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Purification and Characterization of Select Glycoproteins of Almonds (Prunus Dulcis L.)

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PURIFICATION AND CHARACTERIZATION OF SELECT
GLYCOPROTEINS OF ALMONDS (*Prunus dulcis* L.)

By

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<thead>
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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>B-ME</td>
<td>β-mercaptoethanol</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BSB</td>
<td>Borate saline buffer</td>
</tr>
<tr>
<td>CBBR</td>
<td>Coomassie brilliant blue R</td>
</tr>
<tr>
<td>Con A</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>DEAE</td>
<td>Diethylaminoethyl</td>
</tr>
<tr>
<td>DI</td>
<td>Distilled</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>HF</td>
<td>Hydrogen fluoride</td>
</tr>
<tr>
<td>HNL</td>
<td>Hydroxynitrile lyase</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse radish peroxidase</td>
</tr>
<tr>
<td>IEF</td>
<td>Isoelectric focusing</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IgE</td>
<td>Immunoglobulin E</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>NC</td>
<td>Nitrocellulose</td>
</tr>
<tr>
<td>NDND-PAGE</td>
<td>Nondenaturing nondissociating polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>NFDM</td>
<td>Nonfat dried milk</td>
</tr>
<tr>
<td>pAb</td>
<td>Polyclonal antibody</td>
</tr>
<tr>
<td>PBS-T</td>
<td>Phosphate buffer saline-Tween 20</td>
</tr>
<tr>
<td>PH</td>
<td>Prunasin hydrolase</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinyl difluoride</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature °C</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris-buffered saline-Tween 20</td>
</tr>
<tr>
<td>Tris</td>
<td>2-Amino-2-hydroxymethyl-propane-1,3-diol</td>
</tr>
<tr>
<td>2D</td>
<td>Two dimensional</td>
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ABSTRACT

Almonds (Prunus dulcis L.) are the most widely consumed tree nuts in the USA besides USA also being the number one global producer of almonds. Although almonds can be consumed without any adverse effects by the majority of the population, a few susceptible individuals develop allergic symptoms following the ingestion of almonds. Almond allergies are the third most common of all tree nut allergies, affecting ~15% of the tree nut allergic population in the USA. Several proteins of almonds have been identified as being allergenic, including almond major protein (AMP) or amandin which is a major allergen of almonds. However, although several glycoproteins of almonds have been biochemically characterized, the potential allergenicity of most almond glycoproteins are yet to be elucidated. In the present study select almond glycoproteins were partially purified and characterized.

Glycoproteins which comprise less than 2% of the total soluble proteins of almonds were purified and separated from the non-glycoprotein fraction using affinity chromatography and were further resolved in to 3 peaks when passed through a gel filtration column. The 3 glycoprotein peaks (glycoproteins A, B and C) were partially characterized biochemically and immunologically in this study.

SDS- PAGE analysis under reducing conditions showed that both glycoproteins A and B had 3 major peptide bands in addition to several minor peptides. The 3 major bands of glycoprotein A had molecular masses of ~13 kDa, ~22 kDa and ~44 kDa. The major peptides of glycoprotein B were found to have molecular weights of ~12 kDa, ~34 kDa and ~62 kDa. Glycoprotein C was composed of a single major peptide of ~62 kDa and also of several minor peptides ranging from ~11 kDa to ~55 kDa. The major peptides at ~62 kDa of glycoproteins B and C were identified as prunasin hydrolase and hydroxynitrile lyase, respectively by N-terminal amino acid sequencing.

All three glycoproteins A, B and C were immunoreactive with polyclonal antibodies raised against whole almonds in rabbit. However, none of the 3 glycoproteins were recognized by the monoclonal antibodies 4C10 and 4F10 raised against AMP of almonds. Dot blot analysis of glycoproteins with human IgE from almond allergic patients resulted in the recognition of glycoprotein B by 6 of the 11 (53%) patient sera tested. 3 of the 11 samples (27%) reacted with
glycoprotein A while most patient sera did not show any reactivity with glycoprotein C. Deglycosylation resulted in a significant loss of immunoreactivity of all 3 glycoproteins indicating the possibility of carbohydrate moieties playing a role in their immunoreactivity.
CHAPTER 1

INTRODUCTION

Almonds (*Prunus dulcis* L.) belong to the *Rosaceae* family, which is of great economic importance since it also includes peaches, pears, plums, prunes, raspberries, nectarines, cherries, apricots and apples (Britannica, online encyclopedia, 2010). United States is the largest global producer of almonds, with California being the major producer. In 2010 global almond production was 799 366 metric tons, of which 637 706 metric tons (80%) were produced in the USA (Almond Almanac, 2010). It is predicted that global almond production will increase by almost 12% (900 000 metric tons) in 2011 with the USA producing a record 750 000 metric tons (USDA, 2010c).

With regard to the nutritive value of almonds, they contain a significant amount of proteins (up to 25%) and lipids (~55%) of which 90% is accounted for by two monounsaturated fatty acids, oleic (52-67%) and linoleic (22-39%) (Sathe, 1993). A significant amount of fiber (12%) is also present in almond seeds. They are also a good source for several minerals including phosphorus, calcium, magnesium, potassium, zinc, selenium and manganese. Almonds are comparatively a rich source for vitamin E (26.2%), other than that they also contain several vitamin B complexes (USDA, 2010a).

Almonds are the most widely consumed tree nuts in the USA and per capita use of almonds has increased over the years with 0.7 pounds (29% of all tree nuts) being used in 1990 to 1.4 pounds (38% of all tree nuts) in 2009 (USDA, 2010b). Almonds are consumed raw, blanched, or roasted and are commonly used in confectionery and baking. They are also widely used in meat, poultry, fish, and vegetarian dishes of Asia (Britannica, online encyclopedia, 2010).

Although almonds are safely enjoyed by most people, consumption of almonds can result in the development of allergic symptoms in certain susceptible individuals. These symptoms can range from mild discomfort to severe life threatening reactions leading to anaphylactic shock or even death (Asero et al., 2007). Classified as a tree nut, almonds are included in the eight groups of foods that have been identified by the FAO as being major contributors to IgE mediated food
allergies where ~1.1% of children and 0.5% of the adult population in the USA are allergic to tree nuts (Sicherer et al., 2010). Of all tree nut allergies, almond allergies are the third most common in the USA, with ~15% of tree nut allergic individuals showing reactivity against almonds (Sicherer et al., 2001; Sicherer et al., 2003; Sicherer et al., 2010).

Previous studies conducted by Sathe (1993), has revealed that a single protein (almond major storage protein-AMP or amandin) dominates the total soluble protein composition of almond seeds. Furthermore, AMP has been identified as a major allergen of almonds by Roux et al., (1999). In addition to AMP, several other proteins of almonds including conglutin γ, 2S albumin, Pru du 6, profilin (Pru du 4), a 60s ribosomal protein, and a lipid transfer protein of almond has been identified as being allergenic proteins (Poltronieri et al., 2002; Tawde et al., 2006; Abolhassani and Roux, 2009; Willison, 2009; Willison et al., in press).

However, with regard to the glycoproteins of almonds, although several of them have been characterized biochemically (Altmann et al., 1998; Dreveny et al., 2001; Grover et al., 1977; Taga et al., 1984) the potential allergenicity of these glycoproteins is yet to be elucidated. With increasing consumption as well as the increased use of almond as a food ingredient, the potential risk of susceptible individuals being exposed to almonds is also higher. Therefore, characterization and identification of almond glycoproteins is essential to improve our current understanding and management of almond allergic patients. Consequently, this study was undertaken to purify and characterize select glycoproteins of almonds.

The specific aims of the study include:

1. Identification of select almond glycoproteins
2. Biochemical characterization of a selected glycoprotein
3. Immunoreactivity of selected glycoproteins
CHAPTER 2

REVIEW OF LITERATURE

2.1. TREE NUTS

Tree nuts are popular globally and are cultivated in diverse climatic conditions. Other than peanuts (*Arachis hypogaea*), which are groundnuts, commercially important tree nuts include almond (*Prunus dulcis*), cashew (*Anacardium occidentale*), Brazil nut (*Bertholletia excelsa*), hazelnut (*Corylus avellana*), macadamia (*Macadamia integrifolia*), pecan (*Carya illinoinsensis*), pine nut (*Pinus pinea*), pistachio (*Pistachia vera*), and walnut (*Juglans regia*) (FAO, 2009).

Global production of walnuts exceeds that of all other tree nuts, with a total of 1 282 240 metric tons grown around the world in 2009/10. China is the world’s largest producer of walnuts (560 000 metric tons), while the USA ranks second with 396 440 metric tons. Almond production ranks a distant second with 799 195 metric tons grown around the world within the same time period. The USA ranks first in almond production accounting for ~80% of global production (639 570 metric tons) (USDA, 2010c). With regard to the tree nuts that are imported to the USA, cashew ranks first (125 415 metric tons), followed by pecan (59 473 metric tons) and Brazil nut (10 971 metric tons) respectively (USDA, 2010b).

Nutritionally, tree nuts are an excellent source of proteins and lipids. Although tree nuts have a high lipid content, saturated fatty acids account for about only 5 to 8% of the total lipid content and they do not contain cholesterol. Nevertheless, they are rich in mono unsaturated fatty acids (MUFAs) and poly unsaturated fatty acids (PUFAs). The high content of unsaturated fatty acids in nuts is desirable because of their possible health benefits. Especially it has been suggested that PUFAs and MUFAs may have a beneficial effect on blood serum lipid profile by decreasing undesirable low density cholesterol VLDLs and LDLs (Abbey et al., 1994; Kris-Etherton et al., 1999). Cashew nuts and walnuts as well as peanuts are rich in linoleic acid whereas almonds, hazelnuts, macadamia and pistachios are rich in oleic acid. Although only 15% of the total energy provided by nuts comes from carbohydrates, they are a good source of dietary
fiber ranging from 4 to 11% by weight (USDA, 2010a). Tree nuts also contain significant amounts of micronutrients such as manganese, copper, phosphorus, selenium and zinc. Vitamin E is present in considerable amounts in most tree nuts especially almonds and they also contain thiamine, niacin and riboflavin (Kris-Etherton et al., 1999; USDA, 2010a).

Based on several research studies, it is suggested but not proven that eating 1.5 oz 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). Furthermore, tree nuts are increasingly being used in weight management diets due to their capacity to suppress appetite and fat absorption (Coates and Howe 2007).

### 2.2. ALMONDS

Almonds (*Prunus dulcis* L.) belong to the *Rosaceae* family, which is of great economic importance since this family also includes peaches, pears, plums, prunes, raspberries, nectarines, cherries, apricots and apples. The almond tree is native to Southwestern Asia and is cultivated extensively in certain favorable regions between 28° and 48° N and between 20° and 40° S. There are two main types of almonds namely, sweet almonds which are the edible type and the non edible bitter almonds (Britannica, online encyclopedia, 2010).

The United States is the largest global producer of almonds, with California being the major producer. The Nonpareil variety is the major commercial variety that is produced in the USA. Carmel, Butte and Monterey are the other main varieties that are produced in the USA. In 2010 global almond production was 799 366 metric tons, of which 637 706 metric tons (80%) were produced in the USA (Almond Almanac, 2010). It is predicted that global almond production will increase by almost 12% (900 000 metric tons) in 2011 with the USA producing a record 750 000 metric tons (USDA, 2010c). 72% (463 553 metric tons) of almonds produced in the USA in 2010 were exported which was a 4% increase compared to the previous year. Western Europe accounted for 43% of the total exports for 2010 compared with 32% for Asia-Pacific and 15% for the Middle East/Africa. Currently almonds are ranked first of the USA specialty crop exports (Almond Almanac, 2010).

