanut proteins that were spiked into a wide range of food products. Recovery of peanut proteins in dark chocolate were poor unless fish gelatin was added to the extraction buffer (0.05 M -Tris/0.2 M-NaCl, pH 8.2) at 10% w/v, as assessed by using a commercially available ELISA test kits for detection of peanut residues (Cortecs, Deeside, UK). An improvement in the detection limit and recovery rates was ascribed to the fish gelatin due
to its high solubility in aqueous solution and high tannin binding capacity. Tannins possess a high binding affinity to proteins and may interfere with extraction of proteins from foods or can bind to antibodies while running the assay, and chocolate has especially high amount of tannins. Similarly, Wallowitz et al. (2004) emphasized that walnut proteins are poorly soluble in saline solution and in mild (pH 7-8) aqueous buffers normally used for protein extraction. Therefore, more robust methods of extraction are required to enable detection of trace quantities of proteins from finished products during food manufacturing using such solvents.

Accordingly, complete characterization of the major allergens is imperative for numerous reasons, including development and standardization of specific IgE assays, improvement of detection kits for the food industry, and development of immunotherapies using recombinant proteins. The food industry and healthcare institutions should both benefit on a practical level by further standardization and optimization of detection kits and diagnostic tests. The producers of new commercial extracts used for prick skin testing and of extracts used in in vitro specific IgE assays can warrant the presence of all major allergens; therefore, avoiding misleading skin tests or specific IgE assays that may give false-negative results due to missing allergens in the diagnostic test (Wallowitz et al., 2006).

The principal goal of the present study was to investigate pecan protein solubility, qualitative composition of pecan protein polypeptides, and to assess the immune reactivity of rabbit and sheep anti-pecan polyclonal IgG antibody (pAb) binding to soluble proteins of selected pecan samples.
MATERIALS AND METHODS

Pecan Sample Preparation

Raw pecans were washed in several changes of deionized reverse osmosis water (ROD) and air-dried. A portion of the washed pecans was dry-roasted at 270°F for 10 min. Raw and roasted pecans were ground at room temperature to uniform particle size using a 16-speed Oster blender (Niles, IL) equipped with pulse control. Samples were frozen at -20°C until further use. A portion of the ground raw pecans was defatted by washing in 1:10 (w/v) ratio with hexane at 50°C for 30 min (repeated 4 times) on a horizontal shaker, and filtered using Whatman #1 filter paper each time with retention of the particles. The defatted samples were then fully air-dried and stored in Falcon 50 mL conical centrifuge tubes (Fisher Scientific, Pittsburg, PA) at room temperature until further use.

Optimization of the Extraction of Soluble Pecan Proteins

Effect of Extraction Time, Temperature, and pH

Pecan protein yields and profiles were monitored over two different time/temperature extraction combinations. Samples of raw and defatted samples were extracted over night at 4°C in ROD water with various pH values (pH 2 – 12) with gentle rocking. Comparable samples were extracted for 1 hour at room temperature (~25°C) in the same extraction buffers on a horizontal shaker. Defatted pecan samples were extracted in two solvents, ROD water having a pH range of 2-12 and aqueous 1M NaCl also having a pH range 2-12 in 1:10 (w/v) ratio. The pH for each sample was adjusted with 1.0 N NaOH or 1.0 N HCl. The soluble proteins were collected after centrifugation for 15 min at 13,600 x g. The supernatant was collected and then filtered through a
0.45μm membrane. The soluble protein content for each sample extract was estimated using the Lowry method (1951) and analyzed with SDS-PAGE under reducing conditions.

**Effect of Ionic Strength**

The influence of pH and ionic strength was evaluated using solvents having various molar concentrations of NaCl. Raw and defatted pecan flours were extracted in aqueous solutions of 0.0, 0.3, 0.5, 1.0, 2.0, and 4.0 M NaCl without pH adjustment for 1 hour at room temperature on a horizontal shaker. Additionally, raw and defatted pecan flours were extracted for 1 hour at room temperature on a horizontal shaker in 1M NaCl aqueous solutions that had the pH adjusted from pH 2-12 with 1.0 N NaOH or 1.0 N HCl. The samples were then centrifuged for 15 min at 13,600 x g. The supernatant was collected and then filtered through 0.45μm membrane. The soluble protein content for each sample extract was estimated using the Lowry method (1951) and analyzed with SDS-PAGE under reducing conditions.

**Effect of Extraction Buffers**

Protein solubility in six different extraction buffers was investigated by extracting defatted pecan flour in 1:10 (w/v) ratio with a buffer for 1 hour at room temperature on a horizontal rocker. The extraction buffers used were: 0.1M NaOH (pH 12.9); 0.125M buffered saline borate (BSB) (pH 8.45); 0.1M Tris-HCl; 0.01 M PBS, 1M NaCl (pH 7.2); 0.01M PBS, 0.5M NaCl (pH 7.2); ELISA Coating Buffer (Bicarbonate buffer, pH 9.6). Samples were then centrifuged for 15 min at 13,600 x g, and the collected supernatant was filtered through 0.45μm membrane. The soluble protein content for each sample
extract was estimated using the Lowry method (1951) and analyzed with SDS-PAGE under reducing conditions.

**Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis**

The separation of proteins from pecan extracts was performed using a modified version of the SDS-PAGE protocol initially developed by Laemmli. SDS-PAGE was used to characterize the qualitative differences in polypeptide profiles among different samples that had been extracted in previously outlined solvents or extraction buffers. Samples were electrophoresed on a 15% polyacrylamide Tris-HCl precast resolving gel (8.6cm x 6.8cm x 1.0mm; Bio-Rad Laboratories, Hercules, CA). Protein samples were prepared by mixing the extract in a 1:1 ratio with Laemmli sample buffer (62.5mM Tris-HCl, pH 6.8, 2%SDS, 25% glycerol, 0.01 % bromophenol blue; Bio-Rad Laboratories) containing 350 mM dithiothreitol (DTT Cleland’s Reagent; Bio-Rad Laboratories). The mixture was heated for 5 min in a boiling water bath, cooled to room temperature, and centrifuged at 13,000 x g for 5 min immediately before loading samples in the SDS-PAGE gel. Stock 10X Tris/Glycine/SDS Buffer (Bio-Rad Laboratories) was diluted in a 1:10 ratio with ROD to achieve the final 1X running buffer. Five µl of standard molecular weight markers (Precision Plus Protein Dual Color Standards, Bio-Rad Laboratories) and 10 µg of each protein from each sample were loaded in separate wells of the gel for each run.

A Bio-Rad Mini Protean® II electrophoresis cell was used for protein separation. The electrophoresis run time was approximately 35 min at constant voltage of 200V. The run was stopped when the leading front bromophenol blue line reached the end of the gel. Proteins were fixed for 30 min using Fixing Solution 5X Concentrate (60% (w/v)
trichloroacetic acid and 17.5% (w/v) 5-sulfosalicylic acid, Sigma-Aldrich, Inc.) and stained with Brilliant Blue G-250 Colloidal Stain (Bio-Rad Laboratories) for 2 hours. The gels were destained for 60 seconds in a destain solution containing 10% acetic acid, 25% methanol and 65% ROD, followed by several changes of 25% methanol to remove residual dye from the gel. Finally, gel images were captured using the Kodak Gel Logic 440 imaging system (Eastman Kodak Company) and analyzed using Kodak 1D v. 3.6.5. software (Kodak Scientific Imaging System, New Haven, CT).

**Immunogen Preparation for Antibody Production**

Raw pecans were washed in several changes with ROD and air-dried. Pecans were then dry-roasted at 270°F for 10 min. The roasted pecans were chopped, frozen, and then ground to a fine particle size using a 16 speed Oster blender (Niles, IL) equipped with pulse control. The ground, roasted pecans were defatted by washing 1:5 (w/v) with ethyl ether (5 times), followed by a 1:5 (w/v) wash with acetone (2 times). The defatted material was filtered after each washing step with retention of the particles. The powdered roasted pecans were thoroughly air-dried and used as immunogens for subsequent immunization of rabbits and a sheep.

**Polyclonal IgG Antibody Production**

Polyclonal IgG antibodies were produced at Covance Research Products, Inc. (Denver, PA). Three New Zealand rabbits and one sheep were immunized with the powdered, roasted and defatted pecan immunogen using standard immunogen protocols. The dry powder was suspended in the appropriate Freund’s adjuvant immediately prior to immunization. Each rabbit received initial subcutaneous injections at multiple sites with a total of 250 µg of protein emulsified in Freund’s Complete
Adjuvant (FCA). Subsequent booster injections were administered at 21-day intervals using 125 µg of protein in Freund’s Incomplete Adjuvant (FIA) subcutaneously. The sheep received an initial intramuscular injection of 1000 µg protein emulsified with FCA, followed by subsequent boosts at 500 µg protein in FIA. Test bleeds of rabbits were collected at 10 and 24 days post-booster injection, whereas, test bleeds of the sheep were collected 10 days after each boost to monitor the antibody production. The IgG antibodies were partially purified from sera using successive precipitation with 50% and 35% ammonium sulfate (Harlow and Lane, 1988), followed by redissolution and extensive dialysis (12,000-14,000 MW, Spectrum, Gardena, CA) against excessive 0.01M phosphate buffered saline (PBS; 0.002 M NaH₂PO₄, 0.008 M Na₂HPO₄, 0.85% NaCl and 0.02% NaN₃, pH 7.4).

Pecan Specific IgG Titer Determination

Titer values of collected sheep and rabbit anti-pecan IgG were determined by a non-competitive ELISA method outlined by Hefle et al. (2001). Maxisorp™ microtiter plates (Nalge Nunc International, Rochester, NY) were used as solid support. Plates were coated with 100 µl of 1.0 µg/ml pecan protein/antigen extract in coating buffer (0.015M Na₂CO₃, 0.035M NaHCO₃, pH 9.6) and incubated overnight at 4°C. Plates were washed on an automated plate washer (Dynatech Laboratories, Inc., Chantilly, CA) with conjugate buffer (0.025 M PBS, 0.5% bovine serum albumin [BSA, RIA grade, USB Corp., Cleveland, OH], and 0.2% Tween 20). Non-specific binding sites were blocked by addition of 350µl of titer blocking buffer [0.1% porcine gelatin (300 bloom, Sigma-Aldrich St. Louis, MO) in 0.01 M PBS] added to each well and incubated for 1h at 37°C. Plates were then washed with conjugate buffer, and 100µl of rabbit anti-pecan IgG or
sheep anti-pecan IgG serially diluted in conjugate buffer was added to each well and incubated for 2 hours at 37°C. After washing, 100µl of diluted commercial anti-immunoglobulin (IgG) secondary antibody conjugated with alkaline phosphatase (goat anti-rabbit, rabbit anti-sheep (Immunopure® R, Pierce Biotechnology, Inc., Rockford, IL) diluted 1:5,000 in conjugate buffer was added to appropriate wells and then incubated for 1h at 37°C. The plates were washed, followed by addition of 100µl/well of p-nitrophenyl phosphate substrate solution prepared by dissolving P-NPP in 20 ml of ROD (p-NPP; Sigma Fast Tablets, Sigma Chemical Company, St. Louis, MO). The plates are then incubated for 30 min at room temperature in the dark, and 100 µl of 1 N NaOH was added to each well to stop the reaction. The absorbance was measured at 405nm on a BioTek ELx808 plate reader (BioTek Instruments, Inc., Winooski, VT) in order to determine pecan-specific IgG antibody binding.

The titer values of the pecan specific IgGs were calculated using GraphPad Prism® software (GraphPad Software, Inc., La Jolla, CA) by determining the log reciprocal of the mid-linear portion of the dilution curve. The protein content of individual (goat) or pooled (rabbit) IgG solutions were determined by the Lowry method (1951).

**IgG Immunoblotting of Rabbit and Sheep Polyclonal Antibodies**

Pecan extracts were separated by molecular weight by SDS-PAGE under denaturing conditions as previously described. Electrophoresis gels were equilibrated in transfer buffer (1X Tris/Glycine Buffer, Bio-Rad Laboratories, 20% Methanol, Analytical Grade, Fisher Scientific) for 15 min and transferred onto a polyvinylidene difluoride (PVDF) membrane (Immoblin-P PVDF membrane, 0.45µm, Millipore Corporationm
Billerica, MA) using a modified method of Towbin et al. (1979) and Harlow and Lane (1988). The transfer was performed for 1 hour at a constant voltage of 70 V.

