CHARACTERIZATION AND FUNCTIONALITY OF CAROB GERM PROTEINS

by

BRENNAN M. SMITH

B.S., University of Idaho, 2006

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Food Science Program
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2009

Approved by:

Co-Major Professor
Fadi Aramouni

Approved by:

Co-Major Professor
Scott Bean

Copyright

Abstract

The biochemical, physical and baking properties of caroubin, the main protein in the carob bean, were characterized. The biochemical properties of caroubin were analyzed using reversed-phase high performance liquid chromatography (RP-HPLC), size exclusion chromatography coupled with multi-angle laser light scattering (SEC-MALS) and micro-fluidics analysis. The physical and baking properties of caroubin were characterized via SE-HPLC, laser scanning confocal microscopy, farinograph mixing, and texture profile analyzer analysis. Using a modified Osborne fractionation method, carob germ flour proteins were found to contain ~32% albumin and globulin and ~68% glutelin with no prolamins detected. When divided into soluble and insoluble protein fractions under non reducing conditions it was found that caroubin contained (~95%) soluble proteins and only (~5%) insoluble proteins. As in wheat, SEC-MALS analysis showed that the insoluble proteins had a greater M_w than the soluble proteins and ranged up to $8x10^7$ Da. These polymeric proteins appeared to play a critical role in protein network formation. Analysis of the physical properties of carob germ protein-maize starch dough showed that the dough's functionality was dependent on disulfide bonded protein networks, similar to what is found in wheat gluten. When baked into a bread these proteins were shown to have a possible improving affect by decreasing staling in gluten-free breads. This was evident when compared to a gluten-free batter bread, and a wheat bread over a five day period.

TABLE OF CONTENTS

LIST OF FIGURES

Chapter 1

Chapter 2

Chapter 3

LIST OF TABLES

Chapter 1

Chapter 3

ACKNOWLEDGEMENTS

I would like to thank first my graduate committee, Dr. Scott Bean, Dr. Fadi Aramouni, and Dr. Tom Herald. Without their aid and support I could not have completed this research. I would also like to thank Dr. Tilman Schober. Although not a member of my committee, Dr. Schober has provided a great deal of knowledge and inspiration to my research and education. Finally, I would like to thank the United States Department of Agriculture Grain Marketing Production Research Center for the financial help throughout my degree and providing a great work environment with outstanding co-workers.

Chapter 1:
Literature Review

Celiac Disease

Introduction

Celiac disease is an autoimmune disorder affecting the upper regions of the small intestine (Godkin and Jewel 1998; Fasano and Catassi 2008). It was first described by Samuel Gee in 1888 (Marsh 1992; Bruzzone and Asp 1999). It is genetically controlled and commonly found amongst close relatives (Leeds et al 2008). The typical symptoms of this disease are diarrhea, bloating, fatigue, and various forms of malnutrition including vitamin and mineral deficiencies (Godkin and Jewel 1998; Green and Cellier 2007; Fasano and Catassi 2008; Weiser and Koehler 2008). Proteins from wheat, rye, and barley instigate this disease by causing the inflammation and subsequent loss of the villi of the intestinal mucosal layer. This is caused by the immune system attacking the cells of the villi in response to gluten. The diagnosis of celiac disease can be difficult with the symptoms similar to other bowel disorders. When diagnosis is achieved there is only one known treatment to stop the symptoms. This is a diet completely devoid of all wheat, rye, and barley (Fasano and Catassi 2001; Weiser and Koehler 2008).

Genetics of Celiac Disease

A majority of the people (95%) diagnosed with celiac disease are carriers of the genes that code for the human leukocyte antigen known as HLA-DQ2 or HLA-DQ8. However, ~5% the celiac population does not have this gene and ~30% of the world's population carries the gene (Karell et al 2003; Van Heel and West 2006). This is because the genetic predisposition to this disease is considered polyfactorial, meaning that several genes and possibly non-genetic factors, such as retroviruses, work together to cause gluten intolerance. It is unknown which combination of genes causes celiac disease. The frequency of the HLA genotype varies greatly

amongst different populations (Wieser and Koehler 2008). HLA-DQ2 genes are prevalent in high levels in Europe, Africa, India, and South and Central America. In South and Central America up to ~90% of some populations carries HLA-DQ2 and in the area around the Pacific Rim, this gene is almost completely absent even among celiac patients (Layrisse et al 2001).

The Role of Wheat Gluten

Wheat proteins have been traditionally split into four fractions based on their solubilities. These fractions are albumins, globulins, gliadins, and glutenins (Osborne 1903). Celiac disease's symptoms are largely instigated by the alcohol soluble proteins or gliadins (Lammers et al 2008). Gliadin along with glutenin, the other functional protein found in gluten, make up wheat's storage proteins. The storage proteins are found throughout the caryopsis's endosperm and provide a nitrogen source for the developing wheat embryo (Hoseney 1998). Gluten's functionality arises due to gliadin's ability to provide extensibility and glutenin's ability to provide elasticity. This somewhat unique trait is utilized in several food systems including gas retention in breads, elasticity of noodles, and can be attributed to soft crumb structures and prolonged freshness of wheat based foods (Cornish et al 2006).

Mechanism of Action

It was once thought that the gliadin fraction of gluten was the major cause of intestinal inflammation because it is resistant to degradation by peptidases and proteases of the stomach due to their high levels of proline. This allows gliadin to pass on to the duodenum and jejunum regions of the small intestine (Green and Cellier 2007; Wieser and Koehler 2008). In these intestinal regions, gliadin has the ability to interact with mucosal cells causing the disruption of the tight junctions between cells (Lammers et al 2008). The disruption allows for large peptides

greater than the typical limit to pass. When this occurs there is a rapid release of cytokine interleukin-15. This causes a large increase of intraepithelial lymphocytes. Tissue transglutaminase will then bind gliadin peptides to antigen HLA-DQ2 or HLA-DQ8. This stimulates T-cells and the release of proinflammatory cytokines. Once T-cells are stimulated, inflammation and a loss of epithelial cells occur. As a result, there is a loss of intestinal nutrient absorption. Because of the immune system activation, IgA and IgG antibodies against glutens are released (Wieser and Koehler 2008). Research has also shown similar responses from the immune system triggered by glutenin. This means that glutenins are also a major contributor to celiac disease (Godkin and Jewell 1998).

Symptoms

Symptoms of celiac disease arise from the damaged intestinal mucosal layer. These symptoms are related to the inflamed and damaged epithelial villi or a secondary mechanism, of which are not well understood. This inflammation leads to the inability to absorb nutrients and causes diarrhea, bloating, and anemia (Wieser and Koehler 2008). Not only do these conditions have devastating effects on celiac patient's quality of life, they are also attributed to many other auto immune disorders (Fasano and Catassi 2008) (Table 1).

Table 1: Symptoms and manifestations of celiac disease. Modified from Fasano and Catassi (2008).

Manifestations secondary to untreated celiac disease	
Celiac disease with classic symptoms	**Celiac disease with non-classic symptoms**
Abdominal distension	Arthritis
Anorexia, irritability	Aphthous stomatis
Chronic or recurrent diarrhea	Constipation
Failure to thrive	Dental enamel defects
Vomiting	Dermatitis herpetiformes
Muscle wasting	Hepatitis
Fatigue	Iron-deficient anemia
	Pubertal delay
	Recurrent abdominal pain
	Short stature
Associated diseases (or secondary to untreated celiac disease?)	
Autoimmune Diseases	**Neurological and psychological disturbances**
Type I diabetes	Ataxia
Thyroiditis	Autism
Sjogren's syndrome	Depression
Others	Epilepsy with intracranial calcifications

Diagnosis and Treatment

With the plethora of symptoms, diagnosis of celiac disease can very difficult and is often falsely diagnosed as another common bowel disorder, such as irritable bowel syndrome. There are several methods used in identifying celiac disease. The types and order of the tests are often determined by visible symptoms (Hopper et al 2007). These tests include, but are not limited to, antibody testing, endoscopy, and genetic testing for the HLA-DQ2 genes (Godkin and Jewell 1998; Korponay-Szabo et al 2003; Wieser and Koehler 2008).

Antibody testing relies on a serological blood test. This screening tests for the presence of tissue transglutaminase titers. Testing for celiac related antibodies has been shown to be greater than 90% effective in identifying celiac disease when followed by a mucosal biopsy and may one day completely replace endoscopy and biopsy testing (Sblattero et al 2000).

Endoscopy is somewhat more invasive than the other screening procedures. In this process an endoscope is passed through the mouth, esophagus, and stomach to the duodenum and jejunum of the small intestine where multiple tissue samples are taken from the mucosal layer. These tissue samples are then observed via microscopy to determine the level of damage to the intestinal villi (Sblattero et al 2000; Wieser and Koehler 2008).

Often times an official diagnosis never occurs but instead people put themselves on a gluten free diet to see if symptoms subside. If this is the case, a true diagnosis becomes more difficult because intestinal villi are repaired within weeks if a gluten-free diet is consumed. Since a biopsy of the intestinal mucosal layer is considered the "golden standard," a diagnosis may never occur unless gluten is replaced in the diet for an extended period of time and a biopsy is done (Gjertsen et al 1994; Godkin and Jewell 1998; Leeds et al 2008; Wieser and Koehler 2008).

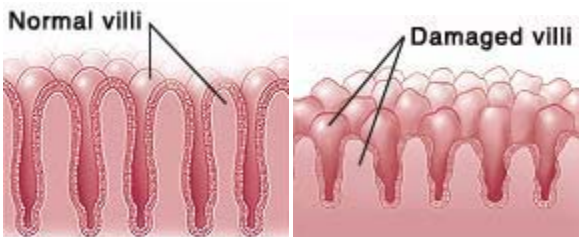

Figure 1: From left to right: Normal intestinal villi and damaged intestinal villi. Taken from Anonymous (2006b)

The only known treatment to combat the symptoms of celiac disease is a diet devoid of wheat, rye, and barley and possibly oats. This is a large commitment that will last a lifetime and within a few weeks the intestines will begin to repair the damage to the mucosal villi and nutrient absorption should be restored to normal. Until this occurs, dietary supplements are

often recommended for people in the recovery period. In severe cases of celiac disease the intestines have been so damaged that a full recovery is not possible (Figure 1). When this happens a lifelong dietary supplementation is required and in very severe cases nutrients must be supplied intravenously (Leeds et al 2008; Wieser and Koehler 2008).

Many celiac patients report a reoccurrence of problems with the disease because of the prevalence of wheat in food products. This makes it almost impossible to exclude gluten completely out of the diet. Gluten can be found in almost all types of foods. It is used commercially as a binder, thickener, and protein substitute. Examples of these products are sausages, soups, ice cream, and soy sauce (Bogue and Sorenson 2008). It was once believed that a threshold level of 200ppm of gluten could be consumed by the average celiac patient daily. Recent research has determined that 50mg of gluten a day over a three month period can significantly reduce the number of mucosal villi in the intestines (Troncone et al 2008). Another study demonstrated that less than 10mg of gluten intake a day is unlikely to cause problems with inflammation (Akobeng and Thomas 2008). The United States along with many other countries have a maximum allowance of 20ppm of gluten in products labeled gluten-free. This may be well over the harmful level for some celiac patients (Wieser and Koehler 2008).

Gluten-Free Market

Introduction

Since the discovery of celiac disease the number of people diagnosed with the disorder is increasing. It is estimated that about 1% of the world's population is actually affected by the disease, but only 1:266 have been diagnosed (Fasano and Catassi 2001; Van Heel and West 2005). With increased knowledge and education of celiac disease coupled with advances in screening procedures, the number of people subsiding on gluten-free diets will continue to increase. For this reason it is important to gain an understanding of the market trends surrounding gluten-free foods.

Market Trends

The gluten-free market is a rapidly growing industry. It was once considered a very small niche market, but in 1996 reports indicated that this market accounted for ~$700 million in sales annually in the United States. It was estimated that the market would grow at a rate of 25% per year to reach annual sales of ~$1.7 billion by 2010 (Gourmet Retailer 2006; Bogue and Sorenson 2008). With the lack of quality and the growing profit potentials in the gluten free market there have been many advances in research and development in order to achieve products that more resemble wheat goods (Schober et al 2007; Bogue and Sorenso 2008). These advances are pushed by consumer demands in the areas of convenience foods, foods with perceived health benefits, low fat foods, organic foods, extending brands, product improvements, new categories, and premium quality foods. These products include but are not limited to: pizza, drinks, dressings, beer, frozen foods, baking mixes, flours, and confectionary products (Bogue and Sorenson 2008). One of the key elements of new product development is

making more flexible meals that can be adapted to different lifestyles, such as meals on the go and microwavable dinners. As these products become more flexible and celiac awareness increases, gluten-free products are becoming more main stream (Wennstrom and Mellentin 2003; Bogue and Sorenson 2008). On a local level, many restaurants now offer gluten-free foods and on a global level, companies have begun to recognize the potentials of the gluten-free market (Wennstrom and Mellentin 2003; Anonymous 2006a; Bogue and Sorenson 2008). Anheuser-Bush, an American based brewing company that distributes internationally has developed a product called Redbridge. Redbridge is a gluten-free beer made with sorghum (AnonymousUSA 2007; Bogue and Sorenson 2008).

Problems in the Gluten-Free Market

One of the major problems seen with these new products is the lack of knowledge by the consumer. Often times, gluten-free foods have many ingredients critical to the product functionality. The unfamiliarity of ingredients that improve sensory quality, such as gums and preservatives, leads to a fearful consumer. To overcome this, companies must convince the consumers of product safety. To achieve this, five strategies have been developed. They are: leveraging hidden nutritional benefits, new category creation, new segment creation, category substitution, and food product make over. This allows for a more educated consumer that has a better understanding of functional ingredients (Wennstrom and Mellentin 2000; Wennstrom and Mellentin 2003; Bogue and Sorenson 2008).

Historically, gluten-free baked goods have relied on cake like batters to achieve the final end product. Batters commonly use gums like, hydoxypropyl methylcellulose (HPMC) and guar gum to increase viscosity and hold carbon dioxide in leavened products. Gluten-free products

tend to be starch based and have problems with staling and water loss over short periods of time (Schober et al 2007).