High in calories, where 1 oz of almonds contain 163 calories, they are also high in proteins (21.2%) and lipids (49.4%) (USDA, 2010a). Except for methionine, all of the essential amino acids are provided by almonds in quantities equal or greater than that is recommended by
the FAO guidelines. Of the total lipid content of almond, ~90% is accounted for by two
unsaturated fatty acids, oleic (52-67%) and linoleic (22-39%) (Sathe, 1993). A significant
amount of fiber (12%) is also present in almond seeds. They are also a good source for several
minerals including phosphorus, calcium, magnesium, potassium, zinc, selenium and manganese.
Almonds are comparatively a rich source for vitamin E (26.2%), other than that they also contain
several vitamin B complexes (USDA, 2010a).

Almonds are consumed raw, blanched, or roasted and are commonly used in
confectionery and baking (Britannica, online encyclopedia, 2010). Almonds are the most widely
consumed tree nuts in the USA and per capita use of almonds has increased over the years with
0.7 pounds (29% of all tree nuts) being used in 1990 to 1.4 pounds (38% of all tree nuts) in 2009
(USDA, 2010b).

2.3. FOOD ALLERGIES

Food allergies are rapidly increasing worldwide, and are among the most common
immunologically related diseases in industrialized countries (Sampson, 2004). A food allergy
can be defined as when an individual reacts to and develops allergic symptoms following the
ingestion of a food that for the vast majority of the population is part of a healthy diet (Asero et
al., 2007). Any food protein has a potential to trigger an allergic response and a single food can
contain one or more allergens (Murch, 2005).

An allergic reaction to a food can induce IgE mediated, non IgE mediated or a mixed
immune response (Sampson, 2005). Nevertheless, IgE mediated food allergies also referred to as
type I hypersensitivity reactions are the most common type. In IgE mediated food allergies,
specific allergens (usually a food protein) cross link the IgE antibodies that are bound to the
membranes of effector cells (cells that produce an end effect such as mast cells or basophils).
This IgE cross linking results in the activation and degranulation of effector cells with the release
of histamine and other mediators of immunity such as inflammatory mediators, which
subsequently leads to allergic responses in susceptible individuals. This type of food allergies
produces immediate symptoms (Roit et al., 2001). The portion of the allergen that cross links
with IgE is referred to as an epitope. Epitopes can be linear, conformational or a combination of
both where linear epitopes contain a contiguous stretch of amino acids and a conformational
epitope contains noncontiguous amino acids which form a three-dimensional/structural motif (Murch, 2005). In non IgE mediated food allergies also referred to as delayed cell mediated allergy or type IV hypersensitivity, symptoms develop hours or even days after exposure to the offending food. This is a cell mediated (T cells) immune response where interactions occur between cells and chemical mediators such as cytokines (Jackson, 2003).

An allergic reaction to a food may evoke symptoms in the gastrointestinal tract, skin and respiratory tract. Gastrointestinal symptoms include swelling of lips and/or tongue, sensation of tightness of throat, nausea, abdominal pain, vomiting and diarrhoea. Symptoms affecting the skin may consist of urticaria, rash or flushing. Respiratory symptoms may affect the larynx, upper and lower respiratory tracts and includes nasal congestion, sneezing, sensation of tightness, asthma and wheezing (Sampson, 2005). Symptoms of food allergies may range from mild discomfort to severe life threatening reactions leading to anaphylactic shock and can occur within a few minutes of consuming the food or may develop after the lapse of several hours (Asero et al., 2007). The most common single cause of anaphylaxis is due to food allergies (Sampson, 2004) where in the USA nearly 90 000 food related anaphylaxis is treated in the emergency departments annually (Clark et al., 2011).

Food allergy remains a major health problem in the USA, where 6-8% of children and 4% (12 million people) of adults are affected by some sort of food allergy (Sampson, 2005). In the USA approximately 150-200 people die annually due to food induced anaphylaxis (Bock et al., 2001; Sampson, 2003). Most cases of food allergies are caused by a relatively small number of food groups and food products (Table 2.1). In addition, allergies to certain foods such as apple, kiwi, hazelnuts, walnuts, celery, carrot, tomato, cherry, and melon are induced by cross reaction with pollen. Identified as the pollen-food allergy syndrome (oral allergy syndrome) this is caused due to plant proteins cross reacting with airborne allergens (Bet v 1, Bet v 2, profilin), mostly birch and ragweed pollen. Allergic symptoms in oral allergy syndrome are limited to oral itching and swelling since most of these allergens are destroyed in the gastrointestinal tract. Moreover, these food proteins that are homologous to pollen are usually liable to heat and thus when either cooked or processed they do not elicit an allergic response in affected individuals (Breiteneder and Ebner, 2000).
The FAO has identified eight groups of foods (“the big eight”) as being major contributors to IgE mediated food allergies which includes wheat, crustaceans, fish, eggs, peanuts, milk, tree nuts and soy bean (Jackson, 2003). Children show allergic reactions mostly to cow’s milk (~41%), egg (~21%), and peanut (~13%), while adults are mostly allergic to crustaceans (~54%), peanut (~16%) and tree nut (~13%) (Sampson, 2004).

Table 2.1. Prevalence of food allergies in the USA

<table>
<thead>
<tr>
<th>Food</th>
<th>Children &lt; 5 years (%)</th>
<th>Adults (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Milk</td>
<td>2.5</td>
<td>0.3</td>
</tr>
<tr>
<td>Egg</td>
<td>1.3</td>
<td>0.2</td>
</tr>
<tr>
<td>Peanut</td>
<td>0.8</td>
<td>0.6</td>
</tr>
<tr>
<td>Tree nuts</td>
<td>0.2</td>
<td>0.5</td>
</tr>
<tr>
<td>Fish</td>
<td>0.0</td>
<td>0.4</td>
</tr>
<tr>
<td>Shell fish</td>
<td>0.1</td>
<td>2.0</td>
</tr>
<tr>
<td>Overall</td>
<td>6.0</td>
<td>3.7</td>
</tr>
</tbody>
</table>

Source: Sampson, 2005.

The most common food allergen in the USA that affects adults is seafood (fish and shellfish) with 2.3% (6.6 million people) of the population being affected (Sicherer et al., 2004). Slightly more than 1% (> 3 million people) of the USA adult population is allergic to peanuts and/or tree nuts. Cow’s milk allergy is the most common food allergy that affects children, with 2% to 3% children showing allergic reactions to cow’s milk, although a significant number of children outgrow their allergy by adolescence (Baral and Hourihane, 2005). Of about the 30 types of allergenic proteins that have been identified in milk, caseins are the most prevalent (Wal, 2001).

Research studies have indicated that in egg and milk allergies, when IgE antibodies recognize specific sequential epitopes the patients are more likely to have persistent allergies, in contrast when IgE antibodies are directed towards conformational epitopes, patients have a higher tendency of developing clinical tolerance (Vila et al., 2001). Moreover, children suffering from atopic dermatitis, allergic rhinitis and/or asthma are more likely to have a food allergy than non atopic children (Eigenmann et al., 1998). The allergenicity of certain foods can be reduced by using different methods of processing. Although food processing may inactivate or eliminate
certain allergens it may not always be possible to determine whether the processing method completely inactivated the targeted food allergens. Therefore, since currently there is no cure for food allergy, avoidance of the offending food is the best defense for allergic patients (Sathe and Sharma, 2009).

2.4. TREE NUT ALLERGIES

Tree nuts are included in the eight groups of foods that have been identified by the FAO as being major contributors to IgE mediated food allergies. In the USA, ~1.1% of children and 0.5% of the adult population are allergic to tree nuts (Sicherer et al., 2010). Although most children outgrow allergies to milk, egg, wheat and soy, only 9% of children outgrow their allergenicity to tree nuts (Fleischer et al., 2005).

In a clinical study undertaken in the UK by Ewan (1996), comprising 62 patients, it was found that the majority of the patients suffered from peanut allergy (47) followed by Brazil nut (18), almond (14) hazel nut (13) and walnut (8). Within the USA, it was found that of tree nut allergic individuals, 34% are allergic to walnut followed by cashew nut (20%), almond (15%), pecan (9%) and pistachio (7%) from a total of 5149 participants of a voluntary registry for peanut and tree nut allergies (Sicherer et al., 2001). In another study conducted in the USA by a random digit dialed telephone survey, it was found that out of the 83 tree nut allergic individuals 62% were allergic to walnut, 44% to cashew, 39% to almond, 35% to pecan, 29% to hazelnut, 30% to Brazil nut and 22% to pistachio (Sicherer et al., 2003). Tree nut allergies are much more serious in comparison to allergic reactions caused by other foods. All fatal allergic reactions by individuals over 6 years of age reported to a USA national registry was found to be caused by either peanuts or tree nuts (Bock et al., 2001).

Several tree nut allergens have been characterized including allergenic proteins of almonds (Poltronieri et al., 2002; Sathe et al., 2002), cashew (Robotham et al., 2005; Sathe et al., 1997; Wang et al., 2002), Brazil nut (Bartolome et al., 1997; Sharma et al., 2010), hazel nuts (Beyer et al., 2002; Lauer et al., 2004; Pastorello et al., 2002) and walnuts (Teuber et al., 1998; Teuber et al., 1999). Most of these tree nut allergens belong to a few selected seed storage protein families. These include 2S albumins which belong to the prolamin superfamily, 7S globulins (vicilins) and 11S globulins (legumins) belonging to the cupin superfamily (Beyer,
2S albumins are heterodimeric structures comprising of a 4 kDa subunit and a 9 kDa subunit. The 7S globulins are trimeric with subunit sizes ranging from 50-70 kDa (Lawrence et al., 1994) whilst the 11S globulins are hexameric with subunit sizes of 60-80 kDa (Adachi et al., 2003). Although the 7S and 11S globulins are a rich source of proteins, they contain low levels of the essential amino acid methionine and cysteine, thus decreasing their nutritional value (Holding and Larkins, 2008). In contrast, the 2S albumins have been shown to have a high cysteine content (6-13% of the total amino acids in 2S) thereby providing a sulfur reserve for seed germination (Youle and Huang, 1981). These storage proteins of seeds are synthesized on the rough endoplasmic reticulum (ER) and are either retained in the ER as protein bodies or are transported via the Golgi complex to specialized protein storage vacuoles (Holding and Larkins, 2008).

In addition to the seed storage proteins, several allergens belonging to other families such as pathogenesis related protein families have been identified and characterized in several tree nuts. These include the lipid transfer proteins (LTPs) in hazel nuts (Pastorello et al., 2002) and walnuts (Pastorello et al., 2004). LTP allergens have been identified in many fruits, vegetables, nuts and seeds, particularly those belonging to the Rosaceae family are important food allergens (Asero et al., 2002).

For a number of these allergens the IgE binding sites including the amino acid sequences of the epitopes have been identified. Epitope mapping of Ana o 1 led to the identification of 11 linear IgE binding epitopes, of which 3 were found to be immunodominant (Wang et al., 2002). Similarly immunoreactive peptides of Ana o 3, a 2S albumin have been identified (Robotham et al., 2005). A major linear IgE epitope and its core amino acids involved in allergenicity has been identified in the 2S albumin (Jug r 1) protein of English walnut (Robotham et al., 2002).

Interestingly, it has been found that although botanically unrelated, allergens within the same group of seed storage proteins show a high degree of homology. For example, it has been revealed in a research study carried out by Poltronieri et al., (2002), that the N-terminal sequence of a 12 kDa allergen of almond shows significant homology to the 2S albumin (Jug r 1) allergen of walnut. Linear epitopes of cashew 2S albumin (Ana o 3) and walnut 2S albumin also show similarity as indicated by a study carried out by Robotham et al., (2005). Identified as a major allergen of hazelnut, Cor a 9 (an 11S globulin) contains an IgE binding epitope, that shares 67%
homology with the 11S globulin, Ara h 3 of peanut (Beyer et al., 2002). This sharing of homologous regions between different tree nuts may be a basis for patients to show allergies against multiple tree nuts. The generation of specific IgE molecules to each tree nut allergen within the same individual could be another reason for an individual to show reactivity against several tree nuts (Aalberse et al., 2001).