The IgG immunoreactivity was evaluated using the following procedure. The membrane was washed three times for 5 min in ROD water with gentle rotation after the transfer to remove residual transfer buffer. The membrane was then soaked in Ponceau S stain for approximately 1 min to verify the transfer of soluble proteins. The Ponceau S stain was then removed by washing the membrane with multiple changes of ROD water. The membrane was blocked with blocking buffer [0.01 M PBS, pH 7.4 containing 0.05% Tween 20 (PBS-T) with 0.2% bovine serum albumin (BSA, RIA grade, USB Corp., Cleveland, OH)] for 2 hours at room temperature with gentle rotation. Rabbit and sheep anti-pecan IgG solutions were diluted 1:10,000 and 1:15,000 (v/v), respectively, with blocking buffer and incubated with the membrane for 1h at room temperature. Unbound IgG was removed by washing the membrane 4 times for 5 minutes each in PBS-T. The secondary antibodies, goat anti-rabbit or rabbit anti-sheep conjugated to horseradish peroxidase (Immunopure®, Pierce Biotechnology, Inc., Rockford, IL) were diluted 1:25,000 (v/v) in blocking buffer and incubated with the membrane for 1h at room temperature with gentle rotation. The membrane was then washed four times with PBS-T to remove unbound secondary antibody, and treated with DAB (3,3’-diaminobenzidine) substrate solution for 15 min at room temperature (Pierce Protein Research, Thermo Fisher Scientific Inc., Waltham, MA) to visualize bound anti-rabbit and anti-sheep IgG. The membrane was then washed in several changes of PBS-T in order to stop the enzymatic reaction. Finally, the membrane images were captured using the Kodak Gel Logic 440 imaging system.
RESULTS AND DISCUSSION

In this study, electrophoresis was performed to characterize the qualitative differences in the soluble pecan protein/polypeptide pattern among a number of extracts with varying pH and extraction buffer ionic strength. One-dimensional SDS-PAGE is a standard procedure used to separate and identify proteins in extracts by their molecular mass regardless of their original charge (Poms et al., 2004). Normally, single bands represent one protein; though, other proteins can migrate at the same molecular weight (Poms et. al., 2004). Pecan proteins were separated and identified by SDS-PAGE and visualized by Brilliant Blue G-Colloidal stain. Effects of time, temperature and pH on defatted pecan protein solubility extracted with ROD water at various pH values (pH 2 – 12) are shown in Figure 2.1 and Table 2.1. No significant difference was observed in polypeptide profiles and protein content between samples that were extracted for 1h at room temperature or at 4°C overnight. Pecan proteins are poorly solubilized in ROD water. Protein solubility was only increased under very alkaline pH (12) values (Figure 2.1). This observation is consistent with reported analysis on soluble proteins in pecan by Venkatachalam et al. (2008). Additional comparison of the two time/temperature conditions is depicted in Figure 2.2 and Table 2.2 where pecan proteins were extracted in 1M NaCl solution with varying pH values. The presence of salt increased the amount of total soluble pecan protein extracted by 2-3 fold and showed distinctly different polypeptide profiles compared with the samples extracted in ROD water. The detected increase in protein solubility could result from less hydrophobic interaction and aggregation between proteins and phenolic compounds that are present at high concentrations in pecans (Venkatachalam et al., 2006; Venkatachalam et al., 2008; Sathe
et. al., 2009). The two time/temperature conditions did not show a significant difference for protein solubilization, and 1h at RT was chosen as the optimum extraction condition for further analysis. To better examine the role of NaCl and pH on protein solubilization raw and defatted pecan samples were extracted at different salt concentrations without pH adjustments and in 1M NaCl having different pH values (2 – 12) (Figures 2.3 and 2.4). Polypeptide profiles between raw and defatted samples showed no significant difference when comparing individual extracts. Low molecular weight proteins tend to be more soluble under increasingly acidic pH and lower salt concentration, while the solubility of high molecular weight proteins begins to increase at 1M NaCl and alkaline pH. It was also observed that pecan proteins start to precipitate out of solution at high levels of salt (>2M NaCl). Again, the amount of total soluble pecan protein content was influenced by the amount of salt present in the extraction buffer (Table 2.3). Soluble pecan proteins are comprised of albumins, globulins, prolaines, and glutelins (Venkatachalam et al., 2008). Albumins are characterized as being soluble in aqueous solutions, globulins are usually readily extracted in saline solutions, and prolaines are soluble in buffers containing water and alcohol mixtures (Westphal et al., 2004). The observations in this study are consistent with the Osborne fractionation of pecan proteins where the alkali glutelins (64.94% of the soluble protein content of pecan) and globulins (22.13%) are the most prominent proteins present, while albumins (2.04%) and acid-soluble glutelins (1.89%) represent the remaining portion of the soluble proteins (Venkatachalam et al., 2008). In our study, protein content was also found to increase with increasing concentrations of NaCl salt present in the extraction buffer.
Figure 2.1 Effect of extraction time, temperature and pH on pecan protein solubility. Defatted pecan flour was extracted in deionized reverse osmosis water (ROD) at pH 2-12. SDS-PAGE separation was conducted under reducing conditions. The number on the top of the gel indicates the pH value of the extraction solution.

Protein load in each lane was approximately 10µg. M- Molecular weight markers

<table>
<thead>
<tr>
<th>Extraction buffer: ROD, (pH 2-12)</th>
<th>1h at Room Temperature</th>
<th>Overnight at 4°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Defatted Pecan Flour</td>
<td>~0.8-1.7</td>
<td>~0.8-1.2</td>
</tr>
</tbody>
</table>

Table 2.1 Soluble pecan protein content estimated by the Lowry method.
Figure 2.2 Effect of extraction time, temperature and pH on pecan protein solubility. SDS-PAGE separation under reducing condition of defatted pecan flour extracted in 1M [NaCl]. The number on the top of the gel indicates the pH value of the extraction solution.

Protein load in each lane was approximately 10µg
M- Molecular weight markers

<table>
<thead>
<tr>
<th>Extraction buffer: 1 M NaCl, (pH 2-12)</th>
<th>1h at Room Temperature</th>
<th>Overnight at 4°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Defatted Pecan Flour</td>
<td>~1.8 – 3.1</td>
<td>~1.4 - 2.2</td>
</tr>
</tbody>
</table>

Table 2.2 Soluble pecan protein content estimated by the Lowry method, extracted in 1M [NaCl] aqueous solution, pH 2 - 12.
Figure 2.3 Effect of NaCl and pH on pecan protein solubility. [A] SDS-PAGE analysis of raw pecan samples extracted in 1M NaCl solution, pH 2-12. Number on the top of the gel indicates the pH value of the extraction solution. [B] SDS-PAGE analysis of various molar [NaCl] (shown on the top of the gel) on protein solubility of raw pecan samples at pH 7.2.

Protein load in each lane was approximately 10µg
M- Molecular weight markers
Figure 2.4 Effect of NaCl and pH on pecan protein solubility. [A] SDS-PAGE analysis of defatted pecan flour samples extracted in 1M [NaCl] solution, pH 2 - 12. Number on the top of the gel indicates the pH value of the extraction solution. [B] SDS-PAGE analysis of various molar [NaCl] (shown on the top of the gel) on protein solubility of defatted pecan flour samples at pH 7.2.

Protein load in each lane was approximately 10µg
M- Molecular weight markers

<table>
<thead>
<tr>
<th>Soluble Protein Content (mg/ml)</th>
<th>Extraction buffer: 1 M NaCl, (pH 2-12)</th>
<th>Extraction buffer: M [NaCl], (0 – 4 M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw Pecan Flour</td>
<td>~1 – 2 mg/ml</td>
<td>~0.5 – 2 mg/ml</td>
</tr>
<tr>
<td>Defatted Pecan Flour</td>
<td>~2 – 2.5 mg/ml</td>
<td>~1 – 4 mg/ml</td>
</tr>
</tbody>
</table>

Table 2.3 Soluble pecan protein content estimated by the Lowry method; 1M NaCl, pH 2 -12 and 0.0-4.0M [NaCl] extracts.
One of the main factors affecting variability among commercial ELISA kits used for detection of allergens is the difference in the effectiveness of solvent/extraction buffers used for the solubilization of allergenic proteins. Therefore, the choice of a suitable extraction solvent is as important as a selection of the specific antibodies used in the kits (Westphal et al., 2004). From a clinical standpoint, standardized extracts and pure allergens are necessary for the diagnosis and therapy of food allergy; therefore, the identification of food allergens is a priority in determining that the extract has all relevant allergens. A good extract used as a starting material for accurate research and clinical analysis should have sufficiently high protein concentration with low lipid and carbohydrate content (Pastorello and Trambaioli, 2001). Extraction optimization and further characterization of the soluble proteins becomes vital to ensure that all relevant allergens are present for clinical analysis in such tests as skin prick tests.

PBS, high salt buffer, Tris buffer, and Carbonate buffer with high pH values are frequently used extraction buffers in immunoassays (Westphal et al., 2004). Results of Westphal et al. (2004) study show that extraction of allergenic proteins from peanut, soybean, and sesame seed was greatly influenced by the buffer used which also had an impact on the detection of allergens using different commercial ELISA kits. Additionally, Sathe et al. (2009) evaluated the influence of extraction and protein content of selected nut seeds to demonstrate the significance of the extraction solvent used for protein solubilization using buffered saline borate (BSB), Tris-HCl, SDS, NaOH, PBS with 0.85% NaCl, standard PBS with 0.4M NaCl, and buffer D (Tris base, citric acid, cysteine, ascorbic acid, polyethylene glycol, EDTA, glycerol, pH 8). There is great variability in the chemical composition including the protein, lipid, carbohydrate, moisture, ash, tannin,
and phytate levels among various seeds (Venkatachalam and Sathe, 2006). The optimized extraction conditions for nut seeds are dependent on the particular nut and they need to be evaluated and determined for each individual nut. Moreover, each extraction buffer may interact with each of these components differently affecting the level of extractable proteins.

Figure 2.5 qualitatively demonstrates the effectiveness of extracting proteins from both raw and defatted pecan samples in six different buffers. The majority of soluble pecan proteins were located between 10 and 200 kDa, with the most prominent bands at 12, 16, 18, 36, 50, and 75 kDa. The most effective solution used for extraction of the highest concentration of pecan protein was 0.1M NaOH (Table 2.4) followed by BSB and 0.01M PBS, 1M NaCl (pH 7.2). While NaOH had solubilized the most pecan proteins, especially in defatted flour, the molarity and high pH value (12.9) of the buffer may irreversibly denature proteins and alter the native protein properties by deamidation of glutamine and asparagine side chains (Sathe et al., 2009), which can also be noticed by the significant differences in polypeptide profiles of the extracts (Figure 2.5). The polypeptide profiles of pecan samples extracted in 0.1M NaOH resemble a smear with only a few distinct bands observed in defatted pecan flour. BSB and 0.01M PBS, 1M NaCl (pH 7.2) show similar polypeptide patterns with BSB being slightly more effective in the solubilization of pecan proteins. These results observed in our study are also consistent with the assessment of pecan proteins reported by Venkatachalam et al. (2008) and Sathe et al. (2009).
**Figure 2.5** SDS-PAGE analysis of the effect of various extraction buffers on pecan protein solubility.

Protein load in each lane was approximately 10µg
Raw pecan-Tris-HCL, 0.1M max load ~12 µl; 3.9 µg
M- Molecular weight markers
<table>
<thead>
<tr>
<th>EXTRATION BUFFER</th>
<th>RAW PECAN (mg/ml soluble protein)</th>
<th>DEFATTED PECAN FLOUR (mg/ml soluble protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 M NaOH (pH12.9)</td>
<td>12.0</td>
<td>30.2</td>
</tr>
<tr>
<td>0.125 M BSB (pH8.45)</td>
<td>2.4</td>
<td>8.5</td>
</tr>
<tr>
<td>0.1M Tris-HCl</td>
<td>0.65</td>
<td>1.3</td>
</tr>
<tr>
<td>0.01M PBS, 1M NaCl (pH 7.2)</td>
<td>1.3</td>
<td>5.9</td>
</tr>
<tr>
<td>0.01M PBS, 0.5 NaCl (pH 7.2)</td>
<td>0.28</td>
<td>1.9</td>
</tr>
<tr>
<td>ELISA Coating Buffer (pH 9.6)</td>
<td>3.3</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Table 2.4 Soluble pecan protein content estimated by the Lowry method employing different extraction solvents.

The foremost reason why saline extraction buffers of neutral pH are commonly used in detection of food allergens is due to their compatibility in immunoassays, which helps in maintaining antibody properties and antigenicity of the extracted proteins as well as providing the optimal pH range for the antibody-antigen interaction (Westphal et al., 2004). In our study, the BSB provided a slightly higher extraction of pecan proteins as compared to the 0.01M PBS, 1M NaCl, pH 7.2, all further analysis of pecan proteins was conducted using crude extract of defatted pecan flour in 0.01M PBS, 1M NaCl, pH 7.2 as
starting material due to its compatibility with ELISA tests, and this buffer also provides a good starting buffer of further purification of specific pecan proteins.

Among the other major issues in the detection of allergens in food is that their solubility and/or structure can be altered by processing methods (Westphal et al., 2004). Pomés et al. (2006) showed that roasting increased the extraction efficiency of Ara h 1, a major peanut allergen, where soluble proteins in both raw and roasted peanut samples were extracted using PBS, 1M NaCl, pH 7.4 and 1% non-fat dry milk at 60°C. On the other hand, Westphal et al. (2004) demonstrated that less protein was extracted from dark roasted peanut flour than from light roasted peanut flour affecting the detectability of relevant allergens. Immunoreactivity of proteins could also have changed due to the processing methods either by causing denaturation or aggregation of proteins resulting in their poor extractability. These findings indicate that detection assays could provide misleading results by underestimating the presence of allergens in food extracts, and extraction buffers should be optimized for each allergenic food to maximize the amount of protein extracted from that particular food.

Immunoblotting is often coupled with gel electrophoresis to determine the presence, specificity, and quantity of antibodies from polyclonal antisera raised against the antigens present in the extract (Harlow and Lane, 1988). Pecan proteins were separated by SDS-PAGE and their immunoreactivity was determined by protein transfer to PVDF membrane and probing it with semi-purified pooled antibodies from three rabbits and a sheep. The immunogen we used for raising anti-pecan rabbit and sheep polyclonal anti-pecan antibodies (pAb) was roasted pecan; we then assessed the quality
The protein content of pecan extracts prepared using a standard PBS with low salt concentration (0.15M NaCl) and PBS with high salt concentration (1M NaCl) are shown in Table 2.5. SDS-PAGE analysis of pecan extracts (Figure 2.6C) shows the differences in polypeptide patterns, where only 5 very weak bands are observed in the roasted pecan sample extracted using the low salt PBS buffer, while peptide profiles in the other samples are quite similar. The major polypeptide bands are located at 12, 16, 18, 36, 50, and 75 kDa. The effect of roasting does not seem to affect the general profile of proteins (as observed in the roasted pecan sample extracted in the high salt PBS), but there is a slight difference in the staining intensity and bandwidth of several bands in 37 and 20-15 kDa range. The anti-pecan protein IgG profiles of both sheep and rabbit pool (Figure 2.6A and 2.6B) were comparable in all samples where pecan proteins were extracted in high salt PBS. It also appeared that the sheep antisera bound to lower molecular weight proteins to a greater extent than the rabbit antisera. However, a significant reduction in antibody binding was observed in the roasted pecan sample extracted in low salt buffer.
<table>
<thead>
<tr>
<th>EXTRATION BUFFER:</th>
<th>RAW PECAN (mg/ml)</th>
<th>DEFATTED PECAN FLOUR (mg/ml)</th>
<th>ROASTED PECAN (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01M PBS, 1M NaCl (pH 7.2)</td>
<td>1.3</td>
<td>5.9</td>
<td>1.4</td>
</tr>
<tr>
<td>0.01 M PBS, 0.15 M NaCl (pH 7.2)</td>
<td>0.4</td>
<td>1.1</td>
<td>0.6</td>
</tr>
<tr>
<td>0.125 M BSB (pH 8.45)</td>
<td>2.4</td>
<td>8.5</td>
<td>4.7</td>
</tr>
</tbody>
</table>

Table 2.5 Protein content of Pecan Samples estimated by Lowry.