To overcome staling problems, resent research fed by consumer demands has been pushed in the direction of developing breads that are closer to wheat. One method being used to achieve this is supplementation of bread with proteins. It is hypothesized that protein networks within baked goods interact with starch and aid in the prevention of rapid staling and moisture loss. The lack of protein networks in gluten-free breads forces the system to be batter based. This can be problematic for processing and does not allow for shaping or hand forming, but relies solely on the shape of the pan in which it is baked (Schober et al 2008). Recent advances have attempted to overcome these limitations by producing bread dependent on HPMC and a protein network of maize prolamin (zein). While this system does allow for molding its major limitation is that all ingredients must be kept above zein's glass transition of ~ 40 °C until baking (Schober et al 2008). No known quality or staling work has been completed on these zein based breads.

The driving force behind the evolution of gluten-free markets and scientific research is the push for products that are more similar to wheat based products. As a result the gluten free market has become a billion dollar industry that is constantly striving for better quality products to help improve the lives of celiac patients.

Gluten-Free Bread

Introduction

Gluten-free breads may contain a number of ingredients. Many of these ingredients fit into major functional classes. These are cereals and cereal like flours that do not contain gluten, non-wheat starches, salt, sugar, yeast, chemical leavening agents, hydrocolloids, soy, egg, and enzymes to name a few. While gluten-free breads do not contain all of these ingredients within one formulation, they usually are starch or flour based. When flour is used, starch is commonly used in addition. The starch, flour, or mixture of the two, typically contains a hydrocolloid and can contain some sort of protein or combination of protein supplementation such as egg and soy (Arendt et al 2008). The addition of enzymes has been used with some success in increasing bread quality via protein cross linking. However, the use of some of these enzymes has been found to be somewhat controversial with an emphasis on transglutaminase (Goesaert et al 2005; Leeds et al 2008; Wieser and Koehler 2008).

Bread that is not dependant on a gluten network is a very fragile system. It is prone to falling and poor crumb structure (Schober et al 2008). Milling techniques and flour handling have been shown to have an effect on bread quality. This is due to changes in particle size, flour components, and starch damage (Hoseney 1998; Arendt et al 2008). Taking this into account, when whole flours are used, the non-starchy components can have a negative effect on bread quality (Schober et al 2007). This has been attributed to bran and coarse pieces of flour disrupting the ability of a hydrocolloid to efficiently retain gas. The negative aspects of flours are often overcome by addition of starches. This is due to the small uniform particle size of

starch coupled with its uniform functional properties (Hoseney 1998; Schober et al 2007; Arendt et al 2008).

Flours

There are several flours considered safe for consumption by celiac patients. These flours are derived from both cereal and non-cereal sources. There is a large variation in content and functional properties between types, such as: protein, ash, moisture, nitrogen free extract, lipids, starch gelatinization temperatures, and functionality of proteins. Each bread system has been optimized for use with specific flours. Rice flour and sorghum four are commonly used and several others can be observed in table 2.

Table 2: Common Gluten-free flours.

Gluten-free flours	
Rice flour	Maize flour
Sorghum flour	Tapioca flour
Arrow root flour	Millet
Potato flour	Buckwheat flour
Soy flour	Amaranth flour

Starch

Starch, a major component of gluten-free breads, can be isolated from almost any cereal, tuber, or plant material high in starch with the exception to wheat, rye, and barley (Arendt et al 2008; Wieser and Koehler 2008). Each plant has its own unique starch granule that varies in size, shape, and chemical and physical properties. A granule of starch is made of two components, amylose and amylopectin, that are present in varying amounts depending on the starch source (Hoseney 1998). Some of the more common starches isolated for use in gluten free foods are from corn, potato, rice, and cassava.

Starch functions in baking systems in many ways. It has the ability to absorb large amounts of water. When heated in combination with water, starch will gelatinize. Gelatinization within a bread system causes the starch granules to become partially soluble and swell, while maintaining a granular appearance. Once this occurs, amylose and amylopectin are able to easily form hydrogen bonds. This coupled with the leaching out or solublizing of amylose allows for a continuous network that envelops and sticks the gelatinized starch granules together (Hoseney 1998; Arendt et al 2008). This is important in bread making because it has a direct effect on loaf volume and structural integrity of the crumb. The retrogradation (crystallization) of gelatinized starch contributes to staling in parallel with water migration by causing firmer crumb structure via an increase in order between polymers of amylose and amylopectin. It is also attributed to leathery crust, less elasticity of the crumb, and flavor loss (Arendt et al 2008).

Hydrocolloids

Hydrocolloids are used in gluten-free baking to improve texture, viscoelastic properties, slow starch retrogradation, act as water binders, aid in gas retention, and to substitute gluten in breads (Arendt et al 2008). There are many types of hydrocolloids all of which have differing functional properties and can contribute differently depending on the system. Hydrocolloids come from hydrophilic polymers of vegetable, animal, microbial, or synthetic material (Hoefler 2004; Arendt et al 2008). Hydroxypropyl methyl cellulose (HPMC) and carboxy methyl cellulose (CMC) are commonly used hydrocolloids in gluten-free bread production. This is because both gums have tested high in their overall acceptance when used in gluten-free breads and are

responsible for higher levels of crumb elasticity when compared to other gluten-free breads (Arendt et al 2008).

It has been suggested that hydrocolloids have two major effects on starch structure in bread. Hydrocolloids may coat starch granules causing a decrease in starch swelling and leaching of amylose to cause an overall increase in crumb firmness which is not desirable. The other effect is the reduction of retrogradation or crystallizing of amylose and amylopectin that may aid in softening the crumb structure (Arendt et al 2008).

Proteins

Protein supplementation in gluten-free breads can be utilized in different ways. One of these is protein network formation. Protein network formation not only has the ability to increase gas retention, it can also change the means in which gluten-free breads are produced. A protein network has the potential to replace the old batter based baking systems with more moldable dough that is not reliant on pans to hold its shape prior to baking. These breads have been made under experimental conditions with zein proteins from maize heated above glass transition (Schober et al 2007). Another method of protein network formation is by cross-linking different types of proteins with transglutaminase to give them viscoelastic properties. However, this method is highly debated because of transglutaminase's ability to amplify the effects of gluten when consumed by a celiac. The exact mechanism causing functionality of these systems is unknown.

Water

The final ingredient that is necessary for all breads is water. Water allows for hydration of flour components and hydrocolloids. Without water these ingredients would remain as a dry

powder. Water is also considered a universal solvent. When placed in water, bread ingredients like salt and sugar readily dissolve to form solutions of ions. The ions of salt and sugar not only change the flavor of products, they can also affect hydration properties of other ingredients, texture, water activity, and yeast activity.

Leavening Agents

Yeast and chemical based leavening agents are critical for achieving leavened baked goods. Their ability to produce carbon dioxide coupled with a system able to prevent the escape of the gas produces a foam with flour and when baked a leavened product is achieved.

The Carob Tree

Introduction

Carob, *Ceratonia siliqua*, is a leguminous shrub native to the Mediterranean region (Batlle and Tous 1997; Wang et al 2001; Dakia et al 2007; Bengoecha et al 2008). It is cultivated throughout the world in most temperate zones that allow for temperatures between ~4 °C and 40 and °C with average rain fall of at least 25 cm (Batlle and Tous 1997). The seeds and pods have been traditionally used as a food thickener and sweetener (Batlle and Touse 1997; Wang et al 2001). In recent times carob's primary use is in the production of carob bean gum and other food and industrial additives (Batlle and Tous 1997; Wang et al 2001; Dakia et al 2007). Carob germ flour is created as a byproduct of carob gum production (Bengoechea et al 2008). The germ flour is primarily used as a protein supplement in animal and pet foods and for dietetic supplements for humans (Batlle and Tous 1997; Dakia et al 2007). However, these proteins have been identified as having viscoelastic properties similar to wheat gluten and have the potential to be used in baked goods to improve final end products and functionality of dough (Bienenstock et al 1935; Feillet and Roulland 1998; Wang et al 2001; Dakia et al 2007; Bengoechea et al 2008). It is of importance that an understanding of the carob tree be obtained in order to exploit it as a valued crop and further its use as a functional food ingredient.

Origins and History

Although the exact origins of the carob tree are unknown, the genesis of the wild carob tree took place somewhere in the Mediterranean, Arabian Peninsula, or the horn of Africa (Batlle and Tous 1997). The questionable origin is due to the widespread cultivation of carob for

food, feed, and animal bedding in pre-historical times. Through observation of wild varieties and archeological records, the first cultivations of carob probably took place in the areas of Turkey, Cyprus, Syria, Lebanon, Israel, Jordan, Egypt, Arabia, Tunisia, and Libya (Hillcoat et al 1980; Batlle and Tous 1997). It is generally accepted that the Greeks are responsible for cultivating the crop in Greece and Italy from seeds taken from the Mediterranean. From here the crop eventually arrived to regions of southern France and Portugal where climates permitted (Batlle and Tous 1997). In more recent times carob was introduced into the United States by the US patent office in 1854 where it was primarily grown in California for ornamental purposes (Schroeder 1952; Batlle and Tous 1997).

Throughout history, carob fruit was easily stored and transported with little problems from pest predation and spoilage. This can be attributed to the high tannin content and low water activity caused by high sugar content and low moisture levels (Batlle and Tous 1997). The high sugar content and rich flavor of the pods makes this crop valuable for use in food, sugar, beverages, and fermented products (Batlle and Tous 1997; Wang et al 2001; Dakia et al 2007). The sugars have been historically collected by crushing the pods and solublizing the sugars to wash them free of the pod. The tannins of the pods were extracted with the sugar to give a dark rich flavored molasses that is still consumed as a dessert topping and food sweetener. During this process the seeds are removed and after extraction the pods are sun dried the use as animal bedding (Batlle and Tous 1997; Wang et al 2001).

Production

Today carob is grown throughout the world where climate permits. The global carob crop production was estimated to be 310,000 tons in 1997 and declining (Batlle and Tous 1997).

The largest production areas are from the southeastern portions of Europe and the Mediterranean where Spain, Italy, Portugal, and Greece account for 70% of the total production. Only 7.5 % of the total world production took place in other areas such as the North and South America, Africa, and Australia. The drops in productions can be accounted for by cultural advances in rural communities where the carob pod has been commonly consumed (Batlle and Tous 1997). Without an efficient way to harvest the pods, which accounts for a majority of the carob farming expenses, carob harvest may be becoming too costly as the price of labor increases. For whatever reason, the total production of carob dropped by 340,000 tons over a 52 year period spanning from 1945 to 1997 (Batlle and Tous 1997).

Ceratonia siliqua grows in regions between 30° and 40° longitude in the southern hemisphere and between 30° and 40° longitude in the northern hemisphere (Batlle and Tous 1997). It can withstand temperatures of 40 °C for long periods of time with little rain fall. However, it is not able to withstand temperatures below -7 °C and receives significant damage at temperatures of -4 °C with different varieties being able to withstand different temperature extremes (Batlle and Tous 1997). The soils best suited for growth are very high in calcium, basic and can be up to 3% salt (Winer 1980). The moisture requirements to bear fruit are between 25 cm and 50 cm annually with 50 cm to 55 cm of rainfall needed to produce a commercial crop (Batlle and Tous 1997). Irrigation shows significant increases in crop yield, but in many regions carob is grown on terrain that is not suitable for other crops and is usually not practical or easily irrigated (Batlle and Tous 1997).

Description

The carob tree is a legume from the family Leguminose and the order Rosales. Unlike other legumes, *Ceratonia siliqua* does not nodulate and therefore does not fix nitrogen on the roots. However, it does have a symbiotic relationship with the fungus, *Arbuscular mycorrhizal*, that allows for the increased uptake of nitrogen from the soil by root colonization. The exact mechanism for this is unknown, but it is believed that this fungus aids in the growth of trees where soil nitrogen deficiencies are present (Batlle and Tous 1997).

Carob trees are a long lived evergreen that grow to a height of about 6m to 10m (Batlle and Tous 1997). The tree has a large semispherical crown with leaves that alternate down the branches in a pinnate fashion. Leaves are 10 cm to 20 cm in length, dark green on the dorsal side and a pale green on the ventral side. The leaves have a thick waxy coating that prevents excessive moisture loss in semi arid climates. In July of alternating years the leaves are shed and replaced with new (Batlle and Tous 1997). The flowers of the carob are small and numerous, arranged in a twisting manner down the inflorescence in numbers of 15 to 20. They are only found on old wood and inflorescences are between 6 and 12 cm in length. Only a few inflorescences bear fruit and there is rarely more than two fruit per inflorescence (Batlle and Tous 1997). The pod or fruit is observable in different conformations depending on the variety. The straighter pods are considered more desirable because of the ease of harvest. Each pod is about is about 10 cm to 30 cm long and 1.5 cm to 3.5 cm wide (Figure 2). Pods which make 90% of the fruit weight are filled with several seeds arranged in a linear non overlapping manner separated by the mesocarp. Seeds are compressed and slightly oblong with dimensions of 8 mm to 10 mm long by 7 mm to 8 mm wide by 3 mm to 5 mm thick. Each seed is covered by a

shiny brown and very hard testa which accounts for 30 to 33% of the seeds weight (Batlle and Tous 1997)(Figure 3).

Figure 2: A group of straight carob pods. Taken from Anonymous (2009a).

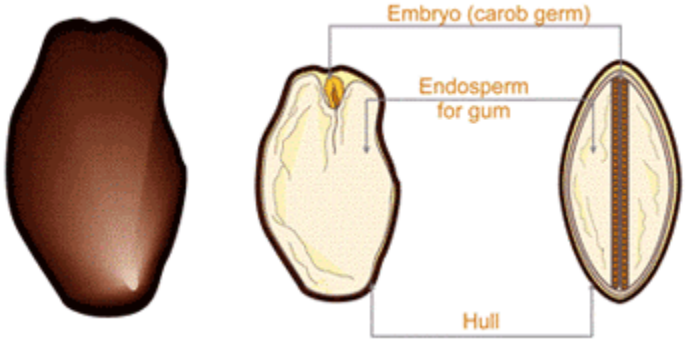

Figure 3: Carob seed with major anatomical features outlined. Taken from Anonymous (2009b).