By using different methods of processing partial or complete removal of a particular allergen is possible. For example research studies has shown that blanching of pecan (Venkatachalam et al., 2006) and walnut (Su et al., 2004) results in a reduction of their immunoreactivity, although this method of processing has no effect on the immunoreactivity of almonds (Venkatachalam et al., 2002).

2.5. ALMOND ALLERGIES

Almond allergies are the third most common allergies to tree nuts in the USA, with ~15% of tree nut allergic individuals showing reactivity against almonds (Sicherer et al., 2001).

Almond allergens

Most of the proteins (≥ 95%) present in almonds are water soluble (Sathe, 1993). The major almond seed storage proteins were studied by Youle and Huang (1981), and they reported that almond seeds contain 25%, 15% and 60% of 2S, 7S and 11S proteins respectively. Studies conducted by Sathe (1993), and Sathe et al., (2001), has also shown that the dominant protein in almond is amandin also referred to as almond major protein (AMP) and that AMP constitutes up to ~65% of the total soluble fraction (Sathe et al., 2002). Amandin is a legumin type of storage protein with a sedimentation value of 14S and a molecular weight of 427 300 ± 47 600. Amandin is an oligomeric protein containing prunin monomers. These monomers form a doughnut shaped trimer and two amadin trimers form a hexameric structure (Albillos et al., 2008). Each prunin monomer is composed of a 42-46 kDa acidic R chain and a 20-22 kDa basic β chain, where the two polypeptides are linked via disulfide bonds (Sathe et al., 2002).

Although several proteins of almonds have been characterized biochemically, relatively few proteins have been identified as allergens. Early research work carried out by Bargman et al.
(1992), showed that IgE from sera of 4 patients out of a total of 8 bound with unidentified almond proteins of molecular mass ~70 and 50 kDa.

AMP has been identified as a major allergen of almonds by Roux et al., (1999) where it is recognized by human IgE from almond allergic patients. Sathe et al. (2002), and Roux et al. (2001), have shown that IgE from almond allergic sera are strongly reactive against the 42-44 kDa peptide bands of amandin. Sequence data has confirmed that these peptides are homologous to the previously reported prunin 1 and 2 cDNA sequences (Garcia-Mas et al., 1995). The 66 kD and 50 kD peptides of amandin that are recognized by human IgE is likely to be the same peptide bands that were identified by Bargman et al. (1992). Research work carried out by Sathe et al. (2001), found that AMP was recognized by human IgE, rabbit polyclonal antibodies and mouse monoclonal antibodies in all 60 different genotypes and inter species hybrids of almonds that were analyzed. Competitive ELISA and Western blotting experiments using rabbit polyclonal antibodies raised against AMP has shown that AMP is slightly cross reactive against cashew globulin, tepary and Great Northern bean phaseolins suggesting the possibility of almond major proteins sharing common antigenic features with some other seed storage proteins (Acosta et al., 1999).

In a recent study carried out by Willison et al., (in press), two prunin (amandin) isoforms, 1 and 2 were cloned and expressed as recombinant proteins rPru du 6.01 and rPru du 6.02 respectively. When these recombinant proteins were probed with almond allergic patient sera, it was found that 50% (9 of 18) of patients were reactive to rPru du 6.01, while 28% (5 of 18) recognized rPru du 6.01. Four patients (22%) were found to be reactive to both isoforms.

In a study conducted by Poltronieri et al., (2002), two IgE binding proteins were identified by N-terminal sequencing as almond 2S albumin and conglutin γ. The N-terminal and an internal peptide sequence of the 12 kD almond 2S albumin was found to show good homology to the 2S albumin (Jug r1) of walnut. Endoproteinase digestion of the 12 kD protein resulted in an immunoreactive 6 kD major peptide fraction and a 2 kD peptide fraction with no IgE binding activity. The sequence of the 2kD peptide showed high similarity (80%) to the sequences near the C terminal of English walnut and Brazil nut 2S albumins. N terminal sequencing of the 45 kD conglutin γ protein showed that it shared 60% homology with conglutin, a heavy chain from lupine seed and 50% homology with the 7S globulin from soybean.
Conglutin \( \gamma \) was found to be composed of two subunits, an N-terminal 28-30 kD heavy chain subunit and a C terminal 17 kD subunit. Besides the almond 2S protein, several other plant 2S proteins have been reported as being major allergens. These include the 2S proteins of English walnut (Robotham et al., 2002), Brazil nut (Pastorello et al., 1998), cashew (Robotham et al., 2005), sunflower (Kelly et al., 2000) and castor bean (Thorpe et al., 1988). However, of the 7S globulin proteins, only a few have been identified as allergens. Peanut 7S protein, Ara h2 (Viquez et al., 2001) and the 7S protein of soybean, \( \beta \)-Conglycinin have been identified as major allergens (Ogawa et al., 1999).

In a research study carried out by Abolhassani and Roux, (2009), an immunoreactive 60s ribosomal protein was identified. When tested by a dot blot immunoassay using sera from eight almond sensitive individuals, four sera (50\%) showed reactivity against the 60s ribosomal protein. Nevertheless, further research needs to be conducted using larger numbers of almond sensitive sera to determine whether this protein represents a major allergen of almond.

Tawde et al. (2006), in their studies reported almond profilin (Pru du 4) as an allergenic protein by screening an almond cDNA library with almond allergic patient sera. Dot blot analysis and ELISA results showed that 33\% (6 of 18 patient sera) reacted with the recombinant Pru du 4 protein while 44\% were reactive with the native form. Furthermore, in the same study it was found that Pru du 4 shows cross reactivity with grass pollen profilin.

In a recent study on almond lipid transfer proteins (LTPs), this protein was reported as a minor allergen. Almond LTP (Pru du 3) reactivity with human IgE was analysed using sera from 25 almond allergic patients out of which 20\% (5 out of 20 patients) of sera were shown to be reactive (Willison, 2009).

The immunoreactivity of certain almond allergens are affected by different processing conditions. In a research study carried out by Roux et al., (2001), it was revealed that roasting of almonds for 30 min at 160 °C resulted in the appearance of new immunoreactive polypeptides with molecular masses ~40 kDa and 75-84 kDa. Amandin and its polypeptides have been shown to retain their antigenic stability and allergenicity following food processing conditions such as roasting, blanching, autoclaving, and microwaving confirming the stability of these polypeptides (Roux et al., 2001; Vekatachalam et al., 2002). The structural stability of amandin has been proven by studies carried out by Albillos et al. (2009). In this study it was shown that although
protein aggregation was observed, the secondary structure of amandin did not undergo any major changes up to 90 °C. Comparatively, the acidic and basic polypeptide chains of amandin had lower thermal stability than the multimeric protein. Amandin belongs to the cupin superfamily of seed storage proteins which are also known as heat stable proteins that can form immunonogenicity-enhancing aggregates (Breiteneder and Mills, 2005).

Almond glycoproteins

Glycoproteins are proteins that contain one or more oligosaccharide (glycan) chains covalently attached to their polypeptide backbones. The carbohydrate content of glycoproteins can range from 1% to over 85% by weight (Murray et al., 2003).

Several glycoproteins of almonds have been characterized biochemically. However, the immunoreactivity of these glycoproteins is yet to be studied. The crystal structure of the almond glycoprotein hydroxynitrile lyase (HNL) has been determined by Dreveny et al., 2001. Almond HNL was one of the first enzymes whose activity was recognized as early as 1837, where this enzyme is involved in a process known as cyanogenesis. During this process, β-glucosidase mediated cleavage of cyanogenic glycosides occurs to form the corresponding carbohydrate and cyanohydrin. The latter is subsequently cleaved by HNL into HCN and the corresponding aldehyde or ketone (Fig 2.1). Almond HNL has a molecular mass of 61 kDa comprising 521 amino acids and contains four N-glycosylation sites at Asn118, Asn135, Asn352, and Asn392.

N-glycosidase of almonds is also a glycoprotein that has been purified and characterized by Taga et al., (1984). This glycoprotein catalyzes the hydrolysis of N-linked oligosaccharide chains from glycopeptides and glycoproteins and is a single polypeptide chain with a molecular weight of 66 800 Da.

The glycoprotein Peptide-N4-(N-acetyl-â-glucosaminyl) asparagine amidase A (PNGase A) of almond has been purified and characterized by Altmann et al. (1998). The enzyme was found to be a heterodimer with subunits of 55 and 27 kDa and was found to be largely resistant to self-deglycosylation.

Almond emulsion β-D-glucosidase, also a glycoprotein has been isolated and characterized by Grover et al. (1977). This bifunctional enzyme catalyzes the hydrolysis of β-D-glucopyranosides and β-D-galactopyranosides. The active enzyme has a molecular weight of 135
180 ± 770 and is composed of two identical subunits each with a molecular weight of 65 150 ± 650.

![Chemical Reaction]

**Figure 2.1.** Hydroxynitrile lyase mediated process of cyanogenesis in plants. Source: Dreveny et al., 2001.

Although immunological data for almond glycoproteins are lacking except for the identification of conglutin γ as a major allergen (Poltronieri et al., 2002), allergenicity of several other glycoproteins have been reported in the literature. These include the glycoprotein βConglycinin of soybean which has been identified as a major allergen (Ogawa et al., 1999). The 7S globulin of Brazil nut has been identified as a glycoprotein and was also shown to be immunoreactive (Sharma et al., 2010). A study carried out by Lauer et al. (2004), has shown hazelnut vicilin (Cor a 11), to be an allergen. However, Cor a 11, which is a glycoprotein has been identified as a minor allergen both with regard to its prevalence and allergic reactivity, and it was also shown that its carbohydrate moieties does not contribute to allergenicity. In addition several wheat and barley glycoproteins have been reported as being allergenic (García-Casado et al., 1996. The identification and immunoreactivity of select almond glycoproteins was undertaken in this study, due to the fact that very little research work has been carried out with respect to the immunoreactivity of almond glycoproteins.
CHAPTER 3

MATERIALS AND METHODS

Materials

Whole almonds of the Nonpareil marketing variety were a gift from the Almond Board of California (Modesto, CA). Chromatography columns and fraction collectors were from Pharmacia, Inc. (Piscataway, NJ). DEAE DE-53 resin (binding capacity 150 mg protein/mL) and PVDF membranes were from Whatman, Inc. (Piscataway, NJ), while Concanavalin A Sepharose 4B (bead size ~90 µm, binding capacity 20-45 mg porcine thyroglobulin/mL medium), Sephacryl S200 (25-75 µm diameter in wet form, fractionation range $5 \times 10^3$-2.5 x $10^5$ MW) and S300 HR (25-75 µm diameter in wet form, fractionation range $1 \times 10^4$-1.5 x $10^6$ MW) were from GE Healthcare (Piscataway, NJ). Electrophoresis and immunoblotting supplies were from Hoefer Scientific Co. (San Francisco, CA) and Fisher Scientific Co. (Pittsburgh, PA). Whatman 3MM filter papers and nitrocellulose membranes (0.2 µm) were from Schleicher & Schuell Bioscience, Inc. (Keene, NH). X-ray films (BioMax XAR film) were from Eastman Kodak Co. (Rocheser, NY). $^{125}$I labeled goat mouse anti-human IgE was from Hycor 19 Biomedical Inc (Garden Grove, CA). Human sera with a history of almond hypersensitivity were provided by Dr. Suzanne Teuber (University of California Davis, Davis, CA). Ninety-six well polyvinyl microtiter ELISA plates were from Costar (Cambridge, MA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific Co. (Pittsburgh, PA) unless otherwise stated and were of reagent or better grade.