Figure 2.6 Immunoblotts of pecan extracts probed to [A]-Sheep and [B]-pooled Rabbit antisera, and [C]-SDS-PAGE analysis of extracts.

Lane 1- Roasted pecan protein extracted in 0.01 PBS, 1M NaCl
Lane 2- Defatted pecan protein extracted in 0.01 PBS, 1M NaCl
Lane 3- Raw pecan protein extracted in 0.01 PBS, 1 M NaCl
Lane 4- Roasted pecan extracted in 0.01 PBS, 0.15M NaCl
Lane 5- Molecular weight markers
To observe the immune response of immunized animals during the immunization and booster period, a non-competitive ELISA using a titration technique in which the concentration and the strength of antibodies are determined by incubating serially diluted sera with a known amount of antigen. The titer value comparison (Table 2.6) and titration curves (Figure 2.7 and 2.8) additionally indicate the quality of antibodies from both the sheep and rabbit antiserum pool. Using the high salt buffer, the sensitivity of allergen detection by non-competitive ELISA format was significantly improved compared to the levels of antigens detected using the low salt buffer. These results suggest that the low salt buffer failed to extract enough protein that could be detected by the anti-pecan antibodies. The antisera were highly robust and gave sufficiently high titer values when the high salt buffer extract was used as the coating antigen in the titer analysis.
Figure 2.7 Antibody Titer Titration Curve for Rabbit Polyclonal Sera. Titer values were determined as the log reciprocal of the mid-linear portion of the dilution curve using a plate coated with 1.0µg/ml pecan protein (A-D).

A- Raw Pecan extracted in 0.01 M PBS, 1 M NaCl
B- Roasted Pecan extracted in 0.01 M PBS, 1 M NaCl
C- Defatted Pecan extracted in 0.01 M PBS, 1 M NaCl
D- Roasted Pecan extracted in 0.01 M PBS, 0.15 M NaCl
**Figure 2.8 Antibody Titer Titration Curve for Sheep Polyclonal Sera.** Titer values were determined as the log reciprocal of the mid-linear portion of the dilution curve using a plate coated with 1.0μg/ml pecan protein (A-D).

A- Raw Pecan extracted in 0.01 M PBS, 1 M NaCl  
B- Roasted Pecan extracted in 0.01 M PBS, 1 M NaCl  
C- Defatted Pecan extracted in 0.01 M PBS, 1 M NaCl  
D- Roasted Pecan extracted in 0.01 M PBS, 0.15 M NaCl
<table>
<thead>
<tr>
<th>Sample Extracts</th>
<th>Sheep</th>
<th>Rabbit</th>
</tr>
</thead>
<tbody>
<tr>
<td>A- Raw Pecan in (0.01 M PBS, 1 M NaCl)</td>
<td>79,799</td>
<td>10,715</td>
</tr>
<tr>
<td>B- Roasted Pecan in (0.01 M PBS, 1 M NaCl)</td>
<td>48,529</td>
<td>103,514</td>
</tr>
<tr>
<td>C- Defatted Pecan Flour in(0.01 M PBS, 1 M NaCl)</td>
<td>59,429</td>
<td>100,230</td>
</tr>
<tr>
<td>D- Roasted Pecan in (0.01 M PBS, 0.15 M NaCl)</td>
<td>11,776</td>
<td>3,177</td>
</tr>
</tbody>
</table>

**Table 2.6** Comparison of Pecan-Specific IgG Antibody Titer values.

**CONCLUSION**

The results from this study show that pecan proteins are poorly solubilized in ROD water and tend to be more soluble in aqueous buffers containing higher molar concentrations in NaCl salt. Moreover, the ionic strength of the solution has a greater impact on pecan protein extractability than pH. A strong binding of polypeptides in selected samples probed with pooled rabbit and sheep pAb suggests that these pAb can be used for qualitative and quantitative detection of pecan allergens.

Therefore, the presence of clinically relevant allergens is likely to be dependent on the choice of an extraction buffer, which also influences the total soluble protein content and the protein profile of an extract. It is quite important to extract as much of the clinically relevant pecan allergens as possible if using the extract in diagnostic methods such as skin prick tests, or are used to develop in vitro IgE assays for diagnostic tests.
Limited extraction of clinically relevant pecan allergens could result in a false negative clinical diagnosis of pecan allergy. Furthermore, the extract containing the relevant pecan allergens can be used as an immunogen for development of high quality antibodies that can further be used for development of sensitive assays for detection of pecan residues in various food products.
REFERENCES:


Westphal CD, Pereira MR, Raybourne RB, Williams KM. Evaluation of extraction buffers using the current approach of detecting multiple allergenic and nonallergenic proteins in food. J AOAC Internationl 2004; 87 (6) 1458-1465.
CHAPTER 3: ISOLATION AND PURIFICATION OF 2S ALBUMINS FROM PECANS

Abstract

**Rationale:** Numerous food allergens of plant origin belong to the 2S albumin protein family. The 2S albumins are one of the major allergen protein families involved in severe food allergic reactions to nuts, seeds, and legumes. Although tree nut allergies seem to be increasing in prevalence, few allergenic proteins from pecan have been identified. We aimed to purify and isolate 2S albumin from pecan in order to study its allergenicity.

**Methods:** 2S albumins from crude pecan extract were purified using two size-exclusion chromatography steps. Sequence identification of pecan proteins was done by means of LC-MS/MS ion trap mass spectrometry, where protein bands of interest were excised from 1D and 2D SDS-PAGE gels and sent to the National Jewish Medical and Research Center – Mass Spectrometry Facility for analysis. All the peptides and proteins that were identified by mass spectrometry were validated by Spectrum Mill software and the NCBI database was used for plant species search.

**Results:** The two-step purification using size-exclusion chromatography in high salt PBS buffer, pH 7.4 gave single protein fractions corresponding to 2S albumins of pecan seeds. Four distinct peptides were generated through MS/MS analysis that matched the 2S albumin sequence from the NCBI database. 2D electrophoretic analysis of the 2S albumin fraction showed the presence of at least six peptides with pIs ranging between 5.0 and 6.5, with two very distinct polypeptides appearing around pH 6.
Conclusion: This purification protocol allowed us to obtain a highly purified sample of 2S albumins from a natural source that can further be used for characterization and assessment of its allergenic potential.

INTRODUCTION

The prolamine superfamily contains a number of significant allergens of tree nuts, legumes, cereals, fruits, and vegetables. These allergens are further distributed in other subfamilies that include the 2S albumin family of seed storage proteins which includes the conglutin family, the non-specific lipid transfer proteins, and the cereal α-amylase and protease inhibitors (Radauer and Breiteneder, 2007). Moreover, two of the foremost types of proteins present in edible seeds are metabolic and storage proteins (Teuber et al., 2003). The primary function of seed storage proteins is to serve as nitrogen donors for seedling growth during germination (Wallowitz et al., 2006). Anaphylactic reactions to tree nuts are primarily associated with proteins belonging to the 2S albumins, the legumin group (the 11S proteins), and the vicilins group (the 7S proteins) of proteins (Teuber et al., 2003).

Clinical diagnostic practices of diagnosis of tree nut allergies in the U.S. typically utilize a signal skin prick test extract that contains proteins from multiple tree nuts. If a tree nut allergic individual has a positive reaction to the skin prick test, the clinical advice is to avoid all tree nuts. This may be sound advice since cross-contact and comingling of tree nuts can occur during harvest, transport, and/or processing, however; many tree nut allergic individual may be able to tolerate some tree nuts so avoiding all of them may not be warranted and could potentially decrease the tree nut allergic individual’s quality of
life. Of those tree nut allergic individuals who do have clinically diagnosed allergies to multiple tree nuts, it is believed that the high level of reactivity to multiple nuts is due to the fact that a majority of tree nuts contain shared antigenic proteins that are highly homologous in amino acid sequence or are very similar in quaternary and tertiary structure. For example, Brazil nuts, English walnuts, cashews, and hazelnuts all naturally contain 11S legumin seed storage proteins, while hazelnuts and almonds share profilins (Davis et al., 2008). The 2S albumin family of storage proteins seems to be intrinsically allergenic since these proteins have also been identified as major allergens in several species including Brazil nut, peanut, walnut, cashew, almond, hazelnut, oilseed rape, mustard seed, castor bean, sunflower seed, and sesame seed (Garino et al., 2010, Koppelman et al., 2004; Beyer et al., 2002; Moreno et al., 2004; Robotham et al., 2005; Pastorello et al., 2001, Poltronieri et al., 2002). In peanuts, for example, 2S albumins are shown to be more potent allergens than the 7S and 11S globulins (Chen et al., 2011; Porterfield et al., 2009; Koppelman et al., 2004). Additionally, IgE binding from allergic individuals’ sera to 2S albumins from multiple seeds and nuts have also been reported (Beyer et al., 2002; Gonzalez de la Pena et al., 1991; Thorpe et al., 1988; Pastorello et al., 2001; Poltronieri et al., 2002; Teuber et al., 1998).

The 2S albumin proteins are soluble in water at low salt concentrations and are commonly distributed in dicotyledonous plants. The 2S designation refers to the sedimentation constant in Svedberg units, which is a measure of the rate at which a molecule precipitates in a gravitational field and is another criterion used for classification of these proteins (Moreno and Clemente, 2008). The 2S albumins are globular, low molecular weight proteins that contain high levels of arginine, glutamine,
asparagine, and cysteine amino acids. The 2S albumins have a conserved 3D domain comprised of 5 α-helices, and a unique pattern of 8 cysteine residues that form 4-intramolecular disulfide bridges. The only post-translational modification of these proteins includes a proteolytic cleavage of many 2S proteins, leaving a heterodimer that consists of 2 polypeptide chains (4 kDa and 9 kDa) held together by 2 disulfide bonds. The precursor polypeptide is approximately 18-20 kDa molecular weight. The small and large subunits are connected by two intermolecular disulfide bonds, spanning between Cys (1) and Cys (5), and Cys (2) and Cys (3). The other two intra-chain disulfide bonds are present within the large subunit. The bonds connect Cys (4) with Cys (7), and Cys (6) with Cys (8) (Figure 3.1).

**Figure 3.1** Formation of the disulfide bonds between the eight cysteine residues in the 2S albumin protein family (Adapted from Moreno and Clemente, 2008).

The 2S albumins have a high level of polymorphism in the amino acid sequence within the family; in some cases less than 15% of amino acid homology has been observed (Monsalve et al., 2004). Moreover, regions covering the cysteine residues have
the highest amino acid sequence homology, while regions at the C-terminal end of the small subunit, the N-terminal of the large subunit, and between the sixth and seventh cysteine residues within the large subunit show the lowest sequence homology (Moreno and Clemente, 2008). A hypervariable region within the large subunit is another common feature of 2S albumins. This short variable loop is located between the 3rd and 4th \( \alpha \)-helices, and is portrayed as the most essential allergenic site of the 2S albumins (Lehmann et al., 2006). Inconsistency in its length and amino acid composition between species imply that this region does not contribute in determining the folded structure of a protein (Moreno and Clemente, 2008). The allergenicity of 2S albumins could therefore be attributed to their compact structure resulting from the disulfide bonds and the ability of these proteins to interact with membranes (Monsalve et al., 2004).

The identification and characterization of food allergens is a starting point in the management of food allergy in order to assure proper diagnosis, therapy, and detection. The purification of natural forms of allergens is important when studying IgE binding activity since the natural proteins can demonstrate a range of structural characteristics not present in recombinant forms (Pastorello and Trambaioli, 2001). Also, purified allergens are essential for the development of serological methods to be used for diagnosis, thus, minimizing the practice of food challenge tests. Additionally, pure forms of allergens can be used as references for the quantification of their content in foods and for recognizing the relationship between allergenicity and protein structure, stability, food matrix and processing. Finally, individual allergens have been suggested as candidate vaccines for allergen-specific immunotherapy (Sancho et al., 2010).
Recently, component resolved diagnostic tests have received attention in using purified allergens over total protein extracts, which have inherent drawbacks from inconsistent extraction efficiency. Purified allergens provide more standardization and consistency in these applications. Using purified allergens also allows improved sensitivity of in vitro diagnosis, recognition of geographic variances in patterns of sensitization to distinct allergens, association of the clinical symptoms to patterns of sensitization, identification of individual allergens as biomarkers for severity or resolution of food allergies, and identification of individual allergens as markers for cross-reactive allergies (Hoffman-Sommergruber et al., 2008).

Even though in recent years our knowledge on the allergenicity of the 2S albumin family of proteins has grown tremendously, molecular and biochemical information is still lacking on some of the major tree nuts that are consumed throughout the world. This study aims to purify the 2S albumin protein from pecan for further studies on the allergenic potential of this particular protein.
MATERIALS AND METHODS

Pecan 2S Albumin Purification

For the pecan 2S albumin purification, a sample of defatted pecan flour was extracted in 0.01 M PBS, 1M NaCl, pH 7.4 in a 1:10 (w/v) ratio on a horizontal rocker for 1h at room temperature. The extract was clarified by centrifugation for 30 min at 4,200 rpm at room temperature, followed by vacuum filtration using a Stericup Filter Unit (pore size 0.22 µm, Millipore, Billerica, MA). The soluble protein content was estimated by the Lowry method (1951). The filtered crude extract was then centrifuged at 13,000 x g for 10 min using a 0.45 µm Spin-X centrifuge tube filter (Costar, Corning, NY). The clarified extract was loaded onto a high performance Superose 12 10/300 GL column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) pre-equilibrated with elution buffer (0.05M PBS, 0.15M NaCl, pH 7.0) at a flow rate of 0.5 ml/min. The proteins were eluted from the column and collected in 1ml fractions for 50 min using Beckman System Gold HPLC system with a 126NM Solvent Module and a 168NM Detector (GMI Inc, Ramsey, MN). UV absorbance measured at 280 nm and separation and staining of samples in SDS-PAGE were used to detect proteins in the fractions that eluted from the column. Electrophoresis was performed as described in Chapter 2. The 2S albumin fractions were identified by the presence of approximately 10-15 kDa dominant protein bands in the stained SDS-PAGE gel. Fractions of interest were concentrated using Amicon Ultra-0.5 10K Centrifugal Filter Devices (Millipore Corp., Bellerica, MA) according to the guidelines from the manufacturer, where the samples were centrifuged for 15 min at 14,000 x g. To recover the concentrated solute, the filter device containing the sample was turned upside down and the sample was collected in a
clean micro centrifuge tube after spinning for 2 min at 1,000 x g. Samples after centrifugation were then pooled prior to another gel filtration step using the same column in order achieve a high separation of low MW proteins from high MW proteins. Fractions of interest were concentrated using acetone precipitation. One part of sample was mixed with 5 parts of 80% acetone and kept overnight at -20°C. The proteins were then centrifuged at 13,000 x g for 10 min. The supernatant was removed from the pellet, and the pellet was fully air-dried and then re-suspended in 0.01 M PBS, 0.15M NaCl pH 7.4. 