Within the testa, the endosperm of the seed is comprised of carob bean gum (locust bean gum)(Figure 3). Carob bean gum is a galactomannan which consists of (1-4) linked β-D-monnopyranosyl (mannose) with single units of (1-6) linked α-D-galactopyranosyl (galactose).

These sugars are present in a ratio of 3.9:1 respectively with a galactose appearing on about every fourth unit of the mannose chain (Hoefler 2004). The gum is very similar to other gums such as guar gum and tara gum in its properties but with some key differences. Unlike gar gum, carob bean gum is insoluble at room temperature and only undergoes a slight swelling. It becomes fully soluble at 60 °C. This is due to the strong hydrogen bonding that occurs on the long mannose chain. It achieves greater hydrogen bonding than guar gum because it has greater distance between galactose units which allows the different subunits to be in closer proximity to each other. Energy in the form of heat allows for the disintegration of the hydrogen bonding between mannose chains and subsequent hydration (Hoefler 2004). Its molecular weight is between 400,000-1,000,000 and it can tolerate higher salt concentrations than most other anionic hydrocolloids while maintaining solubility (Hoefler 2004).

The embryo accounts for 23 to 25% of the seeds weight (Batlle and Tous 1997). It is composed primarily of protein and fiber with low to moderate amounts of water, lipid, ash, polyphenols, and soluble carbohydrates (Bengoechea et al 2008)(Table 3). The proteins form aggregates linked via non-covalent and disulfide bonding that have molecular weights between ~13 kDa and ~95 kDa with major bands appearing at 95.5, 55, 26.3, and 13.8 kDa (Dakia et al 2007; Bengoechea et al 2008). These proteins have a well balanced amino acid content with all 10 essential amino acids present (Dakia et al 2007).

Table 3: Chemical characterization of defatted carob germ flour. Modified from Bengoechea et al (2008).

Flour component	**% of Flour**
Protein content	48.2 ± 0.24
Lipids	2.26 ± 0.13
Moisture	5.76 ± 0.32
Ash	6.34 ± 0.15
Polyphenols	0.45 ± 0.01
Soluble carbohydrates	2.92 ± 0.03
Total fiber	24.3 ± 0.09

The term caroubin was coined in 1998 by Feillet and Roulland to describe the unique wheat like proteins in carob germ flour. In their study two separate caroubin fractions were observed via extraction and centrifugation. These fractions had nearly identical amino acid profiles and molecular weight distributions. The primary differences came in the form of differing physical traits such as compressibility, elastic recovery, and viscoelastic index as determined by texture profile analysis (Feillet and Rouland 1998). When evaluated by SDS-PAGE and SE-HPLC, the proteins of caroubin had an average molecular weight greater than that of gluten. SE-HPLC demonstrated wheat as having greater amounts of large polymeric proteins than caroubin (Feillet and Rouland 1998).

Osborne protein extractions of the carob germ flours found that carob germ protein was composed of 14.5% albumins, 50% globulins, 3.4% prolamin, and 32.1% glutelins + residue (Plaut et al 1953). Although proteins of the carob germ have similar properties to wheat, these numbers show that the proteins are quite different. Wheat gluten typically has 5% albumin, 10% globulin, 69% prolamin, and 16% glutelin + residue (Osborne 1903). It is generally expected that prolamins in wheat are the major contributors to vicoelastic properties and carob germ

flour had little to no prolamin (Plaut et al 1953; Hoseney 1998). FTIR, NMR, scanning electron microscopy and DSC established that caroubin and gluten do have similar rheological properties, but remain quite different in their functionality (Wang et al 2001).

Processing and Utilization

Upon arrival at a processing facility the carob pods are usually between 10% and 20% moisture. Because the pods need to be at 8% moisture for processing, the carob fruit is stored in environmentally controlled shelters until the pods meet the desired moisture content (Batlle and Tous 1997). The first step in processing is crushing or kibbling of the pods. This frees the seeds from the pods where they can be separated and go on to separate processing. The pods are milled for both food and feed. Animal feeds are produced by milling the kibble to different particles sizes depending on which type of feed is desired. Kibble milled for human consumption is first roasted and milled to a fine powder with the trade name of carob powder (Batlle and Tous 1997). Sugars are also extracted in the form of molasses as mentioned previously (Batlle and Touse 1997; Wang et al 2001). The seeds are usually shipped to a separated processing facility to extract the galactomannons of the endosperm (Batlle and Tous 1997).

The first step in carob gum extracting is removing the thick testa layer surrounding the endosperm and germ. This is a difficult process that can be completed in two different ways. In both methods the final goal is a more friable testa layer that is easily removed. The first of these methods is carbonizing the testa via steeping in sulfuric acid and the second is by dry roasting (Batlle and Tous 1997). Once the seed coat is removed it is milled into a fine powder where it is commonly sold to the leather industry where it is used as a tanning agent due to its

high tannin content (Batlle and Tous 1997). In order to separate the germ from the endosperm, the whole seed excluding the testa is milled so that the endosperm remains in large scale like pieces and the germ is turned into a fine powder (Batlle and Tous 1997)(Figure 4). This can be achieved due to the differences in friability of the two fractions. The germ is much more brittle and reduces in size easily when compared to the endosperm (Batlle and Tous 1997). After separation the germ is used for protein supplementation in both food and feed (Batlle and Tous 1997; Dakia et al 2007). The endosperm goes through another milling step to produce a fine powder that is sold under the trade name carob bean gum or locust bean gum (Batlle and Tous 1997; Hoefler 2004).

Figure 4: From left to right; Photographs of the scale like endosperm and germ flour of carob seeds

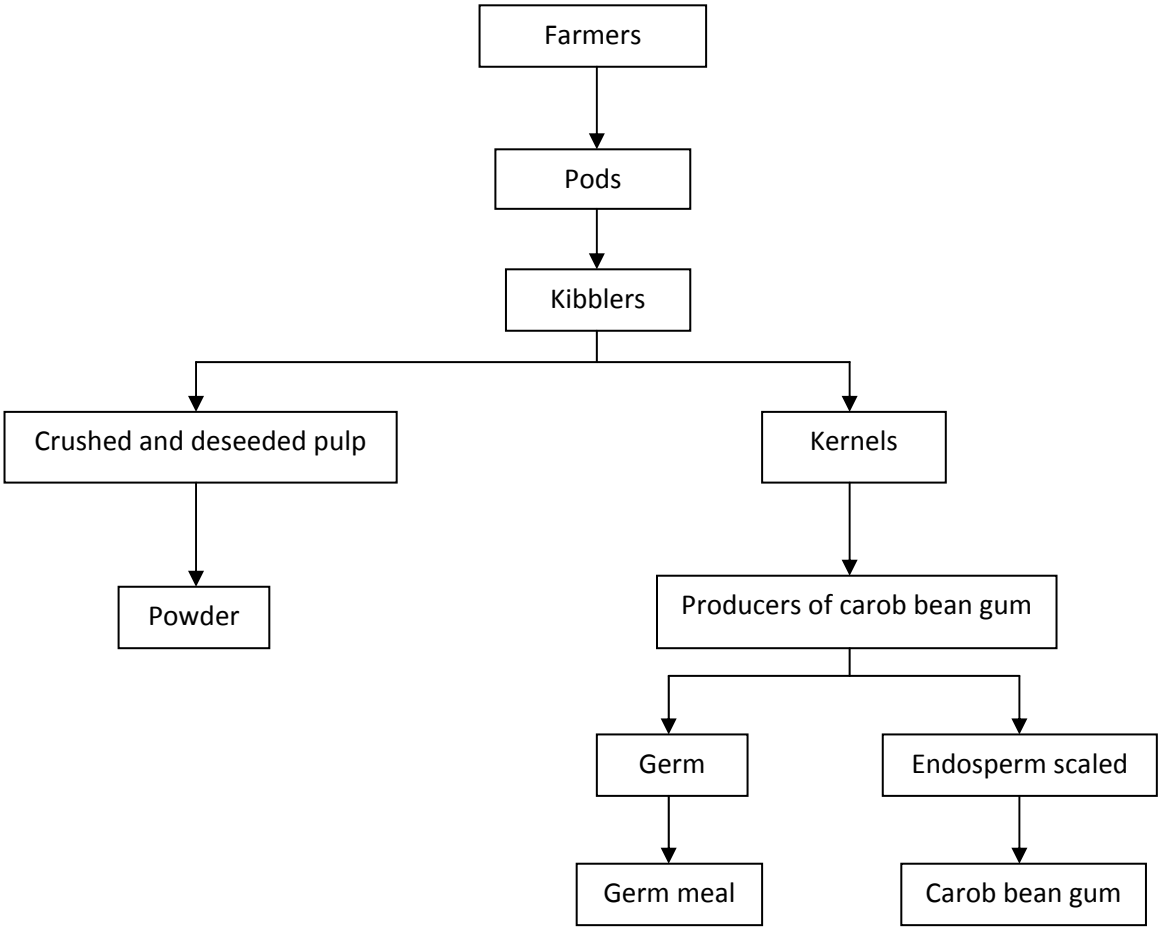

Figure 5: Flow diagram of carob processing. Modified from Batlle and Tous (1997).

Carob bean gum has many uses both industrial and as a food additive due to its textural and hydration properties mention previously. Locust bean gums industrial uses range from concrete strengthening to water binders in explosives and many more (Batlle and Tous 1997). In food systems carob bean gum is recognized as a food thickener, stabilizer and emulsifier. It is a food additive which can be used in the following food categories shown in table 4.

Table 4: Carob bean gum usage levels. Reproduced from Kawamura 2008.

Food Category	Maximum Use Level (%)
Baked goods & baking mixes	0.15
Non-alcoholic beverages & beverage bases	0.25
Cheeses	0.8
Gelatins, puddings, & fillings	0.75
Jams and jellies	0.75
All other foods	0.50

Carob germ flour has been traditionally used as a protein additive in animal feeds and foods for human consumption because of its well balanced amino acid content (Feillet and Rolland 1998; Wang et al 2001). As mentioned previously the carob germ flour was identified as having gluten like properties in a 1935 patent. When used in a yeast leavened bread system containing ~30% carob germ flour and ~70 gluten-free flour, a bread was produced with similar qualities to a European rye bread (Bienenstock et al 1935). Since this time little work has been done to characterize its functional properties when compared to wheat. Until the discovery of celiac disease there was very little data published on the functional properties of carob germ proteins when compared to that of wheat. Prior to celiac disease discovery, high protein wheat carob germ composite breads for diabetics was studied. These breads were of poorer quality than pure wheat breads, but were considered acceptable (Plaut et al 1953). It has been stated in many publications that carob germ protein shows significant potential in gluten-free foods due to its viscoelastic nature and its acceptance as being safe for celiac patients, but no literature could be found on bread products dependant on caroubin for functionality since the 1935 patent.

Literature Cited:

Akobeng, A. K., and Thomas, A. G. (2008). Systematic review: tolerable amount of gluten for people with coeliac disease. *Alimentary Pharmacology and Therapeutics,* 27 (11), 1044–52.

Anonymous. (2006a). New opportunities for gluten-free market. *Food Production Daily* www.foodproductiondaily.com. Accessed April, 4 2009.

Anonymous. (2006b). When your child has celiac disease. *Kramer Company.* Online posting. 10 May 2006. Revolution Health. Last accessed on - 30 Apr. 2008 www.revolutionhealth.com/conditions/digestive/celiacdisease/introduction/child-celiac-disease. Accessed April 4, 2009.

Anonymous. (2007). Anhueser-Busch launches sorghum beer. *Nutra Ingredients USA.* http://nutrain-gredients-usa.com/news.

Anonymous. (2009a). Algarve Property. www.algarveprop.com/customlinks_10_Latest-Features.html. Accessed April 4, 2009.

Anonymous. (2009b). Cargill Texturizing Solutions. www.cargilltexturizing.com/products/hydrocolloids/locust/cts_prod_hydro_loc.shtml. Accessed April 4, 2009.

Arendt, E. K., Morrissey, A., Moore, M. M., Dal Bello, F. (2008). Gluten-Free Breads. *Gluten-Free Cereal Products and Beverages.* Elsevier Inc. New York. 289-311.

Batlle, I., Tous, J. (1997). Carob Tree: *Ceratonia silique* L. Promoting the conservation and use if underutilized and neglected crops. 17. Institute if Plant Genetics and Crop Plant Research, Gatersleben/International Plant Genetic Resources Institute, Rome, Italy.

Bengoechea, C., Puppo, M.C., Romero, A., Cordobes, F., and Guerrero, A. (2008). Linear and non-lenear viscoelasticity of emulsions containing carob as emulsifier. *Journal of Food Engineerin,* 87, 124-135.

Bengoechea, C., Romero, A., Villanueva, A., Moreno, G., Alaiz, M., Millan, F., Guerro, A., and Puppo, M.C. (2008). Composition and structure of carob (*Ceratonia siliqua* L.) germ proteins. *Food chemistry*, 107, 675-683.

Bienenstock, M., Csaski, L., Pless, J., Sagi, A., and Sagi, E. (1935). Manufacture of Mill Products for alimentary purposes and of paste foods and bake products from such milled products. U.S. patent 2,025,705.

Bogue, J., and Sorenson, D. (2008). The marketing of gluten-free cereal products. *Gluten-Free Cereal Products and Beverages.* Elsevier Inc. New York. 393-412.

Bruzzone, C. M., and Asp, E. H. (1999). The cereal science and disease etiology of gluten-sensitive entropothy. *Cereal Foods World*, 44, 109-114.

Cornish, G. B., Bekes, F., Eagles, H. A., Payne, P. I. (2006). Gliadin and Glutenin, The unique Balance of Wheat Quality: Prediction of Dough Properties for Bread Wheats. *American Association of Cereal Chemist International*. 243-280.

Dakia, P. A., Wathelelet, B., and Paquot, M. (2007). Isolation and chemical evaluation of carob (*Ceratonia siliqua* L.) seed germ. *Food Cemistry*, 102, 1368-1374.