Methods

Preparation of defatted almond flour

Whole almonds of the Nonpareil variety were ground in an Osterizer blender (speed setting “grind”; Galaxy model 869-18R) to homogeneous flour and defatted for 8 h using a Soxhlet apparatus and petroleum ether (boiling point range of 38.2-54.3 ºC) as the extraction solvent (flour to solvent ratio of 1:10 w/v). The defatted flour was spread out in a thin layer and
allowed to dry overnight in a fume hood for the removal of residual traces of petroleum ether. Following the overnight drying of the defatted flour, it was further homogenized in an Osterizer blender and passed through a 40 mesh sieve. The defatted flour was stored in screw-capped plastic vials at -20 ºC until further use.

Isolation of almond glycoproteins

Twenty grams of defatted almond flour was extracted in 200 mL of 0.05 M phosphate buffer, pH 7.2 (flour to buffer ratio1/10 w/v) containing 1 M NaCl and 0.01 M β-mercaptoethanol, with continuous magnetic stirring for 1 h at room temperature (RT, ~25 ºC). The resulting slurry was centrifuged at 12 000g for 20 min at 4 ºC and the supernatant was filtered by passing through a Whatman No. 4 filter paper. Glycoproteins were isolated by the method described by Sathe (1991), with slight modifications. Briefly, the supernatant was loaded onto a Concanavalin (Con) A Sepharose 4B (GE Healthcare, Piscataway, NJ) column (2.6 x 7.0 cm) previously equilibrated with 0.05 M phosphate buffer, pH 7.2 containing 1 M NaCl and 0.01 M β-mercaptoethanol. The column flow rate was maintained at 16 mL/h and fractions were collected every 15 min. The column was flushed with equilibration buffer until the absorbance of the fractions reached a baseline at 280 nm. The adsorbed glycoproteins were eluted with the equilibrium buffer containing 0.1M methyl α-D-mannopyranoside. Proteins that eluted from the column were monitored by measuring the absorbance at 280 nm and by gel electrophoresis of aliquots of selected column fractions.

The fractions rich in glycoproteins were pooled (tubes112-148) and dialyzed against 0.05 M phosphate buffer, pH 7.2 containing 0.1 M NaCl for 48 hrs with 6 buffer changes (5L per change). The dialyzate was concentrated using an Amicon ultra centrifugal filter of 3k (MW cutoff, 3000) and loaded onto a Sephacryl S200 (GE Healthcare, Piscataway, NJ) column (2.6 x 74 cm) previously equilibrated with 0.05 M phosphate buffer, pH 7.2 containing 0.1 M NaCl. The flow rate of the column was maintained at 24 mL/h and fractions were collected every 15 min. Proteins eluting from the column were monitored by measuring the absorbance at 280 nm and by gel electrophoresis of selected column fractions.
Glycoprotein A (peak 1)

Fractions rich in glycoprotein A (peak 1) on the gel filtration column (tubes 36 to 40) was pooled, dialyzed against distilled (DI) water for 48 hrs with 6 water changes (5 L per change), and lyophilized.

Glycoprotein B (peak 2)

Fractions rich in glycoprotein B (peak 2) on the gel filtration column (tubes 41 to 50) was pooled, dialyzed against DI water for 48 hrs with 6 water changes (5 L per change), and lyophilized.

Glycoprotein C (peak 3)

Fractions rich in glycoprotein C (peak 3) on the gel filtration column (tubes 52 to 63) was pooled dialyzed against 0.02 M Tris-HCl, pH 8.1 for 48 hrs with 6 buffer changes (5 L per change), and the dialyzate was loaded onto a DEAE DE-53 (Whatman, Inc., Piscataway, NJ) column (2.6 x 10 cm) previously equilibrated with 0.02 M Tris-HCl, pH 8.1. Flow rate was maintained at 24 mL/h and fractions collected every 15 min. The column was flushed with the equilibration buffer until the absorbance at 280 nm reached to baseline. Proteins adsorbed to the column were eluted with a 0-0.5 M NaCl gradient in the equilibration buffer (130 mL each) and flushed with 2 M NaCl in equilibration buffer. Fractions rich in glycoprotein C (tubes 114-120) were pooled, dialyzed against DI water for 48 hrs with 6 water changes (5 L per change) and lyophilized.

All column chromatography was performed at 4 ºC in duplicate. The lyophilized proteins were stored in air tight plastic containers at -20 ºC until further use.

Protein determination

Soluble protein content was determined using the Bradford method (Bradford, 1976). Bovine Serum Albumin (BSA) was used as the standard protein to generate a standard curve (0 to 600 µg/ml).
Electrophoresis

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

Polypeptide profiles of proteins were characterized using SDS-PAGE as described by Fling and Gregerson (1986). Protein samples containing approximately 10-30 µg of proteins were mixed with suitable volumes of SDS-PAGE sample buffer (0.05 M Tris-HCl pH 6.8, 1% SDS w/v, 30% v/v glycerol and 0.01% w/v bromophenol blue as the tracking dye) with (2% v/v β-mercaptoethanol) or without a reducing agent and heated in a boiling water bath for 10 min cooled to RT and electrophoresed. Protein samples were electrophoresed on either 8-25% gradient or 12% monomer acrylamide resolving gel and 4% monomer acrylamide stacking gel. The gels were run at a constant current until the tracking dye migrated to the gel edge and stained overnight with 0.25% w/v Coomassie Brilliant Blue R containing 50% v/v methanol and 10% v/v acetic acid followed by destaining with 50% v/v methanol containing 10% v/v acetic acid until the blue background was completely removed. Pharmacia (Pharmacia Co., Piscataway, NJ) low molecular weight markers were used in each gel run as standards.

*Non-denaturing Non-dissociating-Polyacrylamide Gel Electrophoresis (NDND-PAGE)*

Proteins were separated based on their net negative electrical charge using NDND-PAGE as described by Andrews (1986) and Sathe (1993). Resolving gels were of 3 to 30% w/v linear acrylamide gradient gels with 90 mM Tris, 80 mM boric acid and 2.5 mM Na-EDTA pH 8.4. The stacking gel was of 3% w/v acrylamide. The electrophoresis buffer was 90 mM Tris, 80 mM boric acid and 2.5 mM Na-EDTA pH 8.4. Protein samples containing 10-30 µg were mixed with suitable volumes of the NDND buffer (2 volumes of 0.45 M Tris, 0.4 M boric acid, 12.5 mM Na-EDTA mixed with 1 volume of glycerol containing 0.01% w/v bromophenol blue as the tracking dye). Gels were run overnight at a constant current of 10 mA with tap water cooling and stained overnight with Coomassie Brilliant Blue R followed by destaining with 50% v/v methanol containing 10% v/v acetic acid until the blue background was completely removed. Pharmacia (Pharmacia Co., Piscataway, NJ) high molecular weight markers (thyroglobulin, 669 kDa; ferritin, 440 kDa; catalase, 232 kDa; lactate dehydrogenase, 140 kDa; albumin, 67 kDa) were used in each gel run as standards.
Two dimensional (2D) gel electrophoresis

The charge heterogeneity and electrophoretic mobility of glycoprotein C was determined using isoelectric focusing (IEF) combined with SDS-PAGE. Precast 11 cm Immobiline DryStrip pH 3-11 gradient (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) were used to run proteins in the first dimension. The protein samples containing 20-30 µg of protein were prepared in rehydration buffer (1x ReadyStrip buffer, Bio-Rad Laboratories, Inc., Hercules, CA) and applied along the edge of a channel in a dry and cleaned focusing tray (Protean IEF system, Bio-Rad Laboratories), followed by placing the IPG strip gel side down on to the sample. The IPG strip was overlaid with 1 ml of mineral oil and the sample was allowed to rehydrate for 12 h at 50 µA/strip followed by focusing for 33 000 V-h at 20 ºC. Prior to running the second dimension, the IPG strips were equilibrated in equilibration buffers. Equilibration buffer was 6 M urea, 0.375 M Tris-HCl pH 8.8, 2%SDS, and 20% glycerol. The IPG strip was incubated for 15 min in equilibration buffer I (equilibration buffer containing 2% w/v dithiotreitol-DTT) followed by incubation for 10 min in equilibration buffer II (equilibration buffer containing 2% w/v iodoacetamide). The IPG strips were then kept in SDS-PAGE running buffer for 10 min and laid on top of an 8-25% gradient or 12% monomer acrylamide resolving gel, overlaid with a solution of 1% w/v agarose and once the agarose solidified electrophoresis was allowed to proceed as described above. The gels were stained with Coomassie Brilliant Blue R followed by destaining with 50% v/v methanol containing 10% v/v acetic acid until the blue background was completely removed.

Silver staining

Following SDS-PAGE gels were silver stained to visualize the proteins as described by Westermeier (1993). Gels were immersed in a fixing solution containing 11.4% w/v trichloroacetic acid, 3.4% w/v sulfosalicylic acid and 30% v/v MeOH for 2 h and washed in excess DI water five times for 5 min each. The gels were then incubated for 1h in an aqueous solution containing 25% v/v MeOH and 8% v/v acetic acid followed by treatment of the gels with a solution containing 10% v/v glutaraldehyde for 2 h. The gels were rinsed in excess DI water and washed overnight in DI water. Following the overnight wash, the gels were treated with a 200 mL solution containing 1mL of 15% w/v freshly prepared NaOH, 8 mL of 20% w/v
silver nitrate and ~6 mL of concentrated ammonium hydroxide solution for 15-20 min. The gels
were washed in excess DI water three times for 5 min each and the protein bands were visualized
by immersing the gels in 200 mL of developer solution containing 20 mL of 0.05% w/v citric
acid and 100 μL of formaldehyde. Following the development of the protein bands, the
development was stopped by immersing the gels in a 200 mL solution of 0.05% w/v citric acid
containing 100 μL of 35% w/v aqueous methylamine. The gel was immersed in Kodak fixer
solution (Kodak, Rochester, NY) until the background of the gel was completely removed.

Glycoprotein staining

Following SDS-PAGE, glycoprotein staining of the gels was done to identify the
glycoproteins. The gels were immersed in a fixing solution for 1h containing 50% v/v methanol
and 10% v/v acetic acid followed by washing two times with a solution of 3% v/v acetic acid for
15 min each. The gels were incubated for 1 h in a solution containing 1% w/v periodic acid and
3% v/v acetic acid for 1 h followed by washing four times in a solution of 3% v/v acetic acid for
15 min each. The glycoproteins were developed by immersing the gels in a solution of Schiff’s
reagent for ~15-20 min followed by washing the gels in 3% v/v acetic acid once the
glycoproteins were visualized.

Stokes’ radius and molecular mass

The stokes’ radius and the molecular mass of glycoprotein C was estimated using a
Sephacryl S300 HR (GE Healthcare, Piscataway, NJ) column (1.6 x 76.5 cm), that had been
previously calibrated using high (thyroglobulin, ferritin, catalase, aldolase) and low (albumin,
ovalbumin, chymotrypsinogen A, ribonuclease A) molecular weight standard proteins kits
(Amersham Biosciences, Piscataway, NJ). The column was equilibrated with 0.02 M Tris-HCl,
pH 8.1 containing 0.1 M NaCl for the estimation of the molecular mass and Stokes radius of the
glycoprotein. Fractions were collected every 15 min and the flow rate was maintained at 14
mL/hr. The sample protein was eluted from the column in duplicate and the absorbance was
measured at 280 nm of the collected fractions to monitor the elution of the protein. The Stokes’
radius and the molecular mass of the glycoprotein were calculated based on the standard curve
obtained from the elution of the proteins of known molecular mass through the S300 column.
N-Terminal amino acid sequencing

Based on the band width and the staining intensity, the dominant glycoproteins were identified using N-terminal amino acid sequencing. Following SDS-PAGE, the separated proteins were transferred to a 0.2 µm PVDF membrane as described by Towbin et al., 1979. The N-terminal amino acid sequences of the blotted proteins were determined using an ABI 492 Procise CLC protein sequencer (Applied Biosystems, Inc., Foster City, CA). The sequences were analyzed using the Blast program (National Center for Biotechnology Information, National Institutes of Health, Bethesda, MD, http://www.ncbi.nlm.nih.gov/BLAST/).