The total protein content of the samples was estimated by Lowry method (1951). The final pooled sample was analyzed by SDS-PAGE. Following staining, the gel images were captured using Kodak Gel Logic 440 imaging system and analyzed for presence of minor contaminants and impurities.

**2D Gel Electrophoresis**

Further analysis of protein purity was carried out with 2D gel electrophoresis. The separation of proteins was performed using a modified version of the 2D gel electrophoresis protocol developed by Bio-Rad Laboratories. Isoelectrofocusing (IEF) was performed on 7cm non-linear pH 4-7 strips (ReadyStrip IPG Strips, Bio-Rad Laboratories, Hercules, CA). Each strip was rehydrated in a rehydration buffer (8M Urea, 2% CHAPS, 50 mM DTT, and 0.5% ampholyte), where 50 mM DTT and 0.5% ampholyte were added to the buffer just before running the strip. The final concentration of protein was 25μg in 125μl of rehydration buffer. Focusing conditions were controlled with a multi-step program: the strip was rehydrated for 12h at 50V, Step1: 250V with a run time of 15 min, Step 2: 4000V with run time of 2h, Step 3: 4000V with voltage slope up to 30,000V. The second dimension electrophoresis was conducted using 4–20%
precast polyacrylamide gradient gel (Ready Gel Tris-HCl Gel, Bio-Rad Laboratories, Hercules, CA) in the Bio-Rad Mini-Protean system. Prior to running the SDS-PAGE, the immobilized pH gradient strip was first equilibrated for 15 min in equilibration buffer (6M Urea, 2% SDS, 0.375 M Tris-HCl, 20% Glycerol) with 20 mg/ml DTT, and then equilibrated for another 15 min in equilibration buffer containing 25 mg/ml iodoacetamide. After electrophoresis, protein were fixed on the gels with fixing solution and stained with Brilliant Blue G-Colloidal (Sigma, St. Louis, MO). Images were captured using the Kodak Gel Logic 440 imaging system.

2S Albumin Identification by LC-MS/MS Ion Trap Mass Spectrometry

Stained bands and spots from 1D and 2D electrophoresis were carefully excised using a sterile scalpel blade and placed in microcentrifuge tubes. The samples were analyzed at the National Jewish Health Mass spectrometry facility in Denver, CO. Samples analyses were performed according to the standard protein identification strategy using mass spectrometry described by Shevchenko et al. (1996). Briefly, the candidate proteins excised from the gel followed were reduced using 1.5 mg/ml dithiothreitol (DTT), then alkylated in 10 mg/ml iodoacetamide (IAA). The reduced and alkylated proteins were digested with trypsin overnight and extracted, then peptides were collected from the supernate by using a speed vacuum centrifuge to concentrate and to remove organic solvents. Peptides were chromatographically resolved on-line using a C18 column coupled to a1200 series high performance liquid chromatography instrument (HPLC, Agilent Technologies) and analyzed using a 6340 LCMS ion trap mass spectrometer (Agilent Technologies, Palo Alto, CA). The mass spectrometry systems include a HPLC-chip interface (Agilent Technologies). Raw data was extracted and
searched using the Spectrum Mill search engine (Rev A.03.03.038 SR1, Agilent Technologies, Palo Alto, CA). “Peak picking” was performed within SpectrumMill with the following parameters: signal-to-noise was set at a 5:1 ratio, a maximum charge state of 7 was allowed (z=7), and the program was directed to attempt to “find” a precursor charge state. During searching the following parameters were applied: the NCBI database with pecan was added to a plants database used at the National Jewish facility, carbamidomethylation was included as a fixed modification, and oxidized methionine as a variable modification, collected spectra were compared to tryptic peptides in the database, a maximum of 2 missed cleavage points was used, a precursor mass tolerance of +/- 2.5 and a product mass tolerance of +/- 0.7 was allowed, and a maximum ambiguous precursor charge = 4 was used. Data were evaluated and protein identifications were considered significant if the following confidence thresholds were met: a minimum of 2 peptides per protein, protein score > 20, individual peptide scores of at least 10, and Scored Percent Intensity (SPI) of at least 70% were required. The SPI provides an indication of the percent of the total ion intensity that matches the peptide’s MS/MS spectrum. Standards were run at the beginning of each day and at the end of a set of analyses for quality control purposes.

RESULTS AND DISCUSSION

In order to obtain individual proteins from food sources, purification protocols need to be optimized for each individual protein (Hoffman-Sommergruber et al., 2008). Gel filtration chromatography or size-exclusion chromatography is one of the most common techniques used for separation of proteins. Using gel filtration, proteins are
separated in solution on the basis of their size as the pass through a column bed. Ionic strength, pH, and the composition of the buffer used for solubilizing the proteins are not important factors as long as they do not affect the stability of the proteins and column media (Pastarello and Trambaioli, 2001). The Superose 12 10/300 GL column used in this project has a broad fractionation range and is very suitable for laboratory scale separation of proteins. It possesses high physical and chemical stability that is based on highly cross-linked porous agarose particles. Under normal chromatography conditions, non-specific interactions between proteins and Superose are insignificant when using buffers with ionic strengths in the range 0.15 M to 1.5 M (GE Healthcare, 2002). Sample purity was assessed by 1D SDS-PAGE, and 2D electrophoresis was conducted in order to determine the range of isoforms present. Tandem MS/MS coupled with LC was also used for internal sequence identification and to analyze the sample for possible impurities.

Several important 2S albumin allergens, including Brazil nut (Ber e 1) and yellow mustard (Sin a 1) have been separated with a single gel filtration step (Pastarello et al., 1998; Gonzalez de la Pena et al., 1991) Figure 3.2B shows SDS-PAGE of a pecan crude extract separated using size-exclusion chromatography. The probable 2S albumin bands representing Car i 1, were concentrated in one major peak that eluted from the column around 32-37 min (Figure 3.2A). This peak consisting of fractions 15-18 as indicated in Figure 3.2 was collected from several runs, and concentrated prior to subjecting the sample to another size-exclusion step in order to achieve greater separation of high MW proteins from low MW proteins. A second size-exclusion chromatography step gave resolution of two peaks containing low MW proteins with an elution time around 28-34 min for the higher peak and 34-37 min for the second peak (Figure 3.3A). The
application of the second size exclusion step allowed complete separation of the low MW proteins of interest from high MW proteins (Figure 3.3B). Three major bands assumed to represent 2S albumins were identified with molecular weights of approximately 16, 12, and 9 kDa. After the second chromatography step, each fraction from Figure 3.3B was subjected to further analysis by 2D electrophoresis. The IEF buffer likely did not have sufficient ionic strength to successfully separate proteins in fractions 17 and 18 (data not shown); therefore, protein bands from the 1D gel were sent for sequence analysis, along with the all six observed peptides in fraction 16 to confirm the protein sequence identity of these bands and spots (Figure 3.4). Venkatachalam et al. (2007) have encountered a similar issue when separating crude extracts of pecan cultivars. Two-dimensional separation of fraction 16 shows the possible presence of several isoforms of 2S albumins, or peptides having a different composition of amino acids and charge densities. Based on stain-binding intensity, there were two predominant peptides in the sample having pI values ranging between pH 5.5 and 6.0.

Figure 3.5 and Table 3.1 represent four different peptides corresponding to the pecan 2S albumin sequence found in the NCBI protein database that were identified by LC/MS/MS analysis in fraction 16. Along with peptides matching pecan 2S albumins, fractions 17 and 18 also had peptides identified as pecan 7S vicilin (globulin family) proteins (Table 3.2). The 7S vicilins are described as large trimeric oligomers having individual subunits usually around 40kDa and 70 kDa in size (Teuber et al., 1999). However, the Osborne fractionation of pecan proteins additionally identified a dominant globulin fraction band below 14 kDa on a denaturing SDS-PAGE gel (Venkatachalam et al., 2008). The observation that the soluble fraction of pecan proteins is primarily
comprised of glutelins and globulins, which require high ionic strength for adequate solubilization, can further explain the challenge encountered in 2D SDS-PAGE separation of selected fractions (Sathe et al., 2009; Venkatachalam et al., 2008). In order to obtain a high purity of pecan 2S albumin, fractions 17 and 18 were not included in final pool.

A final concentration of 1.3 mg/ml of purified pecan 2S albumin was obtained by pooling several runs of acetone-precipitated fraction 16 and re-solubilizing the proteins in 0.01 M PBS, 0.15 M NaCl, pH 7.4 (Figure 3.6). The acetone precipitation was used in order to concentrate the protein sample and further purify the sample from undesirable constituents. In order to rule out possible denaturation and alteration of the native structure of proteins, IgE binding assay showed that fraction (15-18) retained their allergenic activity (data not shown).

CONCLUSION

In the present study, the pecan 2S albumin was successfully purified using size exclusion chromatography coupled to a HPLC system. SDS-PAGE analysis of the purified sample indicated the presence of three bands, representing the intact protein (16 kDa), a large subunit (12 kDa), and a small subunit (9 kDa) of the 2S albumin from pecan (Figure 3.6). LC/MS/MS was used to verify the sequence identity of the pecan bands from 1D gel electrophoresis and spots from 2D gel electrophoresis, and aided in determination of the purity of the final pool of purified pecan 2S albumin. The protocol established in this study will allow purification of sufficient quantities of high purity pecan 2S albumin that can be used for further characterization of the proteolytic stability
of the 2S albumin from pecan and assess its allergenic potential before and after extensive in vitro gastrointestinal digestion.
Figure 3.2. Purification of Pecan 2S albumin [A] 1st size-exclusion chromatography profile of pecan extract at 280nm. [B] SDS-PAGE analysis under reducing conditions of 1nd size-exclusion fractions. The number on the top of the gel indicates the fraction collected in the major peak marked with an arrow in Figure 3.2A.

M- Molecular weight markers.
Maximum volume (45µl) of a sample was loaded in each lane.
Figure 3.3 Purification of pecan 2S albumin. [A] 2^nd size-exclusion chromatography profile of pecan extract at 280nm. [B] SDS-PAGE analysis under reducing conditions of 2^nd size-exclusion fractions. The number on the top of the gel indicates the fraction collected in two major peaks marked with arrows in Figure 3.3A.

M-Molecular weight markers
Red circles indicate bands that were excised and analyzed by LC/MS/MS to determine the amino acid sequence.
Maximum volume (45µl) of a sample was loaded in each lane.
Figure 3.4 2D SDS-PAGE analysis of fraction 16 from 2\textsuperscript{nd} size-exclusion chromatography.

M-Molecular weight markers
Red circles indicate spots that were excised and analyzed by LC/MS/MS to determine the amino acid sequence.
Protein load was approximately 25µg.
Figure 3.5 Pecan 2S albumin amino acid sequence from NCBI database (Acc. # AAO32314). Tryptic peptides identified by LC/MS/MS are shown in red. Arrows and the numbers indicate tryptic digestion sites within the sequence.

<table>
<thead>
<tr>
<th>Peptide Scores</th>
<th>Position and Sequence</th>
<th>Scored Percent Intensity (SPI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15.18</td>
<td>80 ((R)QCCQQLSQMEEQCQCEGLR(Q))</td>
<td>78.6</td>
</tr>
<tr>
<td>19.73</td>
<td>103 ((R)QQQQEEGIRGEEEmEEMVQCASDLPK(E))</td>
<td>89.8</td>
</tr>
<tr>
<td>17.49</td>
<td>103 ((R)QQQQEEGIRGEEEmEEMVQCASDLPKECGISSR(S))</td>
<td>76.9</td>
</tr>
<tr>
<td>13.75</td>
<td>128 ((K)ECGISSRSCEIR(R))</td>
<td>86.2</td>
</tr>
</tbody>
</table>

Table 3.1 Summary of peptides identified in fraction 16 by LC/MS/MS Ion trap matching the 2S albumin from the NCBI protein database.
<table>
<thead>
<tr>
<th>Peptide Scores</th>
<th>Position and Sequence</th>
<th>Scored Percent Intensity (SPI)</th>
<th>Protein ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>20.12</td>
<td>80 (R)QCCQQLSQMEEQCQCEGLR(Q)</td>
<td>95.3</td>
<td>2S Albumin</td>
</tr>
<tr>
<td>18.86</td>
<td>103 (R)QQQEEGIRGEEmEEMVQCAS DLPK(E)</td>
<td>84</td>
<td>2S Albumin</td>
</tr>
<tr>
<td>19.98</td>
<td>112 (R)GEEEMEEMVQCASDLPK(E)</td>
<td>87</td>
<td>2S Albumin</td>
</tr>
<tr>
<td>13.69</td>
<td>47 (R)WEFQQCQER(C)</td>
<td>84.3</td>
<td>7S Vicilin</td>
</tr>
<tr>
<td>12.26</td>
<td>171 (R)QQQQCQSR(C)</td>
<td>88.6</td>
<td>7S Vicilin</td>
</tr>
<tr>
<td>14.73</td>
<td>179 (R)CEERFEEEQR(R)</td>
<td>90.1</td>
<td>7S Vicilin</td>
</tr>
<tr>
<td>15.86</td>
<td>238 (R)CEERLEEERQ(R)</td>
<td>92.1</td>
<td>7S Vicilin</td>
</tr>
<tr>
<td>16.97</td>
<td>272 (R)YEQCCQQCER(Q)</td>
<td>90.7</td>
<td>7S Vicilin</td>
</tr>
<tr>
<td>11.66</td>
<td>284 (R)RGQEQQQLCR(R)</td>
<td>75.7</td>
<td>7S Vicilin</td>
</tr>
<tr>
<td>15.05</td>
<td>326 (R)CQTQEQSPER(Q)</td>
<td>85.7</td>
<td>7S Vicilin</td>
</tr>
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</table>

**Table 3.2** Summary of peptides identified in fraction 17 and 18 by LC/MS/MS-Ion trap corresponding to the pecan 2S albumin and 7S vicilin from the NCBI database.
Figure 3.6 SDS-PAGE separation under reducing conditions of pecan allergens and crude protein extract.
M-Molecular weight markers
Lane 1- purified 2S albumin after 2nd size-exclusion chromatography (2 µg protein load)
Lane 2- crude pecan extract (10 µg protein load)
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CHAPTER 4: IN VITRO DIGESTION STABILITY OF SOLUBLE PECAN PROTEINS IN CRUDE EXTRACT AND THE PURIFIED PECAN 2S ALBUMIN, Car i 1

Abstract

**Rationale:** The digestive stability of food proteins in the gastro intestinal tract is viewed as an important characteristic that may increase the allergenic potential of food proteins allowing them to retain their relative integrity, which increases their probability of sensitizing an individual through the mucosal immune system. The 2S albumin family of proteins has been viewed as highly resistant to treatment with various proteases. This study investigates the digestive stability of a crude pecan extract and purified pecan 2S albumin (Car i 1) in simulated gastric fluid (SFG) and simulated intestinal fluid (SIF) using various levels of proteases.