Fasano, A., Catassi, C., (2001). Current approaches to diagnosis and treatment of celiac disease: an evolving spectrum. *Gastroenterology* 120, 636-651.

Fasano, A., and Catassi, C. (2008). Celiac Disease. Gluten-Free Cereal Products and Beverages*. Elsevier Inc*. New York. 1-27.

Feillet, P., and Roulland, T. M., (1998). Caroubin: A gluten-like protein isolate from carob bean germ. *Cereal Chemistry,* 75, 488-492.

Gjertsen, H. A., Lundin, K. E. A., Sollid, L., Eriksen, J. A., and Thorsby, E. (1994). T Cells Recognize a Peptide α-Gliadin Presented by the Celiac Disease-Associated HLA-DQ (α1*0501, β1*0201) Heterodimer. *Human Immunology,* 39, 243-252.

Goesaert, H., Brijs, K., Veraverbeke, W. S., Courtin, C. H., Debruers, K., Declour, J. A. (2005). Wheat flour constituents: how they impact bread quality, and how to impact their functionality. *Trends Food Science Technology.* 16, 12-30.

Godkin, A., Jewell, D. (1998) Viewpoints in Digestive Diseases: The Pathogenesis of Celiac Disease. *Gastroenterology,* 115, 206-210.

Gourmet Retailer (2006). Gluten-free market set to explode. *Gormet Retailer.* September 13-30.

Green, P. H., Cellier, C. (2007). Celiac Disease. *New England Journal of Medicine.* 357 (17), 1731-1743.

Hillcoat, D., Lewis, G., and Vercourt, B. (1980). A new Species of *Ceratonia* (*Leguminosae-Caesalpinoideae*) from Arabia and the Somali Republic. *Kew Bulletin*, 35, 261-271.

Hoefler, A. C. Hydrocolliods. Eagan Press Handbook Series. *Eagan Press*. St. Paul, Minnesota, 2004.

Hopper, A., Cross, S., Hurlstone, D., McAlindon, M., Lobo, A., Hadjivassiliou, M., Sloan, M., Dixon, S., Sanders, D. (2007). Pre-endoscopy serological testing for coeliac disease: evaluation of a clinical decision tool. *BMJ,* 334: 729.

Hoseney, R. C. Principles of Cereal Science and Technology. *American Assosiation of Cereal Chemists, inc*. St. Paul, Minnesota, 1998.

Kawamura, Y. (2008). CAROB BEAN GUM, Chemical and Technical Assessment (CTA). www.fao.org/ag/AGN/agns/jecfa/cta/69/Carob_bean_gum_CTA_69_.pdf. Accessed April 4, 2009.

Karell, K., Louka, A. S., Moodie, S. J., Ascher, H., Clot, F., Greco, L., Ciclitira, P. J., Sollid, L. M., Patanen, J. (2003). Hla types in celiac disease patients not carrying the *DQA1*05-DQB1*02* (DQ2) heterodimer: results from the european genetics cluster on celiac disease. *Human Immunology,* 64 (4), 469-477.

Korponay-Szabo, I., Dahlbom, I., Laurila, K., Koskinen, S., Woolley, N., Partanen, J., Kovacs, J., Mäki, M., Hansson, T. (2003). Elevation of IgG antibodies against tissue transglutaminase as a diagnostic tool for coeliac disease in selective IgA deficiency. *Gut* 52 (11), 1567–71

Lammers, K. M., Lu, R., Brownley, J., Lu, B., Gerard, C., Thomas, K., Rallabhandi, P., Shea-Donohue, T., Tamiz, A., Alkan, S. (2008). Gliadin induces an increase in intestinal permeability and zonulin release by binding to the chemokine receptor CXCR3. *Gastroenterology* 135 (1): 194–204.e3.

Layrisse, Z., Guedez, Y., Dominguez, E., Paz, N., Montagnani. S., Matos, M., Herrera, F., Ogando, V., Balbas, O., Rodriguez-Larralde, A. (2001). Etended HLA haplotypes in Carib American population: the Yucpa of the Perija Range. *Human Immunology*, 62 (9), 992-1000.

Leeds, J. S., Hopper, A. D., and Sanders, D. S. (2008). Coeliac disease. *British Medical Bulletin*, 88, 157-170.

Marsh, M. N. (1992). Gluten, major histocompatability complex, and the small intestine. *Gastroenterology,* 102, 330-354.

Osborne, T. B. (1903). The Proteins of the Wheat Kernel. Carnegie Institute, Washington, D. C.

Plaut, M., Zelcbuch, B., and Guggenhem, K. (1953). Nutritive and Baking Properties of Carob Germ Flour. *Bulletin of the Research Council of Isreal*, 3, 129-131.

Sblattero, D., Berti, I., Trevisiol, C., Marzari, R., Tommasini, A., Bradbury, A., Fasano, A., Ventura, A., and Not, T. (2000). Human recombinant tissue transglutaminase ELISA: an innovative diagnostic assay for celiac disease. *American Journal of Gastroenterology.* 95 (5): 1253–7.

Schober, T., Bean, S., and Boyle, D. (2007). Gluten-Free Sorghum Bread Improved by Sourdough Fermentation: Biochemical, Rheological, and Microstructural Background. *Journal of Agriculture and Food Chemistry.* 55, 5137-5246.

Schober, T., Bean, S., and Boyle, D., Park, S. (2008). Improved viscoelastic zein-starch doughs for leavened gluten-free breads: Their rheology and microsture. *Journal of Cereal Science,* 48, 755-767.

Schroeder, C. A. (1952). The carob in California. *Fruit varieties and Horticulture Digest*, 7, 24-28.

Troncone, R., Ivarsson, A., Szajewska, H., Mearin, M. L. (2008). Review article: future research on coeliac disease - a position report from the European multistakeholder platform on coeliac disease (CDEUSSA). *Aliment. Pharmacol. Ther.* 27 (11), 1030–43.

Van Heel, D. A., West, J. (2006). Recent advances in coeliac disease. *Gut.* 55 (7), 1037-1046.

Wang, Y., Belton, S. B., Bridon, H., Garanger, E., Wellner, N., Parker, M. L., Grant, A., Feillet, P., and Noel, T., (2001). Physicochemical Studies of Caroubin: A gluten like Protein. *Journal of Agriculture and Food Chemistry*, 49, 3414-3419.

Weiser, H., and Koehler, P. (2008). The biochemical basis of celiac disease. *American Association of Cereal Chemist International, inc*., 85 (1), 1-13.

Wennstrom, P., and Mellentin, J., (2000). Functional Foods and the consumer's perception of health claims. *Scandinavian Journal of Nutrition.* 44, 30-33.

Wennstrom, P., and Mellentin, J., (2003). The food and health handbook. London: New Nutrition Business.

Winer, L. (1980). The potential of the carob tree (*Ceratonia Siliqua*). *International Tree Crops Journal*, 1, 15-26.

Chapter 2:
Characterization of the Composition and Molecular Weight Distribution of Carob Germ Proteins

Abstract:

Biochemical properties of carob germ proteins were analyzed using a combination of selective extraction, reversed-phase high performance liquid chromatography (RP-HPLC), size exclusion chromatography coupled with multi-angle laser light scattering (SEC-MALS) and micro-fluidics analysis. Using a modified Osborne fractionation method, carob germ flour proteins were found to contain ~32% albumin and globulin and ~68% glutelin with no prolamins detected. When extracted under non-reducing conditions and divided into soluble and insoluble proteins as typically done for wheat gluten, carob germ proteins were found to be almost entirely (~95%) in the soluble fraction with only (~5%) in the insoluble fraction. As in wheat, SEC-MALS analysis showed that the insoluble proteins had a greater M_w than the soluble proteins and ranged up to $8x10^7$ Da. The lower level of insoluble proteins in carob germ flour may be one reason that carob proteins are only able to form a weak dough.

Introduction:

Celiac disease, an autoimmune disorder affecting the upper regions of the small intestines is gaining increased attention worldwide. With 1:100 to 1:300 people afflicted with celiac disease in certain populations, this disease is considered the most common genetic disease of humans (Fasano and Catassi, 2001; Weiser and Koehler, 2008). The basis of the disorder is an inflammation of the intestinal villi that occurs upon the ingestion of gluten proteins from wheat, rye, barley and possibly oats (Weiser and Koehler, 2008). With the ever increasing awareness and diagnosis of this disease, it is important that gluten-free food alternatives are explored to better the quality of celiac sufferers' lives by identifying food systems with similar functional and quality attributes to wheat and associated proteins.

Carob, *Ceratonia siliqua*, is a leguminous shrub native to the Mediterranean region where its seeds and pods have been traditionally used as a food thickener and sweetener. In recent times, carob's primary use has been in the production of carob bean gum (locust bean gum), molasses and chocolate substitutes. With large quantities of carob bean gum being produced annually an appreciable amount of carob germ flour is produced as a result and marketed as a byproduct of gum production (Batlle and Tous, 1997).

In the 1930's, carob germ flour was found to exhibit gluten like properties (Bienenstock et al 1935). When compared to that of wheat gluten, relatively little work has been done to characterize the proteins of the carob germ since this time. In 1953 carob germ proteins were analyzed for use in high protein cereal products for diabetics (Plaut et al 1953). Using an Osborne fractionation scheme, these researchers reported that carob germ proteins contained 14.5% albumin, 50.0% globulins, 3.4% prolamins, and 32.1% glutelins. Bread baked from carob-

wheat mixtures in this study were of lower volume than 100% wheat flour bread and had a yellow/green color, but were considered acceptable.

Feillet and Roulland (1998) designated the term caroubin for the proteins found in the carob germ. These authors compared wheat gluten and caroubin using SEC and SDS-PAGE. Unreduced caroubin was found to have large polymeric proteins with an overall similar SEC chromatogram as wheat gluten. These authors speculated that the large polymeric proteins of caroubin might have similar functional properties as wheat gluten. Rheological studies indicated that caroubin had visco-elastic properties, however Feillet and Rolulland (1998) pointed out that due to caroubin's low levels of cysteine, the mechanism of this visco-elastic behavior may be different from that of wheat gluten. Wang et al (2001) used FTIR, NMR, scanning electron microscopy and DSC to characterize the properties of hydrated caroubin and wheat gluten. These authors found that hydrated caroubin was capable of forming sheets and fibrils, but that the caroubin was more hydrophilic and that when exposed to water, had less changes to protein structure than did gluten. Bengoechea et al (2008) extensively characterized carob germ proteins using a combination of techniques. They reported that carob germ proteins were composed of aggregates formed both by disulfide bonds and through non-covalent interactions.

All the above previous research on carob germ proteins (i.e. caroubin) has indicated that it has potential as a gluten substitute in wheat-free foods. While this research has shown that caroubin has large polymeric protein fractions, more research is needed to characterize these proteins and compare them to similar proteins in wheat. Thus, the purpose of this research was to explore the biochemical properties of caroubin with similar methods used in analyzing

the polymeric proteins of gluten and identify major similarities and differences when compared to wheat polymeric proteins.

Materials and Methods:

Carob germ flour was graciously donated by Danisco Foods (Kansas City, MO).

Protein Characterization.

Osborne Extraction:

For basic characterization of the proteins in the carob germ flour a modified Osborne fractionation scheme (Osborne 1907) was used which divided proteins into the following classes based on solubility: albumins and globulins, soluble (non-reduced) prolamins, insoluble (reduced) prolamins, and glutelins. Initially, 20 mg of carob germ flour was extracted twice with 1 mL of appropriate solvent for 15 min with continuous vortexing. After each extraction samples were centrifuged for 5 min at 9,300 X *g* and the supernatants pooled in a 1:1 ratio. The albumin/globulin fraction was extracted with a pH 7.8 50 mM Tris-HCL buffer containing 100 mM KCl and 4mM EDTA (Marion et al 1994). Upon the completion of the albumin/globulin extractions, the supernatants were removed and the residue was washed with 1 mL of DI water to eliminate excess salts left by the extraction buffer. The water was discarded. Next, the soluble prolamin fraction was extracted using 1 mL of 50% n-propanol as described above. After this extraction step, 1 mL of 50% n-propanol containing 2% dithiothreitol (DTT) (w/v) was added to the remaining pellet and extracted as above to extract the insoluble (reduced) prolamins. Finally the pellet was extracted with 12.5 mM Na-borate pH10.0 buffer containing 2% SDS (w/v) and 2% DTT (w/v) to extract the glutelins (Fig 1). Extracts were used immediately after extraction for analysis on a lab-on-a-chip system (Agilent, Waldbronn, Germany).

A second modified Osborne extraction procedure was used to prepare samples for SEC-MALLS and RP-HPLC analysis where only albumin/globulin and glutelin fractions were extracted and extractions were done under non-reducing conditions. The above procedure was used except that the extraction with 50% n-propanol and 50% n-propanol + DTT was left out. Glutelins were extracted after removal of albumin/globulins using the pH 10 SDS buffer with sonication (10 watts for 30 seconds) without added reducing agent. Prior to RP-HPLC analysis samples were reduced by adding β-ME (to a final concentration of 2%) to aliquots of the non-reduced extractions and allowed to set for 30 min.

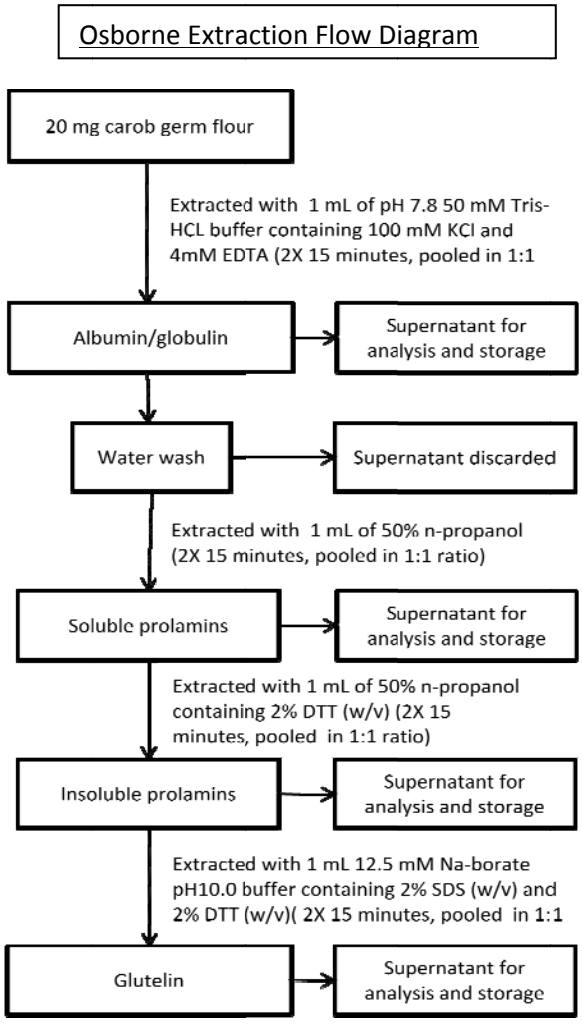

Figure 1. Flow diagram of the sequential Osborne extraction scheme of carob germ flour.