Production of rabbit polyclonal antibody (pAb)

Almond whole protein extract was prepared by vortexing defatted almond flour in borate saline buffer (BSB; 0.1 M H$_3$BO$_3$, 0.025 M Na$_2$B$_4$O$_7$, 0.075 M NaCl, pH 8.45) at a flour/solvent ratio of 1:10 w/v for 1 h at RT. This was followed by centrifugation at 16 000 g for 15 min at RT and the protein concentration of the resulting supernatant was estimated using the method as described by Bradford (1976).

A New Zealand white rabbit was immunized with the BSB extracted almond proteins (1000 µg) in 1mL of TiterMax Gold adjuvant (Sigma Chemical Co. St. Louis, MO) as described by Acosta et al. (1999). Three booster doses of 500 µg each of BSB extracted almond proteins in 500 µL of TiterMas Gold adjuvant was administered at 4 week intervals. A test bleed of 1-2 mL of blood was obtained after 10-14 days of each immunization to check for the pAb titer, which was done using Western blotting. The rabbit was subsequently bled and the serum collected and stored at -20 ºC until further use. Preimmune serum was collected prior to the start of immunization to serve as the control when determining the antibody titer.

Immunoblotting

Following SDS-PAGE the proteins were transferred to a 0.22 µm nitrocellulose membrane as described by Towbin et al. (1979) at 4 ºC for 2-3 hrs and the transferred polypeptides were visualized by brief staining for ~5 min with Ponceau S stain. The unbound sites on the nitrocellulose membrane were blocked by incubating the membrane in Tris-buffered saline (10 mM Tris, 0.9% w/v NaCl, 0.05% v/v Tween 20, pH 7.6) containing 5% w/v nonfat
dried milk (NFDM) for 1 h at RT. Subsequently the membrane was washed with TBS-T for 5 min and incubated with rabbit polyclonal antibody against whole almond diluted 1:10 000 in TBS-T or with an appropriate dilution of monoclonal antibodies (IgG purified monoclonal antibodies raised against amandin, 4C10 and 4F10 used at a dilution of 1: 2 500 and 1: 15 000 respectively) overnight at 4 ºC. Following the overnight incubation, the membrane was washed three times with TBS-T for 15 min each and incubated with the secondary antibody diluted in TBS-T for 1 h at RT. For the polyclonal antibodies, the secondary antibody will be horse radish peroxidase (HRP) labeled goat anti rabbit IgG antibody at 1:40 000 v/v dilution in TBS-T. For the monoclonal antibodies, the secondary antibody will be HRP labeled goat anti mouse IgG antibody at 1:10 000 v/v dilution in TBS-T. The membrane was washed three times with TBS-T for 15 min each following the completion of the incubation period with the secondary antibody. The reactive protein bands were detected by incubating the membrane with a mixture of solution A (containing 100 μL of 250 mM luminol, 44 μL of 90 mM ρ-coumaric acid, 1mL of 1 M Tris-HCl pH 8.5 and 8.85 mL of DI water) and solution B (6μL of 30% hydrogen peroxide, 1 mL of 1 M Tris-HCl pH 8.5 and 8.85mL of DI water) for 5 min and exposing to X-ray film for 30 s to 2 min. For dot blot assays 1 µg of the protein extract was dotted on the membrane and allowed to air dry. The same procedure as outlined above was followed from blocking to development.

The reactivity of human IgE against the glycoproteins were tested by Western blotting and dot blotting using human sera of patients showing strong reactivity to almonds. The transfer of glycoproteins to the membrane was similar to that performed for Western blotting with polyclonal/monoclonal antibodies. Following the transfer, 3mm wide strips of the membrane containing ~20 ug of protein was cut. For the dot blot, 1 μg of protein was dotted on the membrane. Membranes were blocked overnight in 5% NFDM in TBST-T at 4 ºC followed by incubating with almond allergic human sera diluted 1:3 in TBS-T containing 5% NFDM overnight at 4 ºC. Following the overnight incubation, the membranes were washed three times with TBS-T for 30 min each and incubated with the secondary antibody, 125 I-labeled goat mouse anti-human IgE diluted 1:10 in phosphate buffered saline containing 5% NFDM at 4 ºC overnight. The nitrocellulose membranes were washed three times with TBS-T for 30 min each and developed on X-ray film for 4-10 days at -80 ºC.
Enzyme linked immunosorbent assay (ELISA)

Ninety six-well polyvinyl microtiter plates were coated with 50 μL of 10 μg/mL of almond protein extract (soluble proteins of defatted almond flour was extracted in BSB buffer) per well (500 ng almond protein/well) prepared in the coating buffer (48.5 mM citric acid, 103 mM Na₂HPO₄, pH 5.0) and incubated for 1 h at 37 °C. The plate was washed three times with TBS-T and blocked for 1 h at 37 °C with 5% NFDM in TBS-T. The plate was again washed three times with TBS-T and 50 μL of almond pAb diluted 1:40 000 v/v in TBS-T containing 1% NFDM was added to each well and incubated at 37 °C for 1 h. Subsequently the plate was washed three times with TBS-T and incubated with 50 μL/well of the secondary antibody, 1:000 v/v alkaline phosphatase labeled goat anti-rabbit IgG in TBS-T for 1 h at 37 °C. Following the incubation, the plates were washed three times with TBS-T and developed by incubating with 50 μL/well of phosphatase substrate (5 mg/mL p-nitrophenyl phosphate tablet dissolved in 5 mL substrate buffer; 0.0049% w/v MgCl₂, 0.096% v/v diethanolamine, pH 9.8). The reaction was stopped by adding 50 μL of 3M NaOH and the absorbance in each well was measured in a microplate scanning spectrophotometer (KC4, Bio-Tek Instruments, USA) using a 405 nm filter.

Deglycosylation of the glycoproteins

To assay the possible effect of the carbohydrate moieties on the immunoreactivity of glycoproteins, the glycoproteins were deglycosylated and their immunoreactivity tested using immunoblotting techniques (Western blot, dot blot and ELISA). The glycoproteins were deglycosylated as described by Van Holst and Varner (1984). Briefly, 1 mg of the lyophilized glycoprotein was dried overnight in a desiccator. To the dried glycoprotein sample 20 μL of anhydrous methanol and 180 μL of 70% w/v HF in pyridine (Sigma Aldrich, St. Louis, MO) was added and incubated at RT for 90 min followed by the addition of 1mL of ice cold DI water to stop the reaction. The deglycosylated protein was dialyzed against 0.1 M NaCl overnight followed by dialyzing against DI water for 48 hrs. SDS-PAGE of the deglycosylated proteins was done followed by glycoprotein staining to ensure that the glycoproteins were deglycosylated. Immunoblotting of the deglycosylated proteins with rabbit pAb was performed to assess the immunoreactivity of these proteins in comparison to the non deglycosylated proteins.
Data Analysis and Statistics

All statistical analyses were performed using SPSS statistical software (version 15; Chicago, IL). One-way analysis of variance and post-hoc analysis was performed using Tukey HSD to compare means for statistical significance. Results were considered to be statistically significant if \( p \leq 0.05 \). All experiments were carried out at least in duplicate.
CHAPTER 4

RESULTS AND DISCUSSION

Isolation of almond glycoproteins

Almond soluble proteins extracted in 0.05 M phosphate buffer, pH 7.2 containing 1 M NaCl and 0.01 M β-mercaptoethanol from defatted almond flour were loaded onto a Concanavalin A Sepharose 4B column for the separation of glycoproteins, where the glycoproteins bound to the column were eluted with the extraction buffer containing 0.1M methyl α-D-mannopyranoside. The column profile for the separation of the almond glycoproteins is given in Fig 4.1.

![Graph](image)

**Figure 4.1.** Elution profile of almond protein extract off Con A Sepharose 4B column (2.6 x 7.0 cm). Column was equilibrated with 0.05 M phosphate buffer, pH 7.2 containing 1M NaCl and 0.01 M β-ME. Fractions containing glycoproteins (tubes 112-148, 142 mL) were pooled. Inset: SDS-PAGE (under reducing conditions) analysis of fractions (mixed with suitable volumes of SDS-PAGE sample buffer, load per each well, 40 µL) eluted off the column indicated by number on the top of the gel lane. S=protein marker, 0.75 µg, molecular mass of each standard is indicated in the left margin of the inset. L=protein loaded on to the column, 20 µg.
Glycoprotein staining of the SDS-PAGE separated protein fractions was done to confirm that the proteins eluted from the Con A Sepharose 4 B column was glycoproteins (Fig 4.2).

**Figure 4.2.** Glycoprotein stain of almond fractions eluting off Con A Sepharose 4 B column. The numbers on the top of the gel lane indicates tube numbers of the column fractions (mixed with suitable volumes of SDS-PAGE sample buffer, load per each well, 40 µL) eluted off the column. Soy 7S and 11S was used as positive and negative controls respectively, protein load 40 µg. S=protein marker, 0.75 µg. L= protein loaded on to the column, 40 µg.

Fractions rich in glycoproteins were pooled and loaded on to a Sephacryl S200 column for further purification and the glycoproteins resolved in to three peaks (glycoproteins A, B and C) by Sephacryl S200 gel filtration as shown in Fig 4.3.

The third peak off the S200 column (glycoprotein C) was passed through a DEAE DE-53 column, where protein elution was by a linear gradient of 0 to 500 mM NaCl in equilibrium buffer (Fig 4.4). As can be seen from Fig 4.4, glycoprotein peak C eluted as a single peak off the DEAE DE-53 column at a NaCl concentration range of 100-150 mM. Glycoprotein C was passed through the DEAE De-53 column in an attempt to obtain a more purified product by removing the minor impurities. However, as evident from Fig 4.4 this was unsuccessful although we were able to concentrate glycoprotein C. Further purification was also attempted by using reverse phase HPLC, although this purification step also was not successful.
Figure 4.3. Elution profile of almond glycoproteins off Sephacryl S200 column (2.6 x 74 cm). Column was equilibrated with 0.05 M phosphate buffer, pH 7.2 containing 0.1 M NaCl. Fractions containing glycoprotein A (tubes 36-40, 21 mL), glycoprotein B (tubes 41-50, 40 mL) and glycoprotein C (tubes 52-63, 49 mL) was pooled separately. Inset: SDS-PAGE (under reducing conditions) analysis of fractions (mixed with suitable volumes of SDS-PAGE sample buffer, load per each well, 40 µL) eluted off the column indicated by number on top of the gel lane. S= protein marker, 0.75 µg. L= protein loaded on to the column, 20 µg.

Figure 4.4. Elution profile of almond glycoprotein C obtained from S200 column off DEAE DE-53 column (2.6 x10 cm). Column equilibrated with 0.02 M Tris-HCl, pH 8.1. Proteins eluted
with a 0-0.5 M NaCl gradient in the equilibration buffer. Tubes 114 to 120 (31 mL) were pooled to yield glycoprotein C. Inset: SDS-PAGE (under reducing conditions) analysis of fractions (mixed with suitable volumes of SDS-PAGE sample buffer, load per each well, 40 µL) eluted off the column indicated by number on top of the gel lane. S = protein marker, 0.75 µg. OE = almond protein extract in BSB, 20 µg. L = protein loaded on to the column, 20 µg.