**Methods:** The resistance to digestion of soluble pecan proteins and purified Car i 1 was assessed with three levels of pepsin in simulated gastric fluid (SGF). In addition to the treatments with three levels of pepsin in SGF, the stability of the Car i 1 was also examined using two levels of trypsin and pancreatin in simulated intestinal fluid (SIF). The degree of proteolysis was monitored under reducing conditions using SDS–PAGE.

**Results:** The digestion experiments indicated that the high molecular weight proteins of the crude pecan extract were rapidly hydrolyzed and liable to digestion with pepsin in SGF compared to the low molecular weight proteins and polypeptides that remained stable even at high concentrations of pepsin over an extended period of time. The 2S albumin, Car i 1, was found to be resistant to treatment with pepsin, and relatively stable to proteolysis with trypsin and pancreatin with the generation of residual polypeptides.
Conclusion: The digestive stability of the 2S albumin may be a contributing factor that may aid in the allergic sensitization of naïve individuals to pecan. The identification of digestion resistant polypeptides is an important factor contributing to the assessment of food protein allergenicity. The observed digestive stability of Car i 1 in both SGF and SIF allows further evaluation and assessment of the allergenic potential of this protein and its digestion resistant peptides.

INTRODUCTION

Food allergy is becoming an increasing problem in Westernized countries and is also considered a major concern for food safety and public health. Strict avoidance diets are the only option for individuals affected by food allergies, however; widespread use of major allergenic food groups as food ingredients in a large variety of foods poses a great risk for unintentional contamination in processed food (Lehmann et al., 2006). The true prevalence of food allergy ranges between 3% and 4% in the general population, even though more than 20% of the general population in the U.S. believes that they have food allergies. The prevalence of true food allergies is higher in children under the age of 3 years, ranging from a 6-8% primarily due to early exposure and sensitization to cow’s milk, eggs and soy. Many of these children with milk, egg or soy allergies will develop tolerance by the time they reach school age (Sampson, 2005; Taylor et al, 1999).

Routes by which food allergens sensitize an individual are still ambiguous, however; most food allergens are thought to sensitize via the gastrointestinal tract (GIT). Therefore, gut permeability and digestibility are viewed as one of the main factors that affect the allergenic potential of a particular food. Food proteins must possess
physicochemical properties that allow them to preserve their structure from degradation in the gastrointestinal tract in order to cause sensitization (Moreno, 2007). Identified native allergenic proteins and protein fragments produced during digestion can be evaluated for their allergenic properties in order to understand the sensitization and elicitation phases involved in allergic reactions. Even though resistance to pepsin digestion is a common property for some food allergens, currently, no common feature of proteins or protein fragments has been identified as the major cause of sensitization to food allergens (Wickham et al., 2009).

For a food protein to be defined as an allergen it must be capable of inducing an allergic sensitization in naïve individuals which involves interaction of various immune cells that results in the production of specific IgE antibodies, and upon subsequent exposure the food protein must provoke an allergic reaction. The proteins having these characteristics are known as complete food allergens, while the others are referred as incomplete food allergens and are only capable of eliciting clinical symptoms due to their homology to another allergen (Schnell and Herman, 2009). Complete allergens tend to be stable to digestion in the gastrointestinal tract, whereas incomplete allergens are more prone to digestion (Bannon, 2004).

The allergens thought to sensitize via GIT, belonging primarily to the prolamine or cupun superfamily, have for the most part been shown to be resistant to degradation by proteases due in part to their structural stability. A conserved skeleton of cysteine residues which generally form four disulphide bonds provides the structural compactness of the prolamin superfamily, which includes the 2S albumins, non-specific lipid transfer proteins (nsLTP), cereal a-amylase/protease inhibitors and cereal prolamin families. The
2S albumins from mustard, rapeseeds, Brazil nuts, sunflower and sesame seeds have been shown to be particularly resistant to pepsin digestion. Additionally, intact IgE-binding domains were identified in a large digestion resistant fragment of the peanut allergen, Ara h 2, produced upon treatment with trypsin, chymotrypsin or pepsin (Moreno, 2007).

Results from in vitro digestion assays are used to measure the relative resistance of food proteins to proteolysis rather than predicting the allergenic potential of the proteins (Schnell and Herman, 2009). A protein that is resistant to the proteolytic and acidic conditions of the digestive tract has a greater probability of reaching the absorption sites in intestinal mucosa (Astwood et al., 1996). Additionally, digestion assays in simulated gastric fluid (SGF) are currently required as part of the decision tree and a weight of evidence approach used for the assessment of the allergenic potential of transgenic proteins expressed in food crops (Goodman et al., 2008). In the assessment, the pepsin digestion assays are used in combination with other criteria including the source of a gene, sequence homology of novel proteins to known allergens, and serum IgE tests (Goodman et al., 2008; Codex, 2003). Moreover, a protein allergenic potential cannot be predicted with any single test including pepsin resistance assay (Taylor, 2003).

Moreover, factors such as the abundance of the allergen in food (e.g. ovalbumin represents 54% of egg white protein), effects of food processing (e.g. food allergens may retain allergenicity after processing such as baking), and food matrix interactions need to be taken into consideration when assessing the allergenic potential of a particular protein (Astwood et al., 1996; Schnell and Herman, 2009). The effect of the food matrix on protein digestibility was demonstrated in the study by Grimshaw et al. (2003) where a relatively high-fat food matrix prevented the onset of mild oral symptoms prior to the
more severe systemic immune responses in a DBPCFC study with peanut-allergic subjects. The authors believe that the high-fat food matrix (chocolate) may have masked the allergenic epitopes until digestive proteases and acidic conditions during digestion released the emulsified proteins from the fat. It is also plausible that the high tannin and phenolic content of chocolate may have bound allergenic peanut proteins that were later released during digestion. Moreover, a study by van Wijk et al. (2005) showed that the soluble peanut allergens require the adjuvant capacity of food matrix to induce sensitization since the digestion profiles of peanut crude extract and purified Ara h 1 produced very similar digestion profiles. Association of proteins with cell membranes and other lipid structures found in foods has been termed as an important property in promoting the allergenicity of the food proteins (Moreno, 2007).

The pepsin digestion protocols used for determination of the relative stability of a potential allergenic protein typically use levels of pepsin that may be far in excess than levels of pepsin typically found in the stomach during in digestion of a meal. Pepsin secretion in adults has been estimated to be between 20 and 30 k Units of enzyme activity/24 h at 37°C, which is equivalent to approximately 10 mg of pepsin secreted/24 h in commercially available pepsin. Adult dietary consumption of protein is approximately 75 g/24 h which would give a ratio of ~3 mg protein/Unit pepsin secreted compared to ~3 µg protein/Unit pepsin used during digestion assays, indicating that protein normally exceeds pepsin levels during in vivo digestion (Moreno, 2007; Wickham et al., 2009).

As a result of the excess pepsin used in in vitro digestion assays and the lack of the mechanical churning action of the stomach, many of the SGF assay do not actually mimic the gastric digestion in humans but only represents a standardized gastric digestion
model for comparative proteolysis under acidic conditions. *In vitro* digestion assays do have value as they can be used as a convenient tool to characterize food proteins of interest (Schnell and Herman, 2009; Untersmayr and Jensen-Jarolim, 2006). Proteins that are not digested by 90% in 20 min and produce pepsin-stable peptide fragments of more than 5 kDa have an increased probability of causing food allergy (Ofori-Anti et al., 2008). All food allergens evaluated in the study by Astwood et al. (1996) were remarkably more resistant to pepsin hydrolysis when compared to other nonallergenic proteins. Interestingly, some of the most common food allergens such as the major milk allergen (α-Casein), the major fish allergen (parvalbumin), and the major egg allergen (ovalbumin) were easily digested in SGF (Bannon et al., 2003; Untersmayr and Jensen-Jarolim, 2006). Furthermore, nonallergenic proteins such as zein from corn or concanavalin were shown to be very stable to pepsin digestion. Consequently, proteins should have the ability to stimulate the immune system in order to sensitize naïve individuals and/or elicit an allergic reaction, in addition to digestive stability (Moreno, 2007).

*In vitro* gastrointestinal digestion assays need to be combined with immunological assays in order to determine the role of large digestion-resistant fragments and the influence of the food matrix on the stimulation of the immune system (Moreno, 2007). Furthermore, susceptibility to gastric digestion decreases the potential of food proteins to bind IgE, which may increase the threshold dose of allergens required to elicit symptoms in individuals affected with food allergies (Untermayr and Jensen-Jarolim, 2008). Therefore, *in vitro* digestion assays should only be used for assessing protein resistance...
and isolating digestion resistant peptide fragments with potential allergenic epitopes (Moreno, 2007).

Although there are numerous studies that have examined the stability of 2S albumins from various seeds such as peanut, Brazil nuts, sunflower and sesame seed, this information is still lacking on pecan proteins. Only the study conducted by Venkatachalam et al. (2006) had evaluated the susceptibility of soluble pecan proteins to enzymatic digestion in SGF with pepsin and in SIF with pancreatin. Consequently, due to its proven digestive stability and allergenicity in other nut seeds, this study aims to evaluate digestion stability of a pecan protein extract and the purified pecan 2S albumin, Car i 1, using the main gastric enzyme, pepsin, and two intestinal enzymes systems which included trypsin separately and pancreatin which includes several digestive enzymes including amylase, trypsin, lipase, ribonuclease and protease.

**MATERIALS AND METHODS**

**Test Proteins**

Crude pecan extract was prepared from defatted pecan flour by extracting in 0.01 M PBS, 1M NaCl, pH 7.4 in a 1:10 (w/v) ratio on a horizontal rocker for 1h at room temperature. The extract was clarified by centrifugation for 30 min at 4,200 rpm at room temperature, followed by vacuum filtration using Stericup Filter Unit (pore size 0.22µm, Millipore, Billerica, MA). The pecan 2S albumin, Car i 1, was purified as described in Chapter 3.
Proteases

Porcine pepsin purified from the stomach with a specific activity of 2,730 U/mg was purchased from Worthington Biochemical Corporation (Lakewood, NJ, USA, #M8C10390). Trypsin from bovine pancreas with a specific activity of 271 U/mg P (treated with L-(tosylamido-2-phenyl) ethyl chloromethyl ketone, TPCK to prevent contaminating chymotrypsin activity) was purchased from Worthington Biochemical Corporation (Lakewood, NJ, USA, #30P12338). Pancreatin 8X, USP from porcine pancreas with protease a specific activity of 210 USP U/mg was obtained from MP Biomedical, LLC (Solon, OH, USA, #193976). The proteases were used for digestion assays immediately after being dissolved in appropriate buffers in order to prevent possible loss of activity due to auto-digestion.

Pepsin Activity Validation Assay

The proteolytic activity of pepsin was determined before every digestion experiment using the pepsin activity protocol of Ofori-Anti et al. (2008). Solutions used in the assay include: 5% Trichloroacetic acid (TCA) prepared using 6.1 N TCA (Sigma, St. Louis, MO), 300 mM HCl prepared using 6 N HCl (Fisher Scientific, Pittsburg, PA), and simulated gastric fluid (SGF) (0.084 N HCl, 35 mM NaCl, pH 1.2).

A 2.0 % acidified hemoglobin (Hb) solution (Sigma, St. Louis, MO) was made by adding 5 ml of 300 mM HCl to 20 ml of the Hb solution (0.5g of hemoglobin in 20 ml of deionized water). Pepsin was dissolved in SGF fluid to a final concentration of 0.03 mg/ml. The test solution and the blank solution samples were made in three replicates. The test solution was made by adding 0.25 mL of SGF fluid with pepsin to 1.25 mL of acidified hemoglobin. After a 10 min incubation at 37°C, 2.5 ml of 5% of TCA was
added to stop the digestion reaction. The test solution was mixed and allowed to stand for additional 10 min at room temperature. The sample was then filtered through 0.45 µm PTFE syringe filter (Fisher Scientific, Pittsburg, PA) to remove undigested hemoglobin. The blank solution was prepared by adding 0.25 ml of SGF fluid with pepsin to a sample tube containing 1.25 mL of acidified hemoglobin and 2.5 mL of 5% TCA (to prevent pepsin activity). The blank solution was incubated for 10 min at 37°C and allowed to stand for additional 10 min at room temperature before filtering through 0.45 µm PTFE syringe filter. The absorbance at 280 nm was measured for each filtrate. The mean absorbance of the three blank samples was subtracted from the mean of the test solution samples. The difference is then multiplied by the dilution factor (1, when using the 0.03 mg/ml pepsin solution for the assay) and then divided by a constant factor of 0.01 (0.001 change in absorbance at 280 nm per unit of pepsin, unit definition, times 10 min). The result represents the calculated activity units of pepsin activity per mL of the digestion fluid. The specific activity (units/mg solid) is determined by dividing the activity expressed in U/ml with 0.03 mg and compared to the certified value. Acceptable limits for standard deviation is ±23% of company quoted activity per mg.