Polymeric Protein Extraction:

Proteins were extracted (un-reduced) into “soluble” proteins (SP) which typically include all monomeric proteins and smaller polymeric proteins and “insoluble” proteins (IP) which contain the largest polymeric proteins, known in wheat to be correlated to dough strength (Weegels et al 1996; Southan and MacRitchie, 1999). To accomplish this, a sequential procedure was carried out. Soluble proteins were first extracted from 20 mg of carob germ flour with 15 min of continuous vortexing in 1 mL of 50 mM sodium phosphate, pH 7.0 buffer containing 1% SDS (w/v). After 5 min of centrifugation at 9,300 X *g* the supernatant was collected and the extraction procedure was repeated. The supernatants from both SP extractions were pooled in a 1:1 ratio. Insoluble were extracted from the remaining residue using sonication (10 watts for 30 sec in 1 mL of 50 mM sodium phosphate, pH 7.0 buffer containing 1% SDS (w/v). Two extractions were made and supernatants were centrifuged and pooled as described above. Residue proteins (RP) were extracted with the 50 mM sodium phosphate, pH 7.0 buffer containing 1% SDS (w/v) plus 2% DTT (w/v) from the residue remaining after the IP extractions and pooled as above (Fig 2). In some cases, samples were lyophilized and stored frozen at -20 °C until needed.

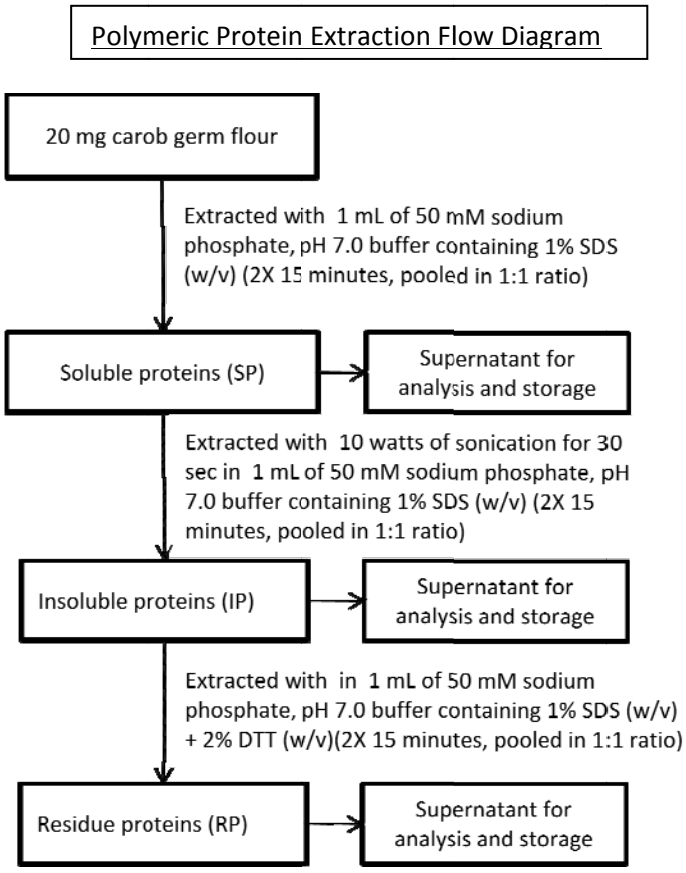

Figure 2. Flow diagram of the sequential polymeric protein extractions of carob germ flour.

Protein Analysis

RP-HPLC:

Osborne fractions were analyzed via RP-HPLC on an Agilent 1100 HPLC system equipped with Poroshell SB300 C8 (Agilent, Palo Alto, CA) column and guard column. Separations were achieved using a linear gradient from 10% acetonitrile/0.1% trifluoroacetic acid (TFA) (w/v) to 90% acetonitrile/0.1% TFA (w/v) over 20 min with a flow rate of 0.7 mL/min and a column temperature of 50^{o}C. Sample detection was by UV at 214 nm and 10 µL of sample was injected for all samples.

SEC-MALLS:

Soluble proteins, insoluble proteins, and residue proteins samples were analyzed via size exclusion (SE) HPLC using an Agilent 1100 HPLC system equipped with a Biosep-4000 column (Phenominx, Torrance, CA) and guard column using 50 mM sodium phosphate, pH 7.0 buffer containing 1% SDS (w/v) as a mobile phase (Bean and Lookhart 1998). Proteins were detected at 214 nm over a 30 min span with a flow rate of 1 mL/min and an injection volume of 20 µL. Column temperature was fixed at 40^{o}C. For characterization of the M_w distributions of SP and IP extracts, SEC-MALLS was conducted using the SEC conditions above with the HPLC system connected to a Wyatt DAWN Helios II multiangle light scattering (MALLS) detector and an Optilab Rex differential refractometer (DR) (Wyatt Technology Corp. Santa Barbara, CA). Scattering angles were normalized using bovine serum albumin (BSA). Temperature of the DR detector was maintained at 25 °C. Dn/Dc of 0.39 was used for all SEC separations of carob protein and was determined as described in Bean and Lookhart (2001).

Micro-Fluidic analysis:

Molecular weight of reduced protein extractions were determined by microfluidic electrophoresis on an Agilent 2100 Bioanalyzer (Lab-on-a-Chip). Protein fractions for the Osborne extractions were analyzed with the Lab-on-a-Chip system as described by the protocols provided from the manufacturer. Briefly, 4.0 µL of sample for each fraction analyzed was mixed into 2 µL Agilent denaturing solution in a 0.5 mL micro-tube. This mixture was vortexed and proteins were set by exposing them to 95^{o}C for 5 min. 84 µL of DI H_20 was added to the protein extraction/denaturing solution mixture and vortexed. Protein 230 Chips with a molecular weight rang of 4.5 kDa to 240 kDa were prepared according to Agilent specifications;

each well was filled with 6 µL of the extraction solutions from above. The prolamin and prolamin reduced extractions were run with the same conditions as above, but with a Protein 80 Chip with a molecular weight range of 5 kDa to 80 kDa to achieve better resolution.

Results and Discussion:

Protein Characterization

The Osborne fractionation protocol was efficient, with ~96% of the total protein being extracted as determined by nitrogen combustion of the residue remaining after all extractions (data not shown). Characterization of the Osborne fractions by microfluidic analysis showed that virtually no prolamins were detected (Fig 3). For the albumin/globulin fraction major bands were observed at ~46kDa and 16kDa with minor bands spanning the range from 7 to 96kDa. Major bands in the glutelins showed nominal M_w of ~96kDa, 46kDa, and 16kDa with minor bands visible throughout this range (Fig 3). In previous work conducted via SDS-PAGE carob proteins were not extracted into different sub-fractions. However, major and minor protein bands appeared in similar molecular weight ranges (Bengoechea et al 2008).

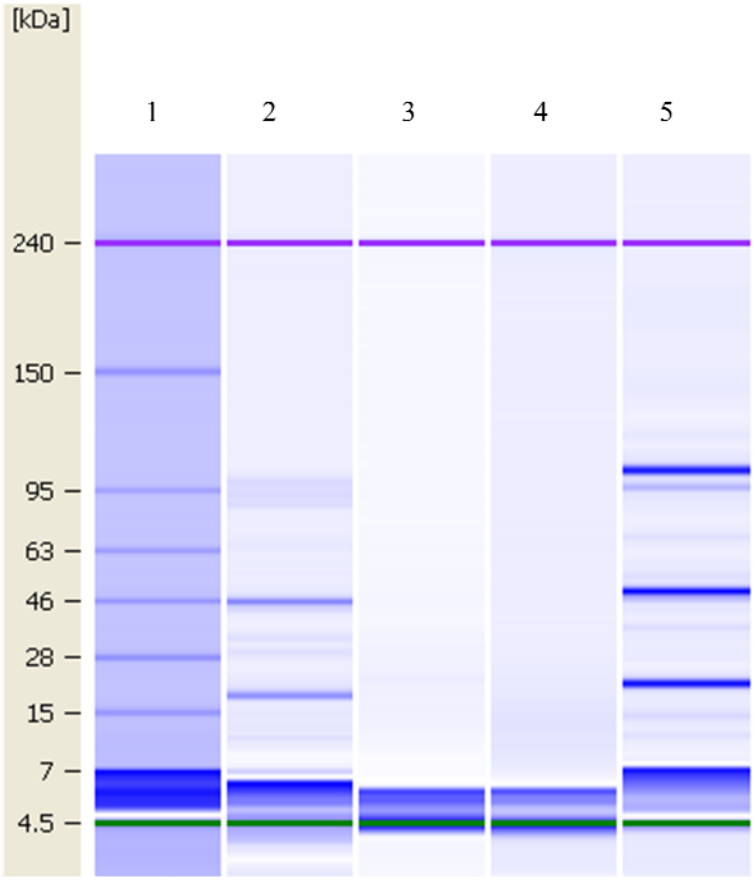

Figure 3. Electropherogram of 1) M_w standards, 2) albumin/globulin, 3) prolamin, 4) reduced prolamin, and 5) glutelin of carob germ proteins. All samples were reduced prior to analysis.

Figure 4 shows the RP-HPLC separations of both the albumin/globulin and glutelin fractions. The albumin/globulin extract contained peaks with a range of elution times with the major peaks eluting at ~9 min. The major peaks in the glutelin extract also eluted at the 8-9 min range with only a few additional minor peaks. The albumin/globulin fraction had more early eluting peaks, indicative of lower surface hydrophobicity (i.e. more hydrophilic), than the glutelin fraction. This would be expected from water and salt soluble proteins. Quantitative data from the RP-HPLC separations revealed that the glutelins were the most abundant protein class comprising ~78% of the total with the albumin/globulin fraction containing the remaining

~22%. These data confirm the previous results of Plaut et al (1953) in that the majority of the proteins extracted were found in the glutelin, albumin and globulin fractions and minimal amounts of prolamin was present. However, Plaut et al (1953) reported that albumins and globulins accounted for the majority of the protein (~65% on a total flour protein basis) with the glutelin making up most of the remainder (~32%). Little information is available on the methodology used by Plaut et al (1953), so it is difficult to speculate on the reasons between these differences.

Wheat typically contains ~10 – 15% albumins/globulins, 67 - 76% prolamins, and 14 - 18% glutelins (Fu and Sapirstein 1996; Sapirstein and Fu 1998; Lookhart and Bean 2000). Caroubin contained no extractable prolamins. Prolamins in wheat are rich in proline and glutamine and this fraction is thought to contribute significantly to wheat gluten functionality. The absence of this protein fraction means that there are large differences in amino acid content between wheat gluten and caroubin. These differences may change the three dimensional structure of proteins which in turn changes their physical and chemical properties. This is one reason why carob germ proteins may function differently than wheat.

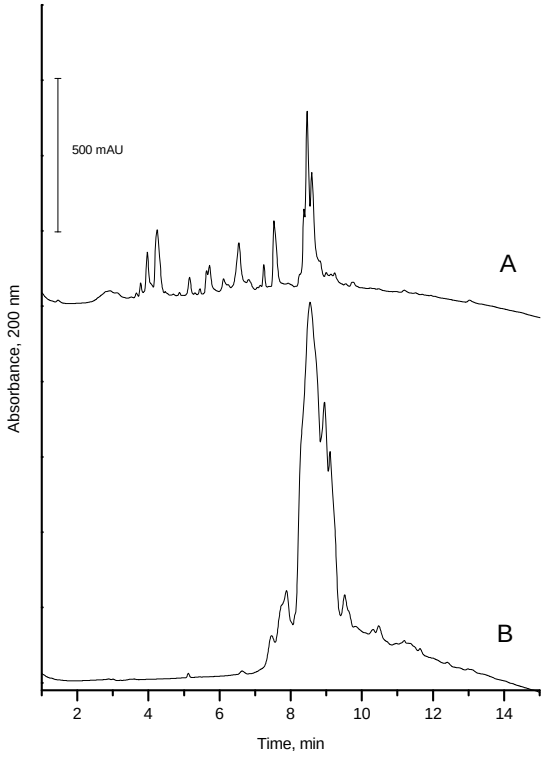

Figure 4. RP-HPLC separations of A) reduced albumin and globulin extract, and B) reduced glutelin extract of carob germ protein.

SEC analysis of the non-reduced albumin/globulin and glutelin fractions showed major differences between the two protein classes in their molecular weight distribution (Fig 5). The albumin/globulin fraction had proteins eluting across a wide time range, indicating a wide M_w distribution. Relatively low amounts of the early eluting high M_w material was seen in the albumin/globulin fraction. Little change was seen in the chromatograms when the samples were reduced, indicating low levels of disulfide bonded polymers present in these proteins (Fig 5A). The glutelin fraction, on the other hand, had high levels of early eluting peaks indicating

polymers of high M_w. Upon reduction, the majority of the early eluting peaks disappeared with subsequent appearance of new peaks eluting later in the chromatogram, indicating that the early eluting peaks were large polymers linked through disulfide bonds (Fig 5B).

The cumulative molecular weight distribution curves show that the albumin/globulin fraction contained proteins of very small M_w that range from ~$0.5x10^7$ g/mol. The glutelin fraction however, contained mostly large M_w proteins up to ~$8x10^7$ and small amounts of lower molecular weight proteins (Fig 6).