As evident from Fig 4.1, glycoproteins comprise only a very small fraction of the total soluble proteins of almonds. Only ~79 mg of glycoproteins were purified from 20 g of defatted almond flour. Table 4.1 summarizes typical yield data of almond glycoproteins. Thus, typically of the total soluble proteins in almonds, only ~1.4% is represented by glycoproteins.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract loaded on Con A column</td>
<td>5500</td>
</tr>
<tr>
<td>Glycoproteins loaded on S200 column</td>
<td>111</td>
</tr>
<tr>
<td>Glycoprotein A off S200</td>
<td>10</td>
</tr>
<tr>
<td>Glycoprotein B off S200</td>
<td>43</td>
</tr>
<tr>
<td>Glycoprotein C loaded on DEAE DE-53</td>
<td>31</td>
</tr>
<tr>
<td>Glycoprotein C off DEAE DE-53</td>
<td>26</td>
</tr>
</tbody>
</table>

aData are for a typical preparation starting with 20 g of defatted almond flour extracted with 200 mL of 0.05 M phosphate buffer, pH 7.2 containing 1 M NaCl and 0.01 M β-mercaptoethanol.

**Biochemical characterization of selected glycoproteins**

**SDS-PAGE**

SDS-PAGE gel electrophoresis in the presence and absence of a reducing agent (β-mercaptoethanol) was performed to characterize the protein profiles of the three glycoproteins (Fig 4.5). Glycoprotein A and B under reducing conditions has several major bands in contrast to a single major band for glycoprotein C. Glycoprotein A is composed of peptides ranging from ~11 kDa to ~72 kDa under both reducing and non-reducing conditions. Under reducing conditions 3 major bands are visible for glycoprotein A at ~13 kDa, ~22 kDa and ~44 kDa (indicated by arrows in Fig 4.5;i). Of these the ~13 kDa band is present under both reducing and non-reducing conditions although the other two bands show a different mobility under non reducing conditions (Fig. 4.5).
Glycoprotein B in the presence of β-ME, is composed of three major bands at ~12 kDa, ~34 kDa and ~62 kDa. In addition glycoprotein B has several minor bands ranging from ~11 kDa to ~55kDa. The major band ~12 kDa of glycoprotein B is present under both reducing and non reducing conditions.

Glycoprotein C has a single major polypeptide at ~ 62 kDa and also several minor polypeptides ranging from ~11kDa to ~55kDa under reducing conditions. Electrophoresis of glycoprotein C under non reducing conditions results in the appearance of 2 bands ~135 kDa and ~170 kDa. This suggests the possibility of either the presence of disulphide linkages for this polypeptide or the formation of aggregates.

**N-Terminal amino acid sequencing**

The major peptide band of glycoprotein B at ~62 kDa and the major band of glycoprotein C also with a molecular mass of ~62 kDa (indicated in Fig 4.5; i by vertical arrows) were subjected to N terminal amino acid sequencing following the transfer of the electrophoresed proteins to a PVDF membrane. The ~62 kDa peptide of glycoprotein B showed 92% identity and similarity (Table 4.2) to the prunasin hydrolase isoform PH C precursor of black cherry (*Prunus*...
The N terminal sequence of the peptide of glycoprotein C showed 100% similarity to the hydroxynitrile lyase of almonds. A similar result for N-terminal sequencing of almond hydroxynitrile lyase has also been reported by Jansen et al. (1992).

Table 4.2. N-terminal amino acid sequences of selected peptides of glycoproteins

<table>
<thead>
<tr>
<th>Peptide band</th>
<th>N-terminal sequence</th>
<th>Sequences producing significant alignment</th>
<th>% identity</th>
<th>% similarity</th>
</tr>
</thead>
</table>
| 62 kDa band of glycoprotein B | A R T D P P I V (X)A T L | 1. Prunasin hydrolase isoform PH C precursor of *Prunus serotina* (AAL35324)  
2. Putative prunasin hydrolase isoform PH-L1 precursor of *Prunus serotina* (AAF34651)  
3. β-glucosidase of *Prunus avium* (AAA91166)  
4. Prunasin hydrolase isoform PH C precursor of *Prunus serotina* (AAL07434) | 92         | 92           |
| 62 kDa band of glycoprotein C | L A T T S D H D F S Y L S F A Y D A | 1. Almond hydroxynitrile lyase (3GDN_A; 1JU2_A)  
2. R-oxynitrile lyase isoenzyme I precursor of *Prunus dulcis* (AAL11514)  
3. R-mandelonitrile lyase precursor of *Prunus serotina* (P52706) | 100        | 100          |

Prunasin hydrolase of black cherry (*Prunus serotina*) has been purified by Li et al. (1992) where they were able to purify three isozymes of prunasin hydrolase namely PH I, PH IIa and PH IIb. PH I and PH IIb were reported to be monomeric glycoproteins with a molecular weight of 68 kDa while PH IIa was reported as being a dimeric glycoprotein with a molecular mass of 140 kDa. The prunasin hydrolase that co purified with glycoprotein B in the present study is present as a major band when electrophoresed with a reducing agent (Fig 4.5; i), ~62 kDa. However, when SDS-PAGE of glycoprotein B in the absence of a reducing agent is carried out this ~62 kDa band is not present (Fig 4.5; ii), thereby indicating the possibility that the prunasin hydrolase of almonds contains disulphide linkages.
Almond hydroxynitrile lyase has been purified by several research groups (Jansen et al., 1992; Kaul and Mattiasson, 1987) and its crystal structure has been determined by Dreveny et al. (2001). They reported that the 61 kDa single chain glycoprotein of 521 amino acids contains four N-glycosylation sites at Asn118, Asn135, Asn352, and Asn392. A disulphide bridge between residues Cys 399 and Cys 450 was also reported by these authors. The presence of a disulphide linkage was suggested in the present study based on the SDS-PAGE gel profiles under reducing and non reducing conditions (Fig. 4.5).

**NDND-PAGE**

The glycoproteins were separated based on their net negative charge using NDND-PAGE gel electrophoresis. NDND-PAGE allows the separation of proteins in their native forms where the proteins are not denatured or reduced. As indicated in Fig 4.6, the major band of glycoprotein C (hydroxynitrile lyase) can be seen ~62 kDa confirming that this is composed of a single polypeptide. This is in agreement with previous data where hydroxynitrile lyase has been identified as a single chain glycoprotein (Dreveny et al., 2001). Glycoproteins A and B as can be seen in Fig 4.5 has several bands and NDND-PAGE (Fig 4.6) indicates that both of these glycoproteins are composed of more than one polypeptide.
**Figure 4.6.** NDND-PAGE of almond glycoproteins. S=protein marker. OE=whole almond protein extract. A, B and C=glycoproteins A, B and C respectively. Protein load 30 µg.

**2D gel electrophoresis**

The charge heterogeneity and electrophoretic mobility of glycoprotein C was determined using isoelectric focusing (IEF, pH range 3-10) combined with SDS-PAGE (Fig 4.7). IEF of glycoprotein C showed that the major protein band (hydroxynitrile lyase) separated into 4-5 bands based on the heterogeneity of the charge. Electrophoresis in the second dimension shows that these bands have similar molecular masses. This may indicate the presence of several isoforms that differ from each other based on their electrical charge or the possibility of impurities present in the protein preparation.

**Figure 4.7.** Two dimensional (IEF+SDS-PAGE) gel electrophoresis of glycoprotein C. Directions of first (pH range 3-10) and second dimensions are indicated in horizontal and vertical arrows respectively. S=protein marker, 0.75 µg. Protein load in first dimension was 30 µg. C=glycoprotein C (30µg) loaded directly on SDS-PAGE.
Stokes’ radius and molecular mass

Glycoprotein C was loaded onto a previously calibrated Sephacryl S300 HR gel filtration column (1.6 x 76.5 cm) to determine the Stokes’ radius and the molecular mass of hydroxynitrile lyase. The regression equation for the standard proteins for the calculation of the molecular mass was \( y = -0.4045x + 2.3757 \) and \( r = 0.958 \), where \( x = \log \text{MW} \) and \( y = K_{av} \) \( [K_{av} = (V_e - V_o)/(V_t - V_o), V_e = \text{elution volume in mL, } V_o = \text{void volume in mL, } V_t = \text{total gel bed volume in mL}] \). The Stokes’ radius of the glycoprotein hydroxynitrile lyase was calculated based on the regression equation for standard proteins, \( y = 0.0122x + 0.192 \) and \( r = 0.9545 \) where \( x = \text{Stokes’ radius} \) and \( y = (-\log K_{av})^{-1/2} \). The molecular mass and the Stokes’ radius for the first peak eluting off the calibrated S300 HR column was found to be 172.1 kDa and 47.1 Å (n=2) respectively. For the second peak of hydroxynitrile lyase eluding off the calibrated S300 HR column the molecular mass and Stokes’ radius were 67.7 kDa and 34.4 Å (n=2) respectively. The molecular mass calculated for the second peak eluting off the calibrated gel filtration column (67.7 kDa) is higher than the reported molecular mass of 61 kDa for hydroxynitrile lyase (Dreveny et al., 2001). Nevertheless, Jansen et al. (1992), using gel filtration, reported a molecular mass of 72 kDa for almond hydroxynitrile lyase.

Figure 4.8. Elution profile of glycoprotein C off calibrated S300 HR column (1.6 x 76.5 cm). Column was equilibrated with 0.02 M Tris-HCl, pH 8.1 containing 0.1 M NaCl. Inset: SDS-
PAGE (under reducing conditions) analysis of fractions (mixed with suitable volumes of SDS-PAGE sample buffer, load per each well, 35 µL) eluted off the column indicated by number on top of the gel lane. S=protein marker, 0.75 µg. L=protein loaded on to the column, 15 µg.

Glycoprotein C eluted off the gel filtration column as two peaks as shown in Fig 4.8. Since Coomassie Brilliant Blue staining of the SDS-PAGE gel of column fractions (see inset of Fig. 4.8) showed that both peaks had similar banding patterns, silver staining of the gel was performed to determine whether there were differences in the protein profiles of the two peaks.

However, as can be seen from Fig 4.9, silver staining also indicated that although the column profile gave two peaks the protein profile was the same. N-terminal sequencing of all four peptides shown in the gel (Fig 4.8 inset and Fig 4.9) was done following the transfer of the electrophoresed proteins to a PVDF membrane for confirmation. All four peptides showed similarity to the hydroxynitrile lyase of almonds (Table 4.2). This indicates the possibility that the two peaks in the column profile represents two different isoforms of the enzyme hydroxynitrile lyase. Currently four isoforms have been reported for almond hydroxynitrile lyase, where they have been shown to have similar molecular masses (~60 kDa) but differ in their sequence and glycosylation sites (Dreveny et al., 2001). Thus, these results and the results obtained for the 2D gel electrophoresis of hydroxynitrile lyase (Fig 4.7) are in accordance with reported data for the molecular weight and the presence of isoforms.

![Silver stain of SDS-PAGE analysis of fractions eluding off calibrated S300 HR column.](image)

**Figure 4.9.** Silver stain of SDS-PAGE analysis of fractions eluding off calibrated S300 HR column. S=protein marker, 0.75 µg. L=protein loaded on to the column 15 µg. Numbers on top
of the gel lane indicates the tube numbers of the fractions (mixed with suitable volumes of SDS-PAGE sample buffer, load per each well, 40 µL) eluding off the S300 HR column.

**Immunoreactivity of selected glycoproteins**

*Immunoreactivity of glycoproteins with polyclonal antibodies*

Western blotting with polyclonal antibodies raised against whole almonds in rabbit was used to assess the immunoreactivity of glycoproteins. As shown in Fig 4.10 all three glycoproteins were strongly reactive with the polyclonal antibodies. Recognition of multiple bands by polyclonal antibodies is to be expected since polyclonal antibodies recognize multiple epitopes.