**In vitro Pepsin Digestion Assay**

The method of Ofori-Anti et al. (2008) was followed for determination of the digestive stability of pecan crude extract and purified pecan 2S albumin proteins with some minor modification.

The simulated gastric fluid (SGF) contained 0.084 N HCl and 35 mM NaCl (pH of 1.2). Buffer solutions used for pepsin digestion included: 200 mM NaHCO3 (pH 11), and 5 X Reducing Laemmli buffer (62.5 mM Tris-HCl, pH 6.8, 2%SDS, 25% glycerol,
0.01 % bromophenol blue, containing 350 mM dithiothreitol (DTT)), reaction buffer without pepsin (SGF) pH 1.2, and SGF + Pepsin at pH 1.2.

The protein reaction mix, P$_{mx}$, enzyme (pepsin) reaction mix, E$_{mx}$, and digestion reaction mix, D$_{mx}$, were prepared by combining 10 µl protein solution and 190 µl SGF (no pepsin), 10 µl H$_2$O and 190 µl SGF+pepsin, and 20 µl protein solution and 380 µl SGF+pepsin, respectively. In addition to the protein solution, a 1:10 dilution of protein solution was also made as a control point to estimate 90% digestion of the targeted protein profile on a SDS-PAGE gel, P$_{1/10}$. The digestion mix contained 380 µl of simulated gastric fluid (SGF) at pH 1.2 and 10 U, 1U, or 0.1U of pepsin per 1 µg of test protein. The protein solution, SGF solution (no enzyme), SGF+pepsin, and water were preheated separately for 10 minutes at 37ºC before the start of the experiment.

Control points for the protein solution were obtained by drawing 40 µl from P$_{mx}$ and mixing with 14 µl of 200 mM NaHCO3 (pH 11.0) and 14 µl of 5X reducing sample (Laemmli) buffer at time zero, following by immediate heating for 10 min at >75ºC. The P$_{mx}$ solution was then incubated for 1h at 37ºC and another 40 µl was drawn and mixed with the NaHCO3 and Laemmli buffer for a control point at 60 min of digestion. The same time points were collected from E$_{mx}$ to assure control points for the enzyme used and to evaluate possible auto-digestion of the enzyme. For the P$_{1/10}$ control, 38 µl SGF+pepsin was added to the NaHCO3 and Laemmli buffer, heated immediately at >75ºC for 10 minutes, and then 2 µl of the 1:10 dilution of protein solution was added to the mixture followed by another heating step. Digestion samples of 40 µl were collected at 0.5, 2, 5, 10, 20, 30 and 60 min intervals from the D$_{mx}$ tube. Acquiring the control at time zero of digestion was accomplished by adding 38 µl of SGF+pepsin to the NaHCO3
and Laemmli buffer and heating for 10 min at >75°C, followed by addition of 2 µl of protein solution and another heating step. Digestion was stopped at the appropriate times by mixing 40 µl from D_mx with 14 µl of 200 mM NaHCO3 (pH 11.0) and 14 µl of 5X reducing sample (Laemmli) buffer. Samples tubes are then heated for 10 min at >75°C. Immediately after heating all samples were stored on ice or at -20°C for later use. SDS-PAGE analysis was conducted as described in Chapter 2, and gel profiles were captured using the Kodak Gel Logic 440 imaging system.

**In vitro Trypsin and Pancreatin Digestion Assay**

The Goodman Laboratory protocol for the digestion of proteins using simulated intestinal fluid (SIF) containing trypsin or pancreatin was followed with minimal modifications (Goodman et al., unpublished data).

Solutions for the simulated intestinal digestion assay included: Simulated intestinal fluid (pH 7.5) that is described in the 26th United States Pharmacopeia (USP 26) as a 0.05 M buffer solution containing potassium dihydrogen phosphate (Rocca Chemical Company, Arlington, Texas, #7109.75-16); and 5 X Reducing Laemmli buffer (62.5mM Tris-HCl, pH 6.8, 2%SDS, 25% glycerol, 0.01 % bromophenol blue, containing 350mM dithiothreitol (DTT)).

The protein reaction mix (P_mx), enzyme (trypsin or pancreatin) reaction mix (E_mx) and digestion reaction mix (D_mx) were prepared by combining 10 µl protein solution and 190 µl SGF (no pepsin), 10 µl H2O and 190 µl SIF+ trypsin or pancreatin, and 20 µl protein solution and 380 µl SIF+ trypsin or pancreatin, respectively. In order to estimate 90% digestion of the targeted protein profile on a SDS-PAGE gel, P_1/10, a 1:10 dilution of the protein solution was made. The digestion mix contained 380 µl of simulated intestinal
fluid (SIF) at pH 7.5 and a ratio of 50 U or 5 U of trypsin or pancreatin per 1 mg of test protein. The protein solution, SIF solution (no enzyme), SGF+trypsin or pancreatin, and water were preheated separately for 10 min at 37°C before the start of the experiment.

Control points for the protein solution were obtained by drawing 40 µl from P_{mx} and mixing with 10 µl of 5X reducing sample (Laemmli) buffer at time zero following by immediate heating for 10 min at >75°C. The P_{mx} solution was then incubated for 2h at 37°C and another 40 µl was drawn and mixed with Laemmli buffer for the control point at 60 min of digestion. The same time points were collected from E_{mx} to assure control points for the enzyme used and to evaluate possible auto-digestion of the enzymes. For the P_{1/10} control 38 µl SGF+trypsin or pancreatin was added to Laemml buffer, heat immediately at >75°C for 10 minutes, and then 2 µl of a 1:10 dilution of protein solution was added to the mixture followed by another heating step. Digestion samples of 40 µl were collected at 2, 5, 10, 20, 30, 60 and 120 min intervals from the D_{mx} tube. The control point at time zero of digestion was prepared by adding 38 µl of SGF+trypsin or pancreatin to Laemml buffer and heating for 10 min at >75°C, followed by addition of 2 µl of protein solution and another heating step. Digestion was stopped at the appropriate times by mixing 40 µl from D_{mx} with 10 µl of 5X reducing sample (Laemmli) buffer. Samples tubes were then heated for 10 min at >75°C. Immediately after heating all samples were stored on ice or at -20°C for later use. SDS-PAGE analysis was conducted following the protocol described in Chapter 2, and the gel profiles were captured using the Kodak Gel Logic 440 imaging system.
RESULTS AND DISCUSSION

The *in vitro* digestion assays (SGF, SIF, or SGF and SIF in tandem) followed by SDS-PAGE analysis are used to evaluate whether or not a protein could be degraded to fragments capable of eliciting an IgE reaction (Fu, 2002). These digestion resistant fragments must be of sufficient size (>3 kDa) and contain at least two IgE binding epitopes in order to cross-link two surface bound IgE antibodies on the mast cells or basophils and cause an allergic reaction (Huby et al., 2000).

As indicated by Moreno (2007), the amount of pepsin used in digestion experiments should be based on enzyme activity instead on weight in order to provide a more dependable measure of the protease used. The multi-laboratory evaluation of an *in vitro* pepsin digestion assay protocol conducted by Thomas et al. (2002) on common allergenic and non-allergenic proteins in SGF used a 10 U of pepsin per 1µg of test protein ratio (3:1, w:w). Moreno (2007) showed that development of multi-phase digestion models provides useful information by stressing the importance of using a physiologically relevant *in vitro* digestion system.

Digestion of pecan crude extract was conducted according to the protocol of Ofori-Anti et al. (2008) using 10 U of pepsin per 1µg of test protein ratio (3:1, w:w) pepsin as indicated by Thomas et al. (2002). The degree of proteolysis was monitored in the presence of a reducing agent by SDS–PAGE. High molecular weight proteins are highly susceptible to proteolysis (Figure 4.1). Molecular weight proteins greater than 15 kDa were degraded within first 30 sec of digestion. A low MW protein fragment, approximately 12 kDa, was partially hydrolyzed based on the comparison to the band intensity of undigested proteins. This digestion resistant fragment seems to correspond to
the heavy polypeptide chain of the native pecan 2S albumin (Sharma, 2010). Rapid hydrolysis of higher MW proteins has also been observed in peanut, almonds, cashews, and walnut (Koppleman et al., 2010; Sathe, 1993; Sathe et al., 1997; Sze-Tao and Sathe, 2000). Bands corresponding to the 40 kDa band represent pepsin. Very slight auto-hydrolysis fragments of pepsin can be observed in the enzyme control lanes. Lowering the concentration of pepsin by 10 and 100 fold (Figures 4.2 and 4.3) did not have significant effect on the pepsin digestion stability of the high MW pecan proteins. Again, complete degradation was observed in the first 30 sec. However, a fragment at approximately 16 kDa remained stable for the first 10 min with a 10 fold dilution of pepsin (Figure 4.2), and was stable throughout the entire digestion time (up to 60 min) using a 100 fold dilution of pepsin (Figure 4.3). Stable protein fragments with MWs less than 10 kDa were detected in digestion at all three levels of pepsin.

The pecan 2S albumin, Car i 1, showed a significant stability to pepsin hydrolysis with each of the three tested levels (Figures 4.4, 4.5, and 4.6). The protein band with the highest molecular weight (~16 kDa) corresponding to a precursor form of the 2S albumin with covalently bound large and small subunit (Sharma, 2010) remained intact for up to 5 min at the high pepsin concentration (Figure 4.4). The same protein band was stable up to 30 min at 1U of pepsin and for the entire digestion time in 0.1 U of pepsin (Figures 4.5 and 4.6). The small subunit (~5 kDa) was partially hydrolyzed by 30 min with 10 U of pepsin, while it remained stable throughout the digestion course in both 1U and 0.1 U of pepsin. Some proteolytic breakdown is observed in large subunit (~ 12 kDa) at 30 min of digestion with 10 U of pepsin (Figure 4.4). However, when estimated on band intensity, the majority of the protein appeared to remain intact. Further, the large subunit remained
intact during 1 h of digestion with 1 U and 0.1 U of pepsin (Figures 4.5 and 4.6).

Findings from this study coincide with the results from digestion experiments on 2S albumins from peanut, sesame seed, and Brazil nut where the 2S albumin proteins of these seeds were also found to remain stable to pepsin digestion for an extended period of time (Sen et al., 2002; Koppleman et al., 2005; Koppleman et al., 2010; Orruno and Morgan, 2011).

With regard to the digestive stability in SIF with both trypsin and pancreatin, Car i 1 appeared to be more susceptible to hydrolysis in these systems than in the SGF system. At a higher concentration of trypsin and pancreatin, 50 U per 1 mg of protein, complete hydrolysis of the 16 kDa band is observed within 5 min of digestion (Figures 4.7A and 4.8A). Substantial hydrolysis of the large subunit was also observed in treatments with both proteases; however, weak bands were still visualized after the 2 h digestion time. The 10 fold dilution of enzyme mixes greatly influenced the susceptibility of Car i 1 to both trypsin and pancreatin. Both the large and small subunit remained stable during the entire digestion time. Generation of new fragments was observed in the 12 kDa range (Figures 4.7B and 4.8B). Additionally, intact Car i 1 protein (16 kDa band) was also observed at the end of the digestion with pancreatin (Figure 4.8B), however, it appeared that the intact protein was degraded by 30 min with trypsin (Figure 4.7B). Similar levels of trypsin were used when examining the digestive stability of the major peanut 2S albumin allergen, Ara h 2, in SIF and observed similar stability patterns (Koppelman et al., 2010, Sen et al., 2002).
CONCLUSION

The *in vitro* gastrointestinal digestion systems used in this study provide insight on the digestive stability of soluble pecan proteins and the purified 2S albumin, Car i 1. Soluble high MW pecan proteins were rapidly hydrolyzed by pepsin; however, low MW fragments remained stable. Thus, complete proteolysis was not observed under all digestion conditions. The pecan 2S albumin showed remarkable stability in SGF, while it was more susceptible to proteolysis with trypsin and pancreatin. The different digestion patterns observed in this study could be due to the different cleavage specificities of all three proteases resulting in the various pecan protein fragments produced after digestion. The significant stability of the pecan 2S albumin protein to extensive gastrointestinal digestion could be a key factor in allergic sensitization to pecan as these stable protein fragments may have more time to interact with the immune system. The relevance and IgE-binding capacity of the remaining peptides remains to be further evaluated and will be described in detail in Chapter 5 of this thesis.
**In vitro Digestion of Pecan Crude Extract in SGF**

**10U of Pepsin**

*Figure 4.1* SDS-PAGE analysis under reducing conditions of SGF digestion of a crude pecan extract with 10 U of pepsin per 1µg of protein extract. The number on the top of the gel indicates time point of digestion.

M-Molecular weight markers  
P-Undigested crude pecan protein  
P_{1/10}-The 1:10 dilution of P + enzyme (pepsin) at time=0  
D-Digestion of P by pepsin at various time points  
E-Enzyme (pepsin)  
Note: The remaining band at approximately 40 kDa represents pepsin
In vitro Digestion of Pecan Crude Extract in SGF

1U of Pepsin

Figure 4.2 SDS-PAGE analysis under reducing conditions of SGF digestion of a crude pecan extract with 1 U of pepsin per 1 µg of protein extract. The number on the top of the gel indicates time point of digestion.

M-Molecular weight markers
P-Undigested crude pecan protein
P<sub>1/10</sub>-The 1:10 dilution of P + enzyme (pepsin) at time=0
D-Digestion of P by pepsin at various time points
E-Enzyme (pepsin)
Note: The remaining band at approximately 40 kDa represents pepsin
**In vitro Digestion of Pecan Crude Extract in SGF**

**0.1U of Pepsin**

![SDS-PAGE analysis](image)

**Figure 4.3** SDS-PAGE analysis under reducing conditions of SGF digestion of a crude pecan extract with 0.1 U of pepsin per 1 µg of protein extract. The number on the top of the gel indicates time point of digestion.