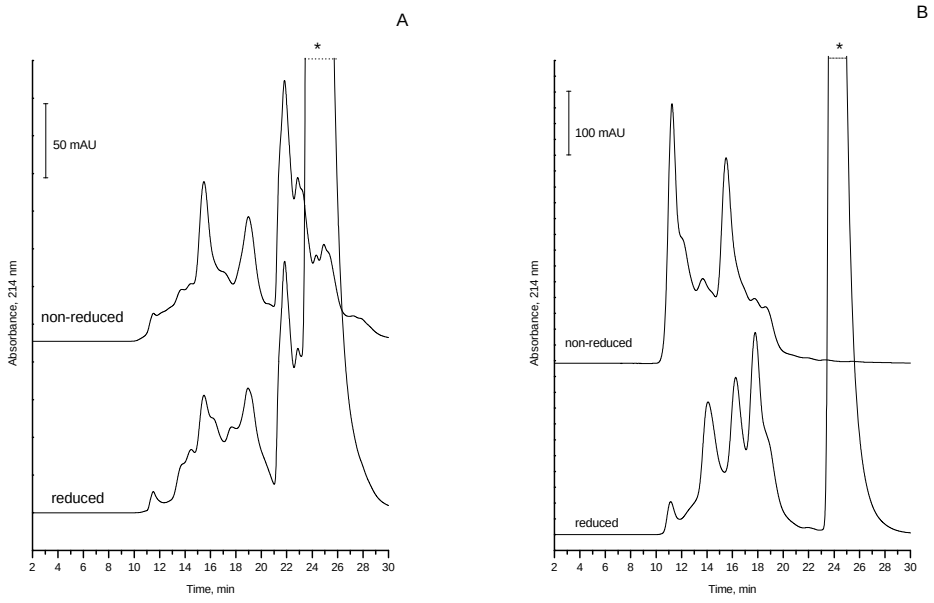

Figure 5. Size exclusion chromatograms of reduced and non-reduced A)albumin and globulins, and B) glutelins of carob germ proteins. The "*" marks the location of the β-ME peak, which has been artificially truncated for scale.

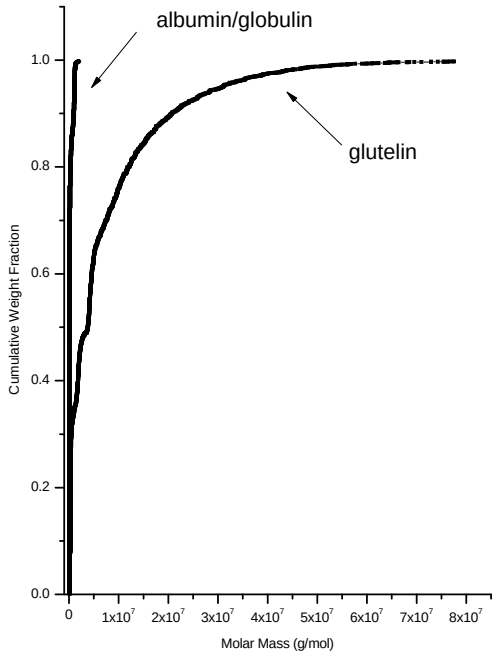

Fig 6. Cumulative molecular weight curves for the non-reduced polymeric peaks of albumin/globulin and glutelins of carob germ proteins.

Fig 8 shows the SEC chromatograms of the SP and IP fractions, both reduced and non-reduced. The SP fraction was found to comprise ~93% of the total protein while, IP ~5%, and RP was ~2% (Fig 7). This is much different than typically found in wheat, where IP typically accounts for 30-50% of the protein depending on the type of wheat and the extraction methodology used (Gupta et al 1993; Ciaffi et al 1996; Bean et al 1998).

Reduction of the SP and IP samples was done to identify disulfide linked polymers in the SEC chromatograms. The SP fraction contained both polymeric and monomeric proteins. Upon reduction, the majority of the proteins eluting from 10-16 min either disappeared or changed demonstrating that these were polymeric proteins linked via disulfide bonds. Furthermore, the large peak eluting at 16-18 min changed with an increase in the peak at ~19 min (Fig 8).

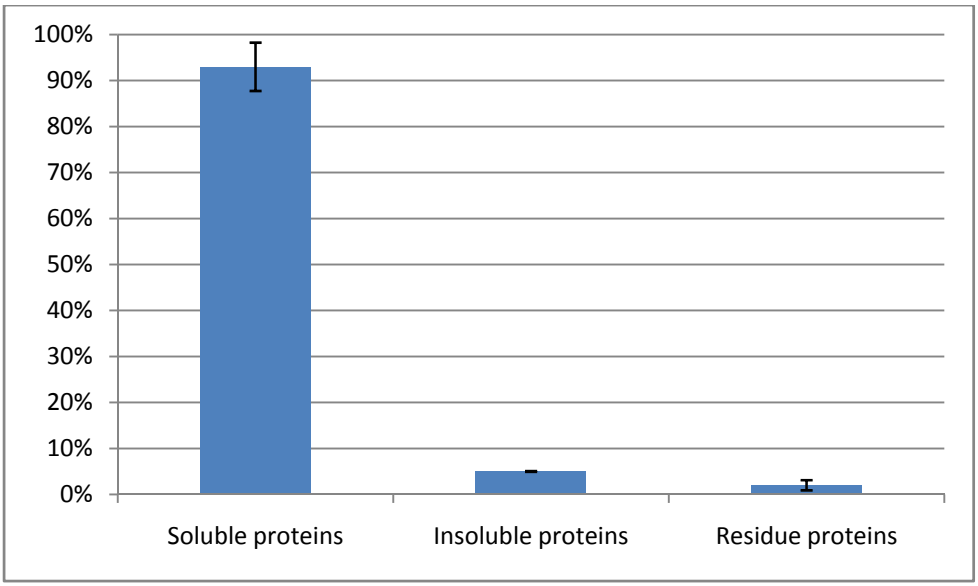

Fig 7. Compositional data of the polymeric protein extraction of carob germ proteins (non-reduced).

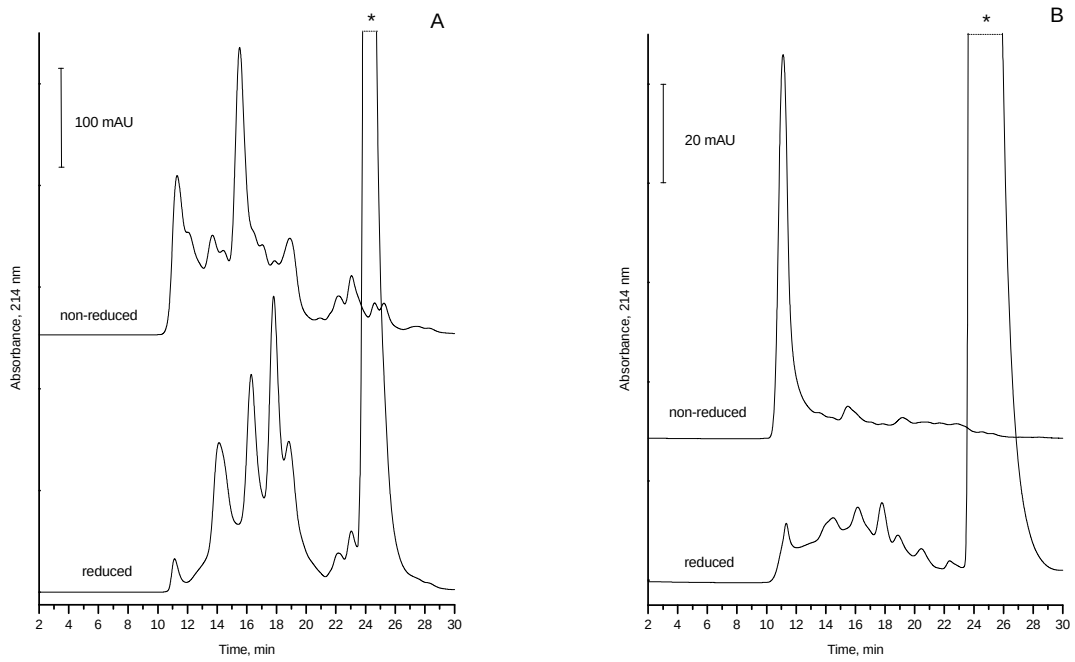

Fig 8. Size exclusion chromatograms of A) non-reduced and reduced soluble proteins (SP), and B) non-reduced and reduced insoluble proteins (IP) of carob germ proteins. "*" marks the location of the β-ME peak, which has been artificially truncated for scale.

The IP extract was composed of mainly large polymers that again largely disappeared upon reduction, demonstrating that the majority of these proteins were disulfide linked. Reduced chromatograms of both the SP and IP were overall similar with some slight differences in the 12-14 min range. This suggests that the polymeric proteins in the SP and IP were composed of the same set of monomers. It is obvious that quantitative differences exist in the reduced SP and IP extracts, e.g. the proteins eluting at 16-18 min were present in much greater proportion to the other proteins than in the reduced IP sample. Comparing the results in Fig 8 to the chromatograms in Fig 5, it is possible to gain some insight into the composition of the SP

and IP. In Fig 5, the albumin and globulins showed only low levels of large polymeric proteins with peaks at 10-12 min with little change in these proteins upon reduction. Conversely, the glutelins showed a large peak in the unreduced samples at 10-12 min that almost completely disappeared when reduced. Since both the SP and IP fractions contained large polymeric protein peaks at 10-12 min when using a solvent system similar to what is used on wheat, it can be concluded that the large polymeric proteins of carob are composed of mainly glutelin. As discussed previously, this may have implications for the functionality of carob germ proteins with respect to visco-elastic dough formation. In wheat, the large functional polymeric proteins are prolamins which have different amino acid compositions and solubility than carob glutelins. Such differences may mean differences in protein structure, hydrophobicity, and charge, which may play a role in protein functionality. Furthermore, the polymeric proteins of carob are much lower in M_w than that of wheat, which will be discussed in more detail below.

The cumulative molecular weight distribution curves as determined by SEC-MALS for the SP and IP fractions show that the curves for both SP and IP are very similar. However, like wheat the IP's contained proteins of higher molecular weight than the SP fraction. When compared to work done by Stevenson et al (2003), Bean and Lookhart (2001), and Carceller and Aussenac (2001) caroubin's overall molecular weight distribution curves resembled that of wheat. However, wheat proteins typically contain proteins in the IP fraction up to ~$1X10^8$. The maximum M_w of the caroubin fraction was ~$7X10^7$ (Fig 9).

These higher molecular weight proteins have been shown to play a major role in glutens functionality (Stevenson et al 2003; Bean and Lookhart 2001; Carceller and Aussenac 2001). Carob germ proteins have been previously shown to have properties similar to that of gluten,

which could provide a means to produce high quality gluten-free food products for the celiac market. Understanding how proteins other than wheat gluten form visco-elastic dough will allow for a better understanding of gluten functionality (Feillet and Roulland 1998). The above results show that carob germ proteins contained mostly (~95%) "soluble" proteins of M_w up to ~$5X10^7$ with only ~5% IP proteins, whereas wheat has been reported to contain 30-50% IP depending on the type of wheat analyzed (Bean et al 2001, Schober et al 2005, Gupta et al 1995). Thus, while caroubin contains polymeric proteins with M_w close to that of wheat, the levels of these largest proteins are very low compared to wheat. This may be one reason that carob is capable of forming only weak dough, i.e. the M_w distribution is skewed to monomeric and smaller M_w polymers. Relating the functionality of SP and IP in carob to that of wheat should be approached with caution however, until more understanding of carob germ proteins can be gained.

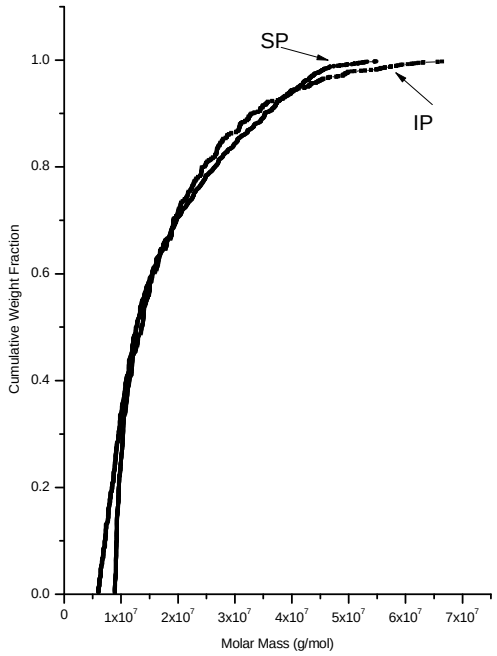

Fig 9. Cumulative molecular weight curves for the non-reduced polymeric peaks of soluble and insoluble proteins of carob germ proteins.

Conclusion:

There are few known proteins capable of dough formation. For this reason caroubins ability to form protein networks is significant in helping us better understand the properties of viscoelastic proteins while providing possible new avenues for future gluten-free foods. While the gluten like properties of caroubin has been reported, the biochemical analysis proved caroubin to be quite different from gluten. It was found that the M_w distribution of carob germ proteins is of lesser M_w and in smaller quantities than that of wheat gluten. Furthermore, in the Osborne extractions caroubin was found to contain no measurable amounts of prolamin, a protein fraction that is attributed to gluten functionality. These major biochemical differences

may be the causative factor in the rheological differences reported by Feillet and Roulland (1998). More research is needed to gain a further understanding of these chemical differences and the chemical interactions that take place during dough formation so that carob may be better utilized.

Literature Cited:

Batlle, I., Tous, J. (1997). Carob Tree: *Ceratonia silique* L. Promoting the conservation and use if underutilized and neglected crops. 17. Institute if Plant Genetics and Crop Plant Research, Gatersleben/International Plant Genetic Resources Institute, Rome, Italy.

Bean S., Lyne, R., Tilley, K., Chung, O., Lookhart, G. (1998). A Rapid Method for Quantitation of Insoluble Polymeric Proteins in Flour. *Cereal Chemistry*, 75, 374-379.