![Western blot image](image)

**Figure 4.10.** Western blot of glycoproteins with polyclonal antibodies raised against whole almonds in rabbit. pAb dilution, 1: 10,000 in TBS-T. S=protein marker. OE=whole almond extract. A, B and C=glycoproteins A, B and C respectively. Protein load in each lane was 25 µg.

*Immunoreactivity of glycoproteins with monoclonal antibodies*

Monoclonal antibodies raised against AMP were used in Western blotting experiments to determine whether these antibodies recognize any epitopes of the glycoproteins. Two
monoclonal antibodies, 4C10 and 4F10 were used in the immunoblotting experiments. However, as can be seen from Fig 4.11, none of the peptides in all three glycoproteins were recognized by either of the monoclonal antibodies, indicating that the specific epitopes recognized by these monoclonal antibodies are not present in the glycoproteins. AMP has been shown to be a non glycoprotein by Sathe et al. (2002), thus any epitopes that are being recognized in AMP, will most likely be not found in the glycoproteins.

Figure 4.11. Western immunoblot of almond glycoproteins with monoclonal antibodies. Dilution factor of IgG purified 4C10 and 4F10 were 1: 2 500 and 1: 15 000 respectively. S=protein marker. OE=whole almond extract. A, B and C= glycoproteins A, B and C respectively. Protein load in each lane was 20 µg.

Immunoreactivity of glycoproteins with human IgE

Sera from 11 patients known to be allergic to almonds were used in a dot blot assay to determine the immunoreactivity of the glycoproteins (Fig 4.12). The patient history including allergies to other tree nuts is summarized in Table 4.3. Most almond allergic patients had also a history of life threatening reactions to nuts other than almonds. Sera from 8 almond allergic patients were used in immunoblotting experiments by Bargman et al., (1992) to identify IgE binding proteins in almonds. Similar to the patient sera used in the present study, most of the patients in the study of Bargman et al. (1992) were also allergic to other tree nuts including Brazil nut, cashew, pecan, pistachio and walnut. In a study carried out by Poltronieri et al.
(2002), using almond allergic sera it was found that hazelnut and walnut proteins competed for IgE binding with almond conglutin γ and 2S albumin.

**Table 4.3.** Patient history of almond allergic subjects

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age</th>
<th>Age of onset</th>
<th>Sex</th>
<th>History</th>
<th>Almond LT</th>
<th>Other nuts LT</th>
<th>Immuno CAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>38</td>
<td>01</td>
<td>F</td>
<td>A, AD, AR</td>
<td>Yes</td>
<td>Cashew, pecan, walnut</td>
<td>Class 3</td>
</tr>
<tr>
<td>2</td>
<td>51</td>
<td>02</td>
<td>F</td>
<td>A, AD, AR</td>
<td>No</td>
<td>Pecan, walnut</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>52</td>
<td>01</td>
<td>F</td>
<td>A, AD</td>
<td>No</td>
<td>Brazil nut, walnut</td>
<td>0.49</td>
</tr>
<tr>
<td>4</td>
<td>38</td>
<td>05</td>
<td>F</td>
<td>A</td>
<td>No</td>
<td>Brazil nut, cashew, hazelnut, macadamia, pecan, pistachio, walnut</td>
<td>1.9</td>
</tr>
<tr>
<td>5</td>
<td>07</td>
<td>03</td>
<td>F</td>
<td>A, AD</td>
<td>No</td>
<td>None</td>
<td>&lt; 0.35</td>
</tr>
<tr>
<td>6</td>
<td>39</td>
<td>01</td>
<td>F</td>
<td>A, AD, AR</td>
<td>Yes</td>
<td>Brazil nut, peanut, pine nut, pistachio, walnut</td>
<td>&lt; 0.35</td>
</tr>
<tr>
<td>7</td>
<td>43</td>
<td>ND</td>
<td>M</td>
<td>A, AD, AR</td>
<td>Yes</td>
<td>Walnut</td>
<td>Class 3</td>
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<tr>
<td>8</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>9</td>
<td>30</td>
<td>Child</td>
<td>M</td>
<td>A, AD, AR</td>
<td>ND</td>
<td>Never eaten other nuts</td>
<td>2.88</td>
</tr>
<tr>
<td>10</td>
<td>35</td>
<td>06</td>
<td>M</td>
<td>A, AR</td>
<td>No</td>
<td>Brazil nut, cashew, hazelnut, macadamia pecan, pistachio, walnut</td>
<td>ND</td>
</tr>
<tr>
<td>11</td>
<td>24</td>
<td>10</td>
<td>F</td>
<td>A</td>
<td>No</td>
<td>None</td>
<td>0.46</td>
</tr>
</tbody>
</table>

A=asthma; AD=allergic rhinitis; AR=atopic dermatitis; LT=has experienced life threatening reactions; ND=no data available.

Glycoprotein B was recognized by 6 of the total of 11 sera tested (53%) followed by glycoprotein A (3 out of 11 samples or 27%). However, glycoprotein C was not recognized by most patient sera except for a slight reactivity shown by the serum obtained from patient 11. However, since food specific IgE antibody levels in sera of many patients can be low these results may not represent the actual immunogenicity of the patients. Furthermore, it has been reported that the strength of an allergic immunoreaction is also dependent on the affinity of IgE towards the epitope. Thus, although somewhat risky, expensive and time consuming, the double-
blind, placebo-controlled oral food challenge (DBPCFC) remains the ‘gold standard’ for food allergy diagnosis (Lin and Sampson, 2009).

**Figure 4.12.** Dot blot of glycoproteins with almond allergic patient sera (i) and with serum sample #11 (ii). Serum used at 3x dilution. #1-11=almond allergic sera. #12=atopic control. #13=control without primary antibody. #14=control without secondary antibody. OE=whole almond extract. A, B and C=glycoproteins A, B and C respectively. GP’s=mixture of all three glycoproteins. Protein dotted on the membrane was 1 µg for (i).

Serum from patient #11 was reactive with glycoprotein B, but was only slightly reactive with the whole almond extract (Fig 4.12; i). This could be due to the patient IgE recognizing an epitope or epitopes of glycoprotein B, and since glycoproteins represent less than 2% of the total soluble proteins of almonds, 1µg of whole almond extract may not contain sufficient epitopes to elicit a positive result. However, when whole almond extract equivalent to 1 µg of glycoprotein
B (110 µg of whole almond extract) was used in the dot blot, a reactivity stronger than with 1 µg of glycoprotein B was observed. This suggests the possibility that the serum IgE recognizes epitope/s in addition to those that are present in glycoprotein B (Fig 4.1; ii).

SDS-PAGE electrophoresed glycoproteins were transferred to a nitrocellulose membrane for Western blotting, in an attempt to identify the peptide bands that are being recognized by human IgE, where serum from patient #11 was used for the Western blot assay. As shown in Fig 4.13, all three glycoproteins have epitopes that are being recognized by human IgE. Both prunasin hydrolase and hydroxynitrile lyase of glycoproteins B and C respectively are being recognized by human IgE. Hydroxynitrile lyase with patient serum #11 shows a much higher intensity of immunoreactivity in the Western blot (Fig 4.13) in comparison to the dot blot assay (Fig 4.12). This could be due to the fact that in Western blotting the protein is denatured using SDS prior to it being transferred to a nitrocellulose membrane and this increases the accessibility of any buried epitopes (Wilson et al., 1991).

**Figure 4.13.** Western blot of glycoproteins with human serum. Human IgE of patient sera #11 was used (at 3x dilution). S=protein marker. OE=almond extract. A, B and C=glycoproteins A, B and C respectively. Protein load in each lane was 20 µg except glycoprotein C was 15 µg.
However, since hydroxynitrile lyase was not identified by any of the human IgE (except for a very low reactivity shown by patient #11) samples, this glycoprotein needs to be tested using a larger number of almond allergic sera before it can be identified as being an allergenic protein. Although glycoprotein B (which includes prunasin hydrolase) was recognized by 53% of human IgE in the dot blot assay, this protein also needs to be tested using a larger number of almond allergic sera before confirming it as a major allergen of almonds.

In a research study conducted by Sathe et al. (2009), it was shown that several specific tree nut seed protein polypeptides recognized by rabbit IgG polyclonal antibodies are also being recognized by IgE of patient sera. Thus, the authors suggest the possibility of using rabbit polyclonal antibodies to study tree nut protein immunoreactivity. Given the expense and difficulty in obtaining human sera, the use of rabbit polyclonal antibodies in at least the initial phases of a research study will be highly beneficial.

**Effect of deglycosylation on the immunoreactivity of glycoproteins**

Glycoproteins were deglycosylated using HF in pyridine and their immunoreactivity was determined using Western blotting, dot blotting and ELISA to assess the effect of carbohydrate moieties on the immunoreactivity of the glycoproteins.

Following deglycosylation, glycoprotein staining of the SDS-PAGE electrophoresed gel was carried out to confirm that the carbohydrate moieties have been removed from the glycoproteins.

As shown in Fig 4.14 (i), glycoprotein staining of the gel confirms that deglycosylation of the samples have occurred. Coomasie Brilliant blue staining of the same gel is shown in Fig 4.14 (ii) to indicate the changes in the protein profiles when the glycoproteins are deglycosylated. As is evident from Fig 4.14(ii), the protein profiles show a difference following deglycosylation. This observation has been also reported by Fryksdale et al. (2002), where they have found that deglycosylation using either enzymatic or chemical methods results in different protein patterns and also a reduction in the complexity of the gel patterns. The smears in the protein profiles of the deglycosylated samples suggest that a certain amount of proteins have degraded following the deglycosylation procedure. Degradation of proteins when a chemical approach is used to deglycosylate proteins has been reported by others as well. Li et al. (1992) used TFMS.
(trifluoromethane sulfonic acid) for deglycosylation of the glycoproteins, prunasin hydrolase and amygdalin hydrolase from black cherry. They reported the formation of high molecular weight aggregates when deglycosylated prunasin hydrolase was analyzed using SDS-PAGE. In the present study, deglycosylation was carried out as described by Van Holst and Varner (1984) using HF. As reported by them, treatment with HF does not denature the proteins if the carbohydrate moieties do not have an influence on the secondary structure of the glycoprotein. Therefore, as suggested by them, if deglycosylation results in the denaturation of the glycoprotein, this confirms that the carbohydrate moieties are essential for the native conformation of the protein backbone.

![ SDS-PAGE electrophoresed glycoproteins and deglycosylated glycoproteins were transferred to a nitrocellulose membrane for Western blotting with polyclonal antibodies raised against whole almond in rabbit. As shown in Figure 4.15 (i), glycoproteins A and B remains immunoreactive following deglycosylation. However, deglycosylated glycoprotein C is not being recognized by the polyclonal antibodies. In glycoproteins A and B, some new peptides are detected. Figure 4.14. Glycoprotein stain (i) and Coomasie brilliant blue stain (ii) of SDS-PAGE of deglycosylated glycoproteins. S=protein marker. A, B and C=glycoproteins A, B and C respectively. deA, de B and de C=deglycosylated glycoproteins A, B and C respectively. + =positive control, Soy 7S. -=negative control, Soy 11 S. Protein load in each lane was 20 µg except controls was 40µg. ](image-url)
being recognized by the polyclonal antibodies following deglycosylation (Fig 4.15; i). This could be due to the recognition of intact epitopes that are now present in peptide fragments by the polyclonal antibodies.

**Figure 4.15.** Western blot (i) and dot blot (ii) of deglycosylated glycoproteins with polyclonal antibodies raised against whole almond in rabbit (dilution factor 1: 10 000 in TBS-T) S=protein marker. OE=almond extract. A, B and C=glycoproteins A, B and C respectively. deA, de B and de C=deglycosylated glycoproteins A, B and C respectively. Protein load in each lane was 15 µg for the Western blot and 0.5 µg of protein was dotted on the membrane for the dot blot.