M-Molecular weight markers
P-Undigested crude pecan protein
P\textsubscript{1/10}-The 1:10 dilution of P + enzyme (pepsin) at time=0
D-Digestion of P by pepsin at various time points
E-Enzyme (pepsin)

Note: The remaining band at approximately 40 kDa represents pepsin
In vitro Digestion of Pecan 2S Albumin, Car i 1, in SGF

**10 U of Pepsin**

![SDS-PAGE analysis](image)

**Figure 4.4** SDS-PAGE analysis under reducing conditions of SGF digestion of pecan 2S albumin, Car i 1, with 10 U of pepsin per 1 µg of protein. The number on the top of the gel indicates time point of digestion.

M-Molecular weight markers
P-Undigested Car i 1 protein
P$_{1/10}$-The 1:10 dilution of P + enzyme (pepsin) at time=0
D-Digestion of P by pepsin at various time points
E-Enzyme (pepsin)

Note: The remaining band at approximately 40 kDa represents pepsin.
In vitro Digestion of Pecan 2S Albumin, Car i 1, in SGF

1U of Pepsin

Figure 4.5 SDS-PAGE analysis under reducing conditions of SGF digestion of pecan 2S albumin, Car i 1, with 1 U of pepsin per 1 µg of protein. The number on the top of the gel indicates time point of digestion.

M-Molecular weight markers
P-Undigested Car i 1 protein
P_{1/10}-The 1:10 dilution of P + enzyme (pepsin) at time=0
D-Digestion of P by pepsin at various time points
E-Enzyme (pepsin)
Note: The remaining band at approximately 40 kDa represents pepsin
In vitro Digestion of Pecan 2S Albumin, Car i 1, in SGF

0.1 U of Pepsin

**Figure 4.6** SDS-PAGE analysis under reducing conditions of SGF digestion of pecan 2S albumin, Car i 1, with 0.1 U of pepsin per 1 µg of protein. The number on the top of the gel indicates time point of digestion.

M-Molecular weight markers
P-Undigested Car i 1 protein
P<sub>1/10</sub>-The 1:10 dilution of P + enzyme (pepsin) at time=0
D-Digestion of P by pepsin at various time points
E-Enzyme (pepsin)
**In vitro Digestion of Pecan 2S Albumin, Car i 1, in SIF (Trypsin)**

[A]

[B]

**Figure 4.7** SDS-PAGE analysis in the presence of DTT of SIF digestion of pecan 2S albumin, Car i 1, with [A] 50 U of trypsin per 1 mg of protein; [B] 5 U of trypsin per 1 mg of protein. The number on the top of the gel indicates time point of digestion.

M-Molecular weight markers  
P-Undigested Car i 1 protein  
P\textsubscript{1/10}-The 1:10 dilution of P + enzyme (trypsin) at time=0  
D-Digestion of P at various time points  
E-Enzyme (trypsin)  
Note: The remaining bands at approximately 75 kDa and 23 kDa represents trypsin fragment
**In vitro Digestion of Pecan 2S Albumin, Car I 1, in SGF (Pancreatin)**

[A]

[B]

**Figure 4.8** SDS-PAGE analysis in the presence of DTT of SIF digestion of pecan 2S albumin, Car i 1, with [A] 50 U of pancreatin per 1 mg of protein; [B] 5 U of pancreatin per 1 mg of protein. The number on the top of the gel indicates time point of digestion.

M-Molecular weight markers  
P-Undigested Car i 1 protein  
P_{1/10}-The 1:10 dilution of P + enzyme (pancreatin) at time=0  
D-Digestion of P at various time points  
E-Enzyme solution (pancreatin)

Note: The remaining bands at approximately 37 kDa in lane D_{120} represent pancreatin fragment
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CHAPTER 5: ASSESSMENT OF IgE BINDING TO PURIFIED 2S ALBUMIN, AND ITS DIGESTION-RESISTANT PEPTIDES

Abstract

Rationale: Pecan seeds can on occasion cause life-threatening, IgE-mediated, anaphylactic reactions in susceptible individuals. The evaluation of IgE antibody binding to epitopes provides information for assessment of allergenic potential of a protein of interest. The purpose of this study is to evaluate the IgE banding capacity and specificity to the pecan 2S albumin, Car i 1, and its digestion resistant peptides.

Methods: After individual serum characterization, a pool of sera from five allergic individuals with positive ImmunoCap scores for pecan was used in a qualitative immunoblot assessment. One dimensional SDS-PAGE analysis and immunoblotting were performed under reducing conditions on Car i 1 and its digestion resistant fragments from SGF and SIF assays to visualize IgE binding.

Results: The IgE antibody binding capacity to digestion-resistant fragments of 2S albumin in SGF was significantly influenced with the pepsin level showing decreased binding to Car i 1 that was digested with higher levels of pepsin, however; the binding was still observed with all three levels of protease. Very weak binding of IgE was observed in trypsin digested fragments. Pancreatin lowered the IgE binding capacity to lesser extent than trypsin.

Conclusion: Characterization of individual serum demonstrated variability in binding to the purified pecan 2S albumin, Car i 1. Qualitative differences in binding comparing purified Car i 1 and its digestion resistant peptides generated in SGF and SIF systems.
were detected, however; digestion-stable peptides produced in all systems retained IgE binding. Most importantly, partial digestion of Car i 1 produced peptides that have the potential to remain immunologically active despite extensive digestion that could play a key role in sensitization of susceptible individuals to pecans.

INTRODUCTION

Among all food allergy mechanisms, IgE-mediated reactions are the most extensively studied and best characterized (Crespo et al., 2006). An allergic reaction to tree nuts is a type I, IgE-mediated, immediate hypersensitivity reaction. The severity of the allergic reactions can occasionally be life threatening. The consumption of pecans and other nuts has been increasing, owing their increase popularity to their potential health benefits and pleasing taste. Increased consumption may also be contributing to the increase in the prevalence of pecan allergies observed in the U.S. Avoidance diets are particularly challenging for allergic individuals due to accidental ingestion and the use of nuts as hidden ingredients in various food products (Sicherer and Sampson, 2000; USDA, 2010). Therefore, the detection of allergens in foods is conducted for a couple of reasons: (1) to detect and quantify the presence of targeted allergen and (2) to determine whether the targeted allergen can possibly provoke clinical symptoms (Sathe et al., 2005).

There is an increasing need to improve the management and treatment of IgE-mediated of food allergy. A prerequisite for type I allergic reactions is the binding of IgE antibodies to specific regions on the surface of an allergen (Albrecht et al., 2009).
Proteins can be defined based on their primary structure (i.e. the amino acid sequence), protein fold, domain structure, and surface structure. The epitope is a part of the surface structure of an allergen that interacts with the antibody on an atomic level, thus indicating the importance of the surface structure of an allergen for antibody binding capacity (Aalberse, 2000). Allergen binding sites recognized by specific IgE antibodies are often categorized as continuous or discontinuous epitopes or as linear or conformational epitopes. The amino acids residues of conformational epitopes are discontinuously distributed over a protein’s sequence and occur in sterical vicinity to one another in the correctly folded protein. The linear epitopes are continuous stretches of protein’s primary amino acid sequence that are recognized by the specific antibodies. In a folded protein, even a linear epitope is forced into a certain conformation by the 3-D structure of the molecule (Albrecht et al., 2009). Moreover, there can be numerous linear and conformational epitopes on a single allergenic protein and all epitopes could possibly bind a specific antibody with particular affinity and avidity (Sathe et al., 2005). Hence, characterization of allergens at a molecular level is necessary to further advance the quality of both in vivo and in vitro immunoassays used for detection of IgE antibodies of defined allergens.

IgE antibody immunoassays are classified into qualitative, semi-quantitative, or quantitative assay according to the National Committee for Clinical Laboratory Standards (NCCLS) guidelines depending on the degree of accuracy of the quantity of IgE antibody that can be detected in an assay (Plebani, 2003). The detection of specific IgE antibodies in in vitro assays is based on using either crude extract or purified
allergens. The allergenic activity of purified proteins can be confirmed by using sera from individuals with proven food allergy in IgE immunoblots, ELISA, and RAST assays (Hoffman-Sommergruber et al., 2008). Western blotting assays are typically used for qualitative characterization and identification of IgE-binding proteins or cross-reactivity between allergens where proteins are transferred onto nitrocellulose or PVDF membranes after separation by SDS-PAGE. This assay provides information on individual protein bands allowing the detection of impurities with IgE reactivity (Sancho et al., 2010).

The food proteins and their digestion resistant fragments are able to induce the immune responses as they pass through the gastrointestinal tract. Immune responses to linear stretches of the amino acid sequence of the allergens seem to be more favored than to conformational epitopes due to the alteration of the native protein fold by the proteases. Linear IgE-binding epitopes in numerous food groups, including peanut, milk, egg, shrimp, and soybean have been identified (Albrecht et al., 2009). In order to effectively bind IgE antibody, Huby et al. (2000) indicated that an allergen must retain at least two IgE binding epitopes. Therefore, as suggested by Moreno (2007), in vitro gastrointestinal digestion assays should be used in conjunction with immunological assays in order to evaluate the role of digestion-resistant fragments on the stimulation of the immune responses.

Immunologically active epitopes have been identified for several major allergens capable of resisting hydrolysis by proteases in SGF and SIF systems. Studies by Sen et al. (2002) and Lehmann et al. (2006) have identified immunologically active digestion resistant peptides of Ara h 2 using sera from peanut allergic individuals. Additionally,
Moreno et al. (2005) were able to locate the IgE-binding epitopes of the Brazil nut 2S albumin following gastric digestion. IgE binding was also noted in digestion resistant fragments of the major crustacean allergen, tropomyosin, obtained from a crude extract of crab after treatment with proteinases (Yu et al., 2011). Nevertheless, all these results only indicate the sensitization and presence of specific IgE antibodies but do not imply that particular peptides will provoke clinical symptoms in allergic individuals. Experiments such as histamine release from basophils are necessary to further evaluate the allergenicity of these digestion resistant peptides. However, information about the structures that resist digestion and their potential to sensitize the immune system is scarce for many allergenic proteins.

The allergenic proteins of the most frequently implicated species causing tree nut allergies, include walnut, almond, and cashew have been extensively studied regarding their isolation, identification, biochemical characterization, and immunological activity (Fleischer, 2007; Sathe et al., 1997; Sze-Tao and Sathe, 2000a; Sze-Tao and Sathe, 2000b; Poltronieri et al., 2002; Sordet et al., 2009). Unlike these tree nuts, there is a lack of information on the identification, biochemical characterization and immunological activity of pecan allergens. The close family lineage of walnut and pecan could be used to explain the cross-reactivity between walnut and pecan, but the identity of the offending allergens has not been thoroughly studied (Teuber et al., 2000). In the only study on the assessment of pecan protein digestion stability, Venkatachalam et al. (2006) observed antigenic peptides of low molecular weight of crude pecan extract in SGF and SIF probed with IgE. The stability of the major allergens of tree nuts to digestion may also play an
important role in development of tree nut allergy where the exposure to undigested allergens may result in allergic sensitization. Understanding the nature of digestion resistant epitopes may offer new directions to treatments of tree nuts allergy. Therefore, this study aims to evaluate the fate of digestion resistant peptides of pecan 2S albumin, Car i 1, and their ability to bind specific IgE antibodies from allergic individuals after extensive digestion in \textit{in vitro} SGF and SIF systems.

\textbf{MATERIALS AND METHODS}

\textbf{Human Sera}

Twelve serum samples were obtained from patients with clinical history of moderate to severe allergic reactions to nuts from the University Medical Center in Utrecht, the Netherlands. Some of the patients specifically recalled having past allergic reactions to walnuts or hazelnuts while others did not specify or could not determine which tree nut had caused their allergic reaction. The sera were tested for sIgE in the ImmunoCap® test (Thermo Fisher Scientific) against peanuts and different tree nuts, including walnut, pecan, Brazil nut, cashew, hazelnut, and pistachio. Serum samples were additionally characterized for specific binding to crude pecan extract and purified pecan 2S albumin by immnoblot assay. In this study, five sera were pooled and used for characterization of digestion-resistant peptides in immunoassays, and serum from a non-allergic individual was used as a negative control.
SDS-PAGE and IgE Immunoblots

IgE immunoblotting was carried out to monitor the binding pattern of serum IgE from food allergic individuals to the allergenic proteins from walnut and pecan crude proteins, purified pecan 2S albumin proteins, and digestion-resistant proteins and peptides from pecan that were blotted onto polyvinyl difluoride (PVDF) membranes (Immunoblin$^\text{TM}$-P$^\text{SQ}$ PVDF membrane, 0.45µm 8.4cm wide X 7 cm long, Millopre Corporation, Billerica, MA).

All samples were separated by SDS-PAGE using the method previously described in Chapter 2. Following electrophoresis, gels were equilibrated in transfer buffer (1X Tris/Glycine Buffer, Bio-Rad Laboratories, 20% Methanol, Analytical Grade, Fisher Scientific) for 15min and then transferred to PVDF membranes for protein blotting. The transfer of proteins was carried out at a constant voltage of 70 V for 80 min.

In order to visualize the transfer of proteins the membrane was washed three times for 5 min in ROD water with gentle rotation. Coomassie Brilliant Blue R-250 staining solution (Bio-Rad Laboratories, Hercules, CA) was used for staining and visualizing transferred proteins. The membrane was stained for 1 min with gentle rotation at room temperature and destained by washing in multiple changes with Coomassie Brilliant Blue R-250 destaining solution (Bio-Rad Laboratories, Hercules, CA). The image of the air-dried membrane was captured using a Kodak Gel Logic 440 Imaging System.

The IgE immunoreactivity was evaluated using the following procedure. After the transfer of proteins to the PVDF membrane, the membrane was washed three times for 5
min in ROD water with gentle rotation to remove residual transfer buffer. The membrane was soaked in Ponceau S (Sigma-Aldrich St. Louis, MO) stain for approximately 1 min to verify the transfer of protein, and the stain was removed by washing the membrane multiple times with ROD water. The membrane was then blocked with PBST (0.01 M PBS with 0.05% Tween 20) containing 5% non-fat fry milk (NFDM) buffer for 2 hours at room temperature with gentle rotation. The human sera was diluted in 1:10 in PBST (PBS with 0.05% Tween 20) containing 2.5% NFDM and allowed to incubate at room temperature for 1 hour before adding to the blocked membrane.