Bean S., Lookhart G. (2001). Factors influencing the characterization of gluten proteins by size-exclusion chromatography and multiangle laser light scattering (SEC-MALLS), *Cereal Chemistry* 78, 608–618.

Bengoechea, C., Romero, A., Villanueva, A., Moreno, G., Alaiz, M., Millan, F., Guerro, A., and Puppo, M.C. (2008). Composition and structure of carob (*Ceratonia siliqua* L.) germ proteins. *Food chemistry*, 107, 675-683.

Bienenstock, M., Csaski, L., Pless, J., Sagi, A., and Sagi, E. (1935). Manufacture of Mill Products for alimentary purposes and of paste foods and bake products from such milled products. U.S. patent 2,025,705.

Carceller, J., Aussenac, T., (1999). Size characterization of glutenin polymers by HPSEC-MALLS. Journal of Cereal Science, 33, 131-142.

Ciaffi, M., Tozzi, L., Lafiandra, D. (1996). Relationships between flour protein Composition determined by size-exclusion chromatography and dough rheological parameters. Cereal Chemistry, 73, 346-351.

Fasano, A., Catassi, C. (2001). Current approaches to diagnosis and treatment of celiac disease: an evolving spectrum. *Gastroenterology* 120, 636-651.

Feillet, P., and Roulland, T. M. (1998). Caroubin: A gluten-like protein isolate from carob bean germ. *Cereal Chemistry,* 75, 488-492.

Fu, B. X., Sapirstein, H. D. (1996). Procedure for Isolating Monomeric Proteins and Polymeric Glutenin of Wheat. *Cereal Chemistry*, 73, 143-152.

Gupta, R., Khan, K., MacRithie, F. (1993). Biochemical basis of flour properties in wheats. I. Effects of variations in the quantity and size distribution of polymeric proteins, Journal of Cereal Science, 18, 23-41.

Gupta, R., Popineau, Y., Lefebvre, J., Cornec, M., Lawrence, G. J., and MacRitchie, F. 1995. Biochemical basis of flour properties in bread wheats. II. Changes in polymeric protein formation and dough/gluten properties associated with the loss of low Mr or high Mr glutenin subunits. J. Cereal Science 21, 103-116.

Lookhart, G., Bean, S. (2000). Cereal Composition of Their Major Fractions and Methods for Identification. *Handbook of Cereal Science and Technology.* Marcel Dekker, Inc. New York. 363-383.

Marion D., Nicolas Y., Popineau Y., Branlard G., Landry J. (1994). A new and improved sequential extraction procedure of wheat proteins. In: Wheat kernel proteins. Viterbo, Italie, pp 197–199.

Osborne, T. B. (1907). The proteins of the wheat kernel. Press of Judd and Detweiler, Inc., Washington, D. C.

Plaut, M., Zelcbuch, B., and Guggenhem, K. (1953). Nutritive and Baking Properties of Carob Germ Flour. *Bulletin of the Research Council of Isreal*, 3, 129-131.

Sapirstein, H. D., Fu, B. X. (1998). Intercultivar Variation in the Quantity of Monomeric Proteins, Soluble and Insoluble Glutenin, and Residue Protein in Wheat Flour and Relationships to Breadmaking Quality. *Cereal Chemistry*, 75, 500-507.

Schober, T., Bean, S., Khun, M. (2005). Gluten proteins from spelt (*Triticum aestivum* ssp. *spelta*) cultivars: A rheological and size-exclusion high-performance liquid chromatography study. *Journal of Cereal Science*, 44, 161-173.

Southan M., MacRitchie F. (1999). Molecular Weight Distribution of Wheat Proteins. *Cereal Chemistry*, 75, 827-836.

Stevenson, S., You, S., Izydorczyk, M., Preston, K. (2003). Characterization of polymeric wheat proteins by flow-flow fractionation/MALLS. *Journal of Liquid Chromatography & Related Technologies*, 26, 2771-2781.

Wang, Y., Belton, S. B., Bridon, H., Garanger, E., Wellner, N., Parker, M. L., Grant, A., Feillet, P., and Noel, T., (2001). Physicochemical Studies of Caroubin: A gluten like Protein. *Journal of Agriculture and Food Chemistry*, 49, 3414-3419.

Weegels, P.L., Hamer, R.J. Schofield, J.D. (1996). Functional properties of wheat glutenin. *Journal of Cereal Science* 23**,** 1–18.

Weiser, H., and Koehler, P. (2008). The biochemical basis of celiac disease. *American Association of Cereal Chemist International, inc*., 85 (1), 1-13.

Chapter 3:
Dough Formation and Bread Quality of a Carob Germ Protein-Starch Breads

Abstract:

Although carob germ flour proteins have been shown to have gluten like properties, relatively little work has been done to determine the factors that influence dough formation with carob germ flour proteins. The primary objective of this research was to define the critical factors influencing dough functionality and how these factors influence quality and staling of carob germ based bread. Farinograph, size exclusion high performance liquid chromatography, and laser scanning confocal microscopy were used to define the critical factors effecting dough formation. A bread formula consisting of 30% carob germ protein flour and 70% corn starch was developed that formed a visco-elastic dough that could be sheeted and handled like a wheat dough. Carob based bread quality was compared to a sorghum based wheat-free bread and a wheat bread. Specific volumes and C-Cell analysis were taken 2 hrs after baking. Staling tests were conducted at 2, 50, and 122 hrs post baking with a texture profile analyzer. When mixed in a farinograph, control carob germ flour dough displayed curves similar to that of wheat. However, when a reducing agent was added to the flour, mixing peaked at the same time as the control, but rapidly broke down. After reduction the dough was batter-like in consistency and visually appeared to have no visco-elastic properties. These results demonstrate the critical nature of disulfide bonds for the formation of carob germ flour dough. Laser scanning confocal microscopy showed that in control dough protein fibrils were formed, but in reduced dough no protein fibrils were visible and protein appeared as a disorderly mass. Carob based bread had a specific loaf volume of ~2.5 mL/g, sorghum ~2.6 mL/g, and wheat ~5.6 mL/g. Crumb structure in the carob bread, was ~82 cells/cm^2 for carob, ~41 cells/cm^2 for sorghum, and ~53 cells/cm^2 for wheat. Carob based bread over a period of 122 hrs had higher

springiness and cohesiveness values than sorghum based breads. Carob bread was significantly harder than sorghum based breads at all testing times. Wheat based breads had better quality properties than both carob and sorghum based breads for all tests and all test times. This study suggests that carob germ proteins have some similar functionality to wheat gluten proteins and when baked into a bread have the potential to prevent some of the staling associated with gluten-free breads.

Introduction:

Since the discovery of celiac disease the number of people diagnosed with the disorder is ever increasing. It is estimated that about 1% of the world's population is affected by the disease, but only 1:266 have been diagnosed (Fasano and Catassi, 2001; Van Heel and West 2005). With increased knowledge and education of celiac disease coupled with advances in screening procedures the number of people subsiding on gluten-free diets will continue to increase. For this reason it is important to understand the technology of gluten-free foods and improve their quality.

The gluten-free market is a rapidly growing industry. It was once considered a very small niche market, but reports in 1996 indicated that this market accounted for ~$700 million in sales annually in the United States. It was estimated that the market would grow at a rate of 25% per year to reach annual sales of ~$1.7 billion by 2010 (Bogue and Sorenson, 2008; Anonymous, 2006). The lack of quality in comparison to wheat based foods is a major problem that must be overcome to obtain products that more closely resemble wheat based foods.

A majority of gluten free breads are produced from cake like batters that can be fermented or chemically leavened. These batters are typically produced from water, a gluten-free starch source, a hydrocolloid, and other ingredients that are added to improve overall bread quality. The negative properties commonly attributed to these breads are rapid staling, poor flavor, and poor texture. It has been hypothesized that an addition of protein network can alleviate some of these problems (Arendt et al 2008; Schober et al 2008)

Bienenstock et al (1935) first described the gluten-like properties of carob germ proteins. Since this time little work has been done in characterizing these proteins and applying

them in food systems. In 1935's patent, breads were made from dough containing carob germ meal blended with other non gluten containing flours. These breads were described as being dense with a strong resemblance to a rye bread. Research completed on carob germ proteins has been completed in the form of protein analysis and composition with little work done in terms of functionality (Plaut 1953; Feillet and Roulland 1998; Wang 2001; Bengoechea et al 2008).

Disulfides bonds were identified as a major contributor to larger polymeric proteins of carob germ proteins by Bengoechea et al (2008). These proteins are known to be a critical factor in wheat glutens functionality. The importance of these disulfide bonds have yet to be identified in carob germ proteins. For this reason, the first objective of this research was to identify the importance of disulfide bonded proteins in the formation of carob germ based gluten-free dough.

Microscopy work completed by Wang et al (2001) identified discrete fibrils of proteins formed by caroubin that may be responsible for protein network formation. It has been hypothesized that the presence of protein networks in bread systems has an anti staling effect. For gluten-free breads, in general, substantial research has been conducted to find non-wheat proteins that will form protein networks, especially via modification of proteins (Gujral et al 2003; Marco and Rosell 2008; Arendt et al 2008). Since carob germ proteins are known to form networks without modification, the second objective of this study was to determine the quality of carob germ-maize starch breads. Furthermore, since these protein fibrils may have an effect on staling, the staling performance of an optimized carob germ-maize starch bread was tested against a batter based sorghum bread and a wheat bread.

Materials and Methods:

Dough Formation and Protein Characterization

In order to determine the importance of disulfide bonds on carob germ flour-maize starch dough formation, dough was mixed by a Farinograph-E (Duisburg, Germany) at 63 rpms for 20 min. For the control dough, 40 g of a mix containing 30% carob germ flour and 70% corn starch was placed in the farinograph's 50 g mixing bowl. One minute of calibration was allowed and 32 g or 80% water on a flour basis was added and allowed to mix. The reduced dough was prepared as the non-reduced, but with the addition of 2% dithiothreitol (DTT) (w/v) to the water prior to mixing.

To observe the changes taking place in protein structure and function throughout mixing, dough samples were collected under similar conditions mentioned previously. However mixing time was extended to 60 min and no reducing agent was used. Samples of dough were collected during separate mixings at time 0, at peak mixing (~4.5 min), and at the end of the mixing curve (60 min). Dough samples were immediately frozen in a -80 °C freezer and lyophilized. Lyophilized dough was ground via mortar and pestle and stored at – 20 °C for subsequent SE-HPLC analysis.

Polymeric Protein Extraction:

Proteins were extracted (under un-reduced conditions) into "soluble" proteins (SP) which typically, in wheat, include all monomeric proteins and smaller polymeric proteins, and "insoluble" proteins (IP) which contain the largest polymeric proteins. A sequential extraction procedure was used so that SP were first extracted from 66.6 mg of lyophilized carob germ dough (20 mg carob germ flour) with 15 min of continuous vortexing in 1 mL of 50 mM sodium

phosphate, pH 7.0 buffer containing 1% SDS (w/v). After 5 min of centrifugation at 10,000 rpm the supernatant was collected and the extraction procedure was repeated. The supernatants from both SP extractions were pooled in a 1:1 ratio. IP were extracted from the remaining residue using sonication (10 watts for 30 sec in 1 mL of 50 mM sodium phosphate, pH 7.0 buffer containing 1% SDS (w/v). Two extractions were made and supernatants were centrifuged and pooled as described above.

SE-HPLC:

The supernatants collected from the polymeric protein extraction were analyzed via size exclusion (SE) HPLC using an Agilent 1100 HPLC system equipped with a Biosep-4000 column (Phenominx, Torrance, CA) and guard column using 50 mM sodium phosphate, pH 7.0 buffer containing 1% SDS (w/v) as a mobile phase (Bean and Lookhart 2001). Proteins were detected at 214 nm over a 30 min span with a flow rate of 1 mL/min and an injection volume of 20 µL. Column temperature was fixed at 40^{o}C.

Laser Scanning Confocal Microscopy (LSCM):

Carob germ protein – maize starch dough was mixed in a farinograph to peak mixing time as described previously except that a weakly alkaline solution of fluorescein 5(6)-isothiocyanate (FITC) was added to the water during mixing and pieces of dough removed and immediately fixed to slides. Samples were taken from control and reduced dough as described above. A Zeiss LSM 5 PASCAL (Laser Scanning Confocal Microscope) was used to image the dough sections as described in Schober et al (2007).

Baking Formulation and Procedure

Carob Bread:

Due to the high protein content of carob germ flour, starch must be used to dilute the protein to form workable dough. Through optimization in preliminary experiments with varying starch varieties, starch percentage, carob germ flour percentage, and water percentage, a final formulation was derived that allowed for the best specific volumes and crumb structure. The optimized bread formulation was similar to that described by Bienenstock et al 1935. Carob bread formulation was: 30% carob germ flour, 70 % corn starch, 1% sucrose, 1.75% NaCl, 2% active dry yeast, and 80% 30^{o}C DI H_20 on a flour basis. Yeast and 30^{o}C DI H_20 were added to a mixing bowl to allow for 5 min of yeast hydration. Corn starch, NaCl, sucrose, and carob germ flour were blended to a homogenous mixture and added to the hydrated yeast mix. The combined blend was mixed for 1 min with a 300 watt Kitchen Aid mixer on its lowest speed with a paddle attachment. The dough was scraped down and mixed for an additional 3 min on the 2nd lowest mixing speed with the paddle attachment. After the paddle mixing, a dough hook was attached and the dough was mixed for an additional 2 min on the 4th lowest mixing speed. Carob dough was made in 350 g batches (flour basis). The dough was then split into 184.75 g (100 g flour) pieces, hand kneaded for 1 min to remove large pockets of air and shaped by hand into an oblong cylinder like shape with rounded ends. The formed dough was placed into 8 cm x 14 cm x 5.5 cm bake pans oiled with non-stick cooking spray. The dough was proofed at 30^{o}C with a 87% relative humidity to a height of 6 cm. Bread was baked in an electric reel oven (National MFG, Lincoln, NE.) at 210^{o}C and removed after 30 min of baking. Bread was placed on

a wire rack and allowed to cool at room temperature (~24.5 ^{o}C) until post baking analysis and storage as described latter.