A dot blot of the glycoproteins and deglycosylated glycoproteins when probed for immunoreactivity with polyclonal antibodies raised against whole almond protein extract in rabbit gave similar results to the Western blot (Fig. 4.15; ii) where deglycosylated glycoproteins A and B retained their immunoreactivity while the immunoreactivity of glycoprotein C was lost upon deglycosylation.

An ELISA assay was also performed to obtain a more quantitative measurement of the effect of deglycosylation on the immunoreactivity of the glycoproteins. The results (Fig 4.16) indicate that there is a significant loss of immunoreactivity with the deglycosylation of all three glycoproteins. Amigo-Benavent et al. (2009), also reports that no visual differences in the
intensity of protein bands was observed between the control and deglycosylated soy β-conglycinin when tested for immunoreactivity with polyclonal antibodies raised in rabbit against total soy proteins. However, they report a slight and statistically insignificant decrease in reactivity when analyzed by ELISA.

![Figure 4.16](image-url) ELISA of deglycosylated glycoproteins using almond polyclonal antibodies. Antibodies raised against whole almond extract in rabbit and used at a dilution of 1: 10 000 in TBS-T. GP A, GP B and GP C=glycoproteins A, B and C respectively. Error bars represents mean (n=3) ± SD. * = significantly different at P<0.05 between treatment groups.

The almost complete loss of immunoreactivity of glycoprotein C following deglycosylation could be either due to the carbohydrate moieties playing a role on epitope recognition or due to the epitope regions being disrupted or destroyed by the chemical method used to deglycosylate the proteins. The degradation of proteins as a consequence of the deglycosylation procedure is evident in the SDS-PAGE profile (Fig 4.14; ii). As reported by Garcia-Casado et al. (1996), when chemicals are used to remove the glycans, they may also alter other groups of the protein. Thus the loss of allergenicity of the glycoproteins following deglycosylation may not be related to the glycans themselves. However, in a study carried out by Garcia-Casado et al. (1996), it was reported that the allergenic glycoproteins WTAI-CM16*, BTAI-CMb* and BMAI-1 of wheat and barley has epitopes that contains both β1→2 xylose and α1→3 fucose and that IgE recognition of these epitopes by allergic patients are lost following enzymatic deglycosylation. Furthermore, it was also shown in the same study that these patient
IgE antibodies were able to recognize other unrelated glycoproteins if they contained N-linked β1→2 xylose and α1→3 fucose glycans, indicating that these carbohydrate moieties play a role in immunoreactivity.

Effect of denaturants on the immunoreactivity of glycoproteins

Several denaturants were used in dot blot and ELISA experiments to test whether these denaturants had any effect on the immunoreactivity of the glycoproteins. As can be seen from the dot blot (Fig 4.17) and ELISA (Fig 4.18) almond protein extract and the glycoproteins were found to show a significant reduction in immunoreactivity in the presence of 0.25 M and 1M β–ME. With the exception of 1% SDS, the ELISA results indicated a significant reduction of immunoreactivity of almond protein extract in the presence of the other denaturants. Research studies have shown that amandin denatures completely at a concentration of 5M urea (Albillos et al., 2009), and this assay shows that at concentrations of both 1M and 6 M urea the immunoreactivity of almond protein decreases significantly. Thus the possibility exists that the epitopes that are being recognized by rabbit polyclonal antibodies are destroyed due to the denaturation of amandin or are of the conformational type of epitopes.

Figure 4.17. Effect of different denaturants on the immunoreactivity of glycoproteins. Probed with antibodies raised against whole almond extract in rabbit and used at a dilution of 1: 10 000
in TBS-T. OE=almond extract. A, B and C=glycoproteins A, B and C respectively. Protein dotted on the membrane was 0.5 μg.

Compared to the untreated control, all denaturants used in the present study had a significant effect on the immunoreactivity of both glycoproteins A and B. With regard to glycoprotein C, with the exception of 0.25 M β-ME, treatment with all other denaturants resulted in a significant reduction in immunoreactivity. Nevertheless, comparatively immunoreactivity of glycoprotein C is much lower than either glycoprotein A or B (Fig 4.18).

**Figure 4.18.** ELISA of glycoproteins treated with different denaturants. Probed with antibodies raised against whole almond extract in rabbit and used at a dilution of 1: 10 000 in TBS-T. Almond OE=almond extract. GP A, GP B and GP C=glycoproteins A, B and C respectively. Error bars represents mean (n=3) ± SD.
CHAPTER 5

CONCLUSIONS

Glycoproteins comprise a very small fraction (< 2%) of the total soluble proteins of almonds. These glycoproteins were fractionated and partially purified from the non glycoprotein fraction using affinity chromatography and were further resolved into 3 peaks when passed through a gel filtration column. The 3 glycoprotein peaks (glycoproteins A, B and C) were partially characterized in the present study.

Biochemical characterization of selected glycoproteins

SDS-PAGE analysis under reducing conditions showed that glycoprotein A was composed of 3 major bands were visible at ~13 kDa, ~22 kDa and ~44 kDa. Glycoprotein B had 3 major bands at ~12 kDa, ~34 kDa and ~62 kDa while glycoprotein C was composed of a single major polypeptide at ~62 kDa.

N-terminal sequencing of the ~62 kDa bands of glycoproteins B and C revealed them to be the enzymes prunasin hydrolase and hydroxynitrile lyase respectively. 2D gel electrophoresis indicated the possibility of the presence of several isoforms of the enzyme hydroxynitrile lyase which is in accordance with previously published data.

In the present study, only partial purification of the glycoproteins was achieved. Further purification and removal of contaminating minor bands were attempted for glycoprotein C although this was not successful. Future work on further purification is feasible, and this would allow for a more complete identification and characterization of almond glycoproteins including the identity of the types of sugars and the number of sugar residues present in almond glycoproteins.

Immunoreactivity of selected glycoproteins

All three glycoproteins were immunoreactive with polyclonal antibodies raised against whole almonds in rabbit, while none of the three glycoproteins were recognized by the monoclonal antibodies 4C10 and 4F10 raised against AMP of almonds.
Glycoprotein B was recognized by 53% of almond allergic sera while 27% of sera reacted with glycoprotein A. However, results indicate that glycoprotein C is not a major allergen. Further studies using a larger number of almond allergic human sera need to be conducted to confirm these glycoproteins as being major allergens.

Deglycosylation of glycoproteins using HF in pyridine resulted in a significant loss of immunoreactivity in all three glycoproteins. However, it remains to be resolved whether the reduction in immunoreactivity is due to the carbohydrate moieties having an influence on the immunoreactivity or whether it was due to the degradation of the antibody binding sites of the epitopes by HF.

The effect of different denaturants on the immunoreactivity of glycoproteins were determined and it was found that compared to the untreated control, all denaturants used in the present study had a significant effect on the reduction of the immunoreactivity of both glycoproteins A and B. With the exception of 0.25 M β–ME, the other denaturants had a significant effect on the immunoreactivity of glycoprotein C.
APPENDIX A

ANIMAL CARE AND USE COMMITTEE APPROVAL MEMORANDUM

Animal Care and Use Committee (ACUC)
101 Biomedical Research Facility
P.O. Box 306431
Tallahassee, FL 32306-4341
Telephone: 644-4262 Fax: 644-5570
Mail Code: 4341 Email: acucoordinator@mail.fsu.edu

MEMORANDUM

TO: Dr. Shridhar Sathe  
Department of Nutrition Food and Exercise Sciences  
Dr. Kenneth Roux  
Department of Biological Science

FROM: Dr. Paul Q. Trombley, Chair  
Animal Care and Use Committee

SUBJECT: Approval of Protocol #1110

DATE: April 11, 2011

"YOUR NEW PROTOCOL IS APPROVED"

The Animal Care and Use Committee approved new Protocol #1110, "Immunological characterization of allergenic seed proteins", for proposed vertebrate animal use at the March 30, 2011 ACUC meeting. You are approved for the following species and numbers for the proposed protocol approval period.

<table>
<thead>
<tr>
<th>Species</th>
<th>Number Animals Approved</th>
<th>Protocol Approval Expiration Date</th>
<th>Rewrite Due</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit (Oryctolagus cuniculus)</td>
<td>78</td>
<td>March 30, 2011</td>
<td>February 1, 2014</td>
</tr>
<tr>
<td>Mice (Mus musculus)</td>
<td>117</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Enclosed for your records are:

✓ A copy of the Committee Comments
✓ A copy of the Protocol and supporting documents

When you order animals on this protocol, please remember to convey the ACUC number to the LAR at 644-4262. In addition, if you do not currently have animal housing or procedural space assigned or should you need additional animal housing or procedural space, please make a request for space in writing to the Biomedical Advisory Committee (BAC) care of Kristin Aufer at kauther@fsu.edu. Animals will not be ordered unless adequate animal housing/procedural space is confirmed by the LAR Facility Manager.

We appreciate your contribution to assuring that animal research at Florida State University complies with federal guidelines and regulations. Let us know if we can be of further assistance.

PQT9jj
Enclosures
APPENDIX B

HUMAN SUBJECTS COMMITTEE APPROVAL MEMORANDUM

Office of the Vice President For Research
Human Subjects Committee
Tallahassee, Florida 32306-2742
(850) 644-8673, FAX (850) 644-4392

RE-APPROVAL MEMORANDUM

Date: 4/20/2010

To: Shridhar Sathe

Address: 1493
Dept.: NUTRITION FOOD AND MOVEMENT SCIENCES

From: Thomas L. Jacobson, Chair

Re: Re-approval of Use of Human subjects in Research
Identification and characterization of tree nut allergens

Your request to continue the research project listed above involving human subjects has been approved by the Human Subjects Committee. If your project has not been completed by 4/13/2011, you are must request renewed approval by the Committee.

If you submitted a proposed consent form with your renewal request, the approved stamped consent form is attached to this re-approval notice. Only the stamped version of the consent form may be used in recruiting of research subjects. You are reminded that any change in protocol for this project must be reviewed and approved by the Committee prior to implementation of the proposed change in the protocol. A protocol change/amendment form is required to be submitted for approval by the Committee. In addition, federal regulations require that the Principal Investigator promptly report in writing, any unanticipated problems or adverse events involving risks to research subjects or others.

By copy of this memorandum, the Chair of your department and/or your major professor are reminded of their responsibility for being informed concerning research projects involving human subjects in their department. They are advised to review the protocols as often as necessary to insure that the project is being conducted in compliance with our institution and with DHHS regulations.

Cc: []
HSC No. 2010.4122


Almond Almanac, 2010. Almond Board of California, Modesto, CA.


Britannica, online encyclopedia. 2010. 


BIOGRAPHICAL SKETCH

EDUCATION

Jun 2011  MS in Nutrition and Food Science
          The Florida State University, Tallahassee, FL

Jun 2006  MS in Biochemistry, Molecular Biology and Gene Technology
          University of Colombo, Sri Lanka

Nov 2002  BS in Biological Sciences
          The Open University of Sri Lanka

PROFESSIONAL EXPERIENCE

Aug 2009-Apr 2011  Graduate Research Assistant
                    Florida State University, Tallahassee, FL

AWARDS AND HONORS

• Betty Watts Memorial Scholarship, Department of Nutrition, Food & Exercise Sciences, FSU, 2010 and 2011.
• D. B. Ellepola Gold Medal for best performance, BS degree, The Open University of Sri Lanka, 2002
• Faculty of Natural Sciences Prize for best performance in Botany, BS degree, The Open University of Sri Lanka, 2002.
• Faculty of Natural Sciences Prize for best performance in Zoology, BS degree, The Open University of Sri Lanka, 2002.

ABSTRACTS/PRESENTATIONS