The blocked membrane was incubated with human sera in a plastic bag overnight at room temperature with gentle rotation. Unbound IgE was removed by washing the membrane 4X for 5 minutes each time in approximately 20 ml of PBST. The secondary antibody, monoclonal anti-human IgE (Mouse Anti-Human IgE (ε chain specific), Southern Biotech, Birmingham, AL) conjugated with horseradish peroxidase (HRP), was diluted 1:1000 with PBST containing 2.5% NFDM and allowed to incubate at room temperature for 1 hour before adding it to the washed membranes. The membrane was incubated with secondary antibody for 1 hour at room temperature with gentle rotation. The membrane was then washed in approximately 20 ml of PBST, four times to remove unbound secondary antibody.

In order to visualize IgE binding a working solution was prepared by mixing equal parts of the Stable Peroxide Solution and the Luminol/Enhancer Solution (SuperSignal West Dura Extended Duration Substrate trial kit for use with horseradish peroxidase label, Thermo Scientific-Pierce Protein Research Products). The membrane
was briefly soaked in the working solution and placed on plastic transparency sheets, and a Kodak Gel Logic 440 image station was used to capture the emitted light. First, a reference image of the molecular weight markers on the membrane was captured using the white light. Images were then captured for at least two exposure times of 3 min and 10 min (longer if necessary), following re-wetting of the membrane with the working solution.

**Dot Blot**

A volume of 2 µl of each concentration \( (10^2, 10^1, 10^0, 10^{-1}, 10^{-2}, 10^{-3}) \) ng per spot) of serially diluted human IgE protein (Abcam Inc., Cambridge, MA) was spotted on a nitrocellulose membrane (Trans-Blot-Transfer Membrane, 0.45 µm pore size, 9 x 12 cm, Bio-Rad Laboratories, Hercules, CA) and blocked with PBST (PBS with 0.05% Tween 20) containing 5% NFDM buffer for 1 hour at room temperature with gentle rotation. The membrane was then washed with PBST buffer two times for 5 min each. The secondary antibody, monoclonal mouse anti-human IgE (Mouse Anti-Human IgE (ε chain specific), Southern Biotech, Birmingham, AL) conjugated with HRP, was diluted 1:1000 with PBST containing 2.5% NFDM and incubated at room temperature for 1 hour before adding it to the washed membranes. The membrane was incubated with secondary antibody for 1 hour at room temperature with gentle rotation. The membrane was then washed in approximately 50 ml of PBST, four times to remove unbound secondary antibody. The IgE was detected with the same detection system and imaged along with the blots to help gauge the signal strength.
RESULTS AND DISCUSSION

One characteristic that seems to be common for many food allergens is their ability to survive digestion by enzymes encountered in the GI tract. The resistance to *in vivo* digestion of allergenic food proteins enhances their potential for causing an allergic reaction in susceptible individuals because the immune responses are more likely to be triggered by intact proteins (Schnell and Herman, 2009). SDS-PAGE separation coupled with immunoblotting is the standard procedure used for allergen separation and identification. Analysis with human IgE is applied predominantly to identify and characterize new allergens (Poms et al., 2004). IgE-binding fragments were detected when tropomyosin was digested by pepsin, trypsin and chymotrypsin (Yu et al., 2011). Additionally, Sen et al. (2002) identified Ara h2 as the most resistant protein in digestion assays when exposed to chymotrypsin. Extensive digestion of Ara h 2 resulted in a 10 kDa digestion-resistant peptide that contained intact binding sites that were recognized by IgE despite the extensive *in vitro* digestion.

In our study we were able to identify IgE reactive digestion-resistant peptides of the pecan 2S albumin, Car i 1. Initially, 12 allergic sera with clinical history to tree nut allergy and positive ImmunoCAP scores to pecan were evaluated and characterized for their specificity of binding to crude pecan extract and purified pecan 2S albumin by immunoblotting. Sera showed great variability in the IgE-binding capacity to crude pecan extract with recognition of protein bands ranging from 5 to 100 kDa. Out of 12 sera assessed, 5 sera showed binding to low MW pecan proteins and purified pecan 2S albumin (Figure 5.1), while the remaining sera primarily recognized high MW proteins.
(data not shown). Non-specific binding to the molecular weight markers was observed in several sera. No apparent IgE binding was obtained using serum from a non-atopic, non-allergic subject, demonstrating the specificity of IgE reactivity in the sera from the 5 subjects used for further evaluation of the pecan proteins. ImmunoCAP values of the 5 sera are indicated in Table 5.1. All of these individuals had intermediate to high levels of specific IgE against pecan proteins and all had specific IgE against the pecan 2S albumin of interest. The selected sera were pooled and used for further assessment of IgE binding to digestion-resistant peptides of the pecan 2S albumin.

Immunoblotting using pooled sera indicated that the purified Car i 1 and its digestion-resistant peptides generated by treatment with pepsin in SGF were IgE reactive (Figure 5.2). Samples treated with pepsin levels at ratios of 10 U, 1U and 0.1 U to1 µg of test protein used in digestion assays generated stable fragments after 60 min of digestion. Figures 5.2A and B represent the SDS-PAGE separation of the samples and membrane staining using Colloidal R-250 staining solution, respectively. Clear differences in binding capacity were observed based on the level of enzyme used for digestion. However, even at the highest concentration of pepsin used in this study, 10 U, some IgE binding was detected (Figure 5.2C, lane C). Koppelman et al. (2010) used the same levels of pepsin for assessment of Ara h 2 stability in SGF. Additionally, Sen et al. (2002) also used a similarly high level of pepsin (enzyme: protein ratio of 1:2 w/w) for assessment of Ara h 2 and they identified IgE reactive digestion-resistant fragments for this 2S albumin peanut allergen. Furthermore, at the lowest level of pepsin, 0.1 U, all three distinct bands of the pecan 2S albumin, Car i 1, were detected as in crude pecan extract protein control,
showing that pepsin at this level had little effect. The 0.1 U of pepsin level could be viewed as an approximation of physiologically relevant enzyme levels (Moreno et al., 2005). Levels of 50 U and 5 U of trypsin and pancreatin per 1 mg of test protein were used for assessment of the digestive stability of the pecan 2S albumin in SIF. Stable fragments were observed up to 60 min and 120 min of digestion time as indicated in the SDS-PAGE analysis (Figure 5.3A, and Figure 5.4A). Figures 5.3 B and 5.4B represent the membrane staining of the digestion samples using Colloidal R-250 staining solution. Intact 16 kDa pecan 2S albumin was not detected at either level of trypsin, and stability of the 12 kDa fragment was greater at 5 U of trypsin. However, at the 5 U level of pancreatin intact 16 kDa pecan 2S albumin was observed at both the 60 min and 120 min digestion time. The 50 U trypsin and pancreatin generated a peptide fragment around 13-14 kDa. Two stable peptides at approximately 14 kDa and 9 kDa showed weak IgE activity using both levels of trypsin and pancreatin (Figure 5.3C, and Figure 5.4C). Enzyme levels of 50 U and 5 U per 1 mg of test protein correlate to an approximate enzyme:protein ratio of 3:1, and 1:3 w/w, respectively, which is at the higher end of levels used by Koppelman et al (2010), Lehmann et al (2006), Sen et al (2002), and Orruno et al. (2011) when evaluating the stability of the 2S albumins from peanut and sesame seed. Observations from these experiments suggest that the 2S albumin from pecan is more susceptible to digestion by intestinal enzymes than to pepsin, however; digestion-resistant peptides possess IgE binding capacity at all levels of enzymes used. No non-specific binding issues were observed in this experiment as indicated by the use of the non-atopic serum control (Figure 5.5).
Finally, we were able to identify that the 2S albumin from pecan was the most digestion resistant protein from pecan and it also retained IgE binding activity despite extensive digestion. This is an important factor contributing to assessment on food protein allergenicity and the 2S albumin may contribute to allergic sensitization of susceptible individuals to pecans. Therefore, the immunoblot data obtained in this study should be interpreted with caution and the actual potency of the peptides identified by the immunoblot experiments should be further evaluated using mediator release assays to determine if the digestion resistant peptides retain their immunoreactivity /potency equally to that of the intact 2S albumin.

CONCLUSION

Great variability in IgE binding profiles to crude pecan extract and the purified 2S albumin, Car i 1, was observed when characterizing the sera of allergic individuals. Five out of 12 sera showed binding to low molecular weight pecan proteins and specifically the purified pecan 2S albumin. Results from this study demonstrated that IgE reactive peptides from the pecan 2S albumin are retained even after extensive in vitro SGF and SIF. Levels of enzymes used for digestion experiments significantly influenced the stability of 2S albumin and the detection of immunologically active peptides. Taking into account estimated physiological conditions for digestion of proteins; these digestion-resistant peptides possess immunologically active epitopes that are likely contributing to sensitization of individuals and on elicitation of reactions upon exposure for those who are sensitized.
Figure 5.1 Individual serum characterization with pecan crude extract and purified 2S albumin.

[A]-SDS-PAGE separation of proteins
[B]-[F]-Immunoblot analysis of pecan proteins probed with individual serum IgE subjects 1-5
[G]-Negative control with nonatopic serum
[H]-Negative control with secondary Ab (Anti-human IgE)
M-Molecular weight markers
2S- Purified Car i 1 (2 µg load)
C- Crude pecan extract (10 µg load)
Note: Number at the bottom of the images specifies the serum from the Table 5.1.
<table>
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<th>Pecan</th>
</tr>
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</tr>
<tr>
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<td>15.6</td>
</tr>
<tr>
<td>5</td>
<td>55</td>
</tr>
</tbody>
</table>

Table 5.1 ImmunoCAP values of the sera used for evaluation of IgE binding to digestion resistant peptide of the pecan 2S albumin, Car i 1.
Figure 5.2 [A] SDS-PAGE, [B] R-250 stain, and [C] Immunoblot analysis of Car i 1 digested with pepsin in SGF probed with pooled IgE sera.

[A]

M- Molecular weight marker
A- Protein control
B- Digestion with 10 U pepsin at time 0
C- Digestion with 10 U pepsin at 60 min
D- Digestion with 1 U pepsin at 60 min
E- Digestion with 0.1U pepsin at 60 min
F- Enzyme control 10 U pepsin
G- Enzyme control 1 U pepsin
H- Enzyme control 0.1 U pepsin
I- Pecan crude extract (10µg load)

Note: Protein load in lane A was 1.7µg
Figure 5.3 [A] SDS-PAGE, [B] R-250 stain, and [C] Immunoblot analysis of Car i 1 digested with trypsin in SIF probed with pooled IgE sera.

[A]

M- Molecular weight marker
A- Protein control
B- Digestion with 50 U trypsin at time 0
C- Digestion with 50 U trypsin at 60 min
D- Digestion with 5 U trypsin at 60 min
E- Digestion with 50 U trypsin at 120 min
F- Digestion with 50 U trypsin at 120 min
G- Enzyme control 50 U trypsin
H- Enzyme control 5 U trypsin
I- Pecan crude extract (10µg load)

Note: Protein load in lane A was 2.3 µg
Figure 5.4 [A] SDS-PAGE, [B] R-250 stain, and [C] Immunoblot analysis of Car i 1 digested with pancreatin in SIF probed with pooled IgE sera.

[A] M- Molecular weight markers
A- Protein control
B- Digestion with 50 U pancreatin at time 0
C- Digestion with 50 U pancreatin at 60 min
D- Digestion with 5 U pancreatin at 60 min
E- Digestion with 50 U pancreatin at 120 min
F- Digestion with 50 U pancreatin at 120 min
G- Enzyme control 50 U pancreatin
H- Enzyme control 5 U pancreatin
I- Pecan crude extract (10 μg load)

Note: Protein load in lane A was 2.3 μg
Figure 5.5 [A] SDS-PAGE, [B] R-250 stain, and [C] Immunoblot analysis of protein and enzymes probed with negative control serum.

[A] SDS-PAGE

M- Molecular weight markers
A- 2S Albumin (2µg load)
B- Pecan crude extract (10µg load)
C- 10 U of pepsin per 1µg of protein
D- 1 U of pepsin per 1µg of protein
E- 0.1 U of pepsin per 1µg of protein
F- 50 U of trypsin per 1mg of protein
G- 5 U of trypsin per 1mg of protein
H- 50 U of pancreatin per 1mg of protein
I- 5 U of pancreatin per 1mg of protein

[B] R-250 stain

[C] Immunoblot analysis

ng IgE/spot
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SUMMARY

Food allergy has a tremendous impact on the lives of millions and the prevalence of tree nut allergy continues to increase in westernized countries throughout the world. An increase in the prevalence of allergic reactions to tree nuts may be due to their increased consumption. No cure for food allergies is currently available, therefore; strict avoidance diets are currently the only option for allergic individuals. Avoidance diets can be particularly challenging for allergic individuals due to accidental ingestion and the use of nuts as hidden ingredients in various food products.

Pecans are one of the major tree nuts consumed and often capable of causing life-threatening, IgE-mediated, anaphylactic reactions in susceptible individuals. Little information is currently available regarding complete characterization of the soluble pecan proteins and their susceptibility to enzymatic digestion in the gastrointestinal tract.

Therefore, the first objective of this study was to examine the effects of various buffers in the preparation of protein extracts that could be applied in diagnostic tests and development of methods for the detection of pecan residues in foods. The ionic strength of the solution had a greater impact on pecan protein extractability than pH. Additionally, the presence of clinically relevant allergens was dependent on the choice of extraction buffer; which also influenced the total protein content and profile of an extract.

2S albumin seed storage proteins are one of the major allergens involved in food allergic reactions to nuts and seeds. Due to its proven allergenicity in several other seeds, the 2S albumin from pecan was successfully isolated and purified in order to study its allergenicity.
Finally, identification of digestion resistant polypeptides is an important factor contributing to assessment on food protein allergenicity. High molecular weight soluble pecan proteins were rapidly hydrolyzed by pepsin, however; the low molecular weight proteins remained relatively stable with generation of low MW peptides that remained stable throughout the pepsin digestion experiments. The pecan 2S albumin showed remarkable stability to pepsin, while it was more susceptible to proteolysis with trypsin and pancreatin. Most importantly, partial digestion of the pecan 2S albumin, Car i 1, produced immunologically active IgE-binding peptides. Taking into account estimated physiological conditions for digestion of proteins; digestion-resistant peptides identified in this study possess immunologically active epitopes that could possibly have an effect on sensitization of individuals upon exposure to pecans.