Batter Bread:

The sorghum based batter was made in 600 g batches (flour basis). Batter bread was made as described previously (Schober et al 2005, 2007). The batter formulation was: 70% sorghum flour, 30% potato starch, 1% skim milk powder, 1.75% NaCl, 1% sucrose, 2% HPMC K4M, 2% active dry yeast and 105% 30^{o}C DI H_2O. Batter preparation and mixing were modified slightly from previous methods in that yeast and 30^{o}C DI H_20 were added to a mixing bowl to allow for 5 min of yeast hydration. All other dry ingredients were blended to obtain a homogenous mixture and added to the hydrated yeast mixture. This adaptation was made due to problems with hydration and dispersion of the pelleted yeast homogenously throughout the batter system. The combined ingredients were mixed for 30 sec with a 300 watt Kitchen Aid mixer on its lowest speed with a paddle attachment and scraped down. The batter was mixed for an additional 2.5 min on the 2nd lowest mixing speed with the paddle attachment. Three oiled bake pans with the dimensions from above received 250 g of batter and were proofed at 30^{o}C with 87% relative humidity to a height of 4.5 cm. Bread was baked in an electric reel oven at 232^{o}C for 30 min. Bread was placed on a wire rack and allowed to cool at room temperature (~24.5 ^{o}C) until post baking analysis and storage as described later.

Wheat Bread:

Wheat breads were prepared in accordance to the optimized straight-dough bread-baking method 10-10B described by the American Association of Cereal Chemists (AACC). Bread

was placed on a wire rack and allowed to cool at room temperature (~24.5 ^{o}C) until post baking analysis and storage as described later.

Bread Analysis

All post baking analyses were taken 2 hrs post baking so equilibrium of moisture and temperature could be obtained (Schober et al 2007). Loaf weights were taken and loaf volumes were measured by rape seed displacement. Bread loafs were sliced at a thickness of 2.5 cm with a cutting jig that insures uniformity in slice surfaces and thickness between slices. Only slices from the center of the bread were taken for analysis to avoid irregularities between slices. Crumb data was collected and analyzed via a CALIBRE C-Cell (CCFRA Technology Ltd., Appleton, Warrington, United Kingdom). After C-Cell analysis, slice crust were removed to minimize its affect on subsequent texture profile analysis.

Statistical Design:

The experiment was designed with modifications from Moore et al (2004). A randomized block design was used to analyze the bread at 2, 50, and 122 hrs post baking. All slices of bread obtained from a given bread type were randomized and sealed in aluminized bags described latter with exception to slices being tested at two hrs post baking, which were analyzed immediately. For each testing time four slices were examined via TPA analysis. As a result a split plot design was achieved with three blocks containing three plots (carob, batter, wheat) that were subdivided into three split plots (hour 2, 50, 122). Analysis of variance was completed with Statistical Analysis Software (SAS). A level of significance was observed at $\alpha < 0.05$.

Storage:

Storage of bread for analysis was carried out as Schober et al (2007) with few modifications in that slices of bread with crust removed were stored in groups of four in aluminized polyester resin bags (Mylar, 10.0" x 14.0", Impak, Los Angeles, CA). Each bag was sealed with an oxygen scavenger with 2000 cm^3 capacity, Impak) to inhibit mold growth. Prior to packing and sealing each bag was sprayed lightly with 95% v/v ethanol for sterilization purposes.

TPA analysis:

Texture profile analysis (TPA) was preformed with a TA,XT.plus (Stable Micro Systems Ltd., Godalming, Surrey, United Kingdom). The analysis of bread slices during staling was done as Schober et al (2007) with modifications. A 25 mm diameter cylindrical plastic probe attached to a 30kg load cell was used for all TPA measurements. A pre-test, test, and post-test speed of 2.0 mm/sec was used with a trigger force of 5.0 g to compress the center of the crumb a distance of 40% of the slice thickness (2.5 cm). Rest time between cycles was 5.0 sec. Slices were analyzed 2 hrs, 50 hrs, and 122 hrs post baking. Cohesivness, hardness, and springiness datas were collected.

Results and Discussion:

Dough Formation

Functionality of disulfide bonds:

Initial experiments in mixographs did not display pronounced or usable mixing curves due to carobs lack of extensibility. For this reason, mixing experiments were conducted with a Farinograph due to its ability to produce a mixing curve with less severe mixing than the

mixograph (Rasper and Walker 2000). Mixing in a farinograph with and without a reducing agent (DTT) showed two distinct curves (Fig 1). Mixing with no reducing agent resulted in a curve that was sustained for approximately 13 min. In the presence of a reducing agent the curve diminished rapidly after full hydration of flour was achieved. After mixing, dough from the non-reduced trial contained some cohesive integrity while dough from the reduced form exhibited little to no observable cohesiveness and resembled a batter. This demonstrates the critical nature of disulfide bonds in the formation and maintenance of carob germ based dough.

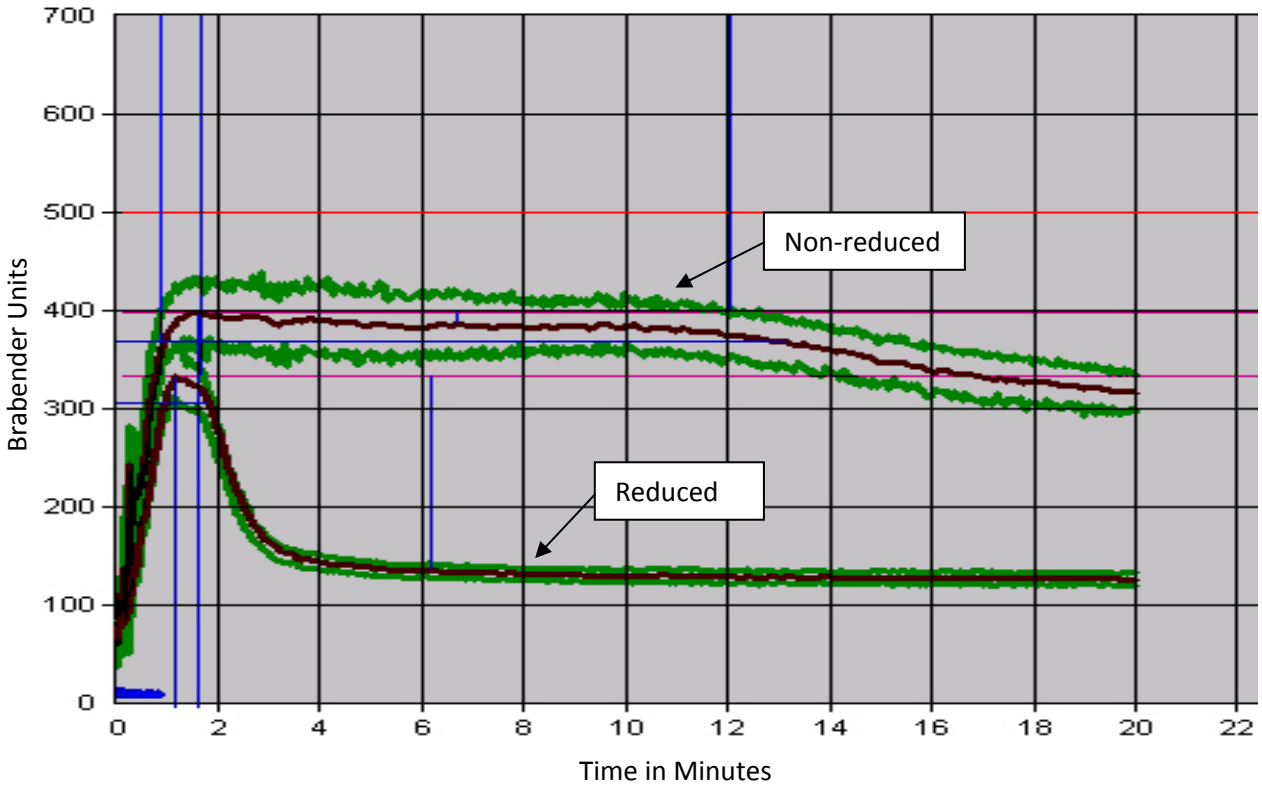

Figure 1: Farinograms of carob germ flour protein – maize starch dough under non-reducing conditions and reducing conditions.

LSCM:

The macro structure of the carob dough and the role of disulfide bonds on this structure can be observed in Fig 2. The reduced dough appeared to be a disorderly mass of starch and protein sheets while the non-reduced dough showed discrete particles with a sheet like appearance. Fibrils linking these discrete particles were easily observed with the 10X objective. These results show that disulfide bonds are necessary to form fibrils and a weak protein network in carob germ proteins. It is well accepted that protein network formation due to disulfide linked cysteins in wheat is one critical property that allows it to have a viscoelastic characteristic. Similar protein structures as described above have been observed in the gluten networks of wheat dough. However, wheat gluten appears to have larger and more numerous fibrils comprising the network. These results confirm the microscopy work completed by Wang et al (2001).

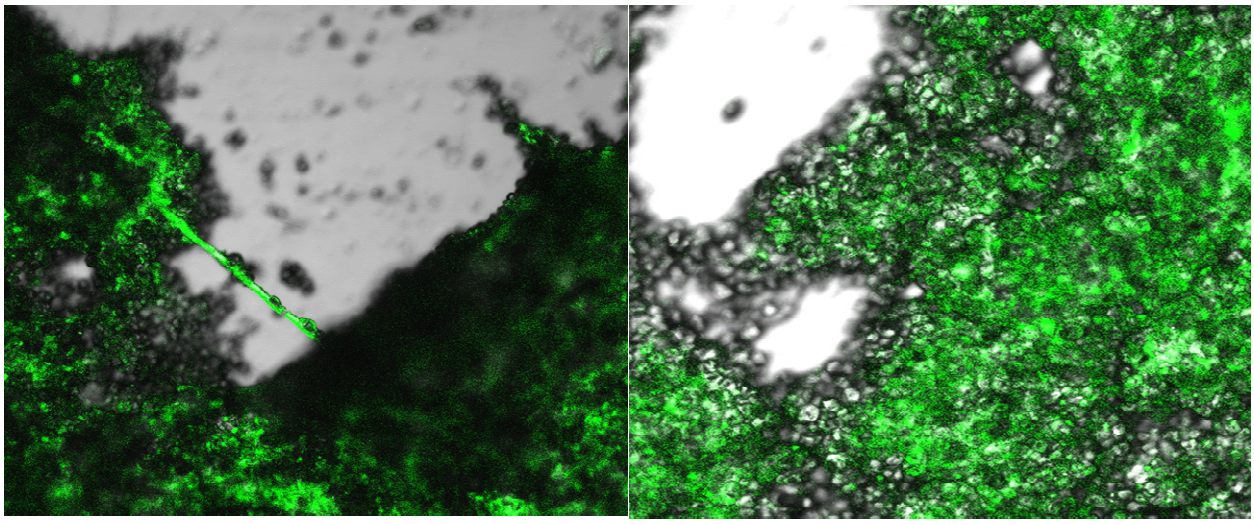

Figure 2. Laser scanning confocal microscopy image of carob germ dough non-reduced (left) and reduced (right). The white regions represent starch and the very dark regions represent protein.

Mixing (SE-HPLC):

While the comparison of IP in carob to that of wheat should be viewed with caution, particularly when working with limited samples, SE-HPLC data during mixing suggest that polymeric proteins in carob are important in the functionality of carob germ proteins similar to that in wheat. This was demonstrated by a shift in elution time for the primary peak of the IP fraction in the SE-HPLC chromatogram when dough samples were collected throughout mixing in a farinograph (Fig 4). This elution time shift shows that mixing for extended periods of time reduces the size and molecular weight of proteins by shear (Fig 4). There were no noticeable changes in the SP fraction throughout mixing. This showed that the changes in peak size and shape of the IP chromatograms were a result of protein size reduction without a change in protein content from one fraction to another (i.e. there was no increase or change in SP as the IP fraction was changing in size (Fig 3 & 4). As this reduction of protein size and molecular weight occurs the amount of force to rotate the farinograph paddles is reduced, meaning that dough strength is directly related to carob germ protein size and molecular weight. Likewise, it appears that these polymers are mainly disulfide linked and disruption of these polymers destroys the functionality of the carob germ proteins (Fig 1 & 2). Furthermore, the dough formed from carob was substantially weaker than that formed from gluten (i.e. it could not be mixed in a mixograph) which may be due to the low levels of the IP. Preliminary experiments in producing more polymers in carob via oxidation did result in shifts in the protein composition to result in more IP (data not shown). Oxidation within a dough system produced firmer dough than a non-oxidized dough that maintained mixing integrity for greater amounts of time in the farinograph. However, bread produced from oxidized dough (data not shown) showed no

significant improvements in specific volume or crumb structure and therefore was not pursued further.

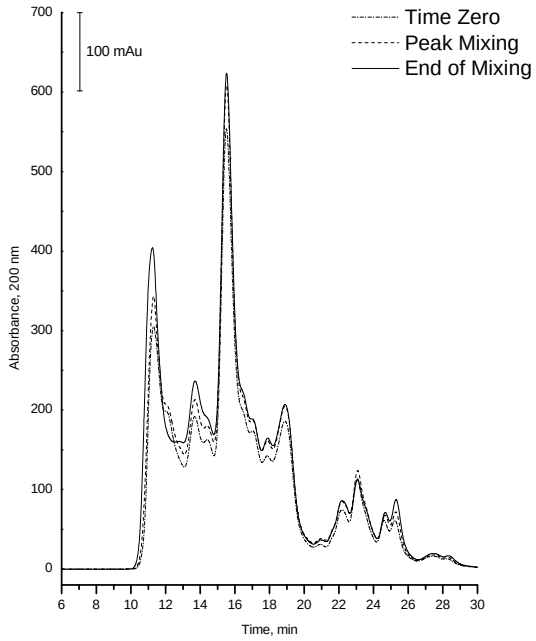

Figure 3. Size exclusion chromatograms of soluble proteins extracted from carob germ based dough taken from different stages of mixing in the farinogram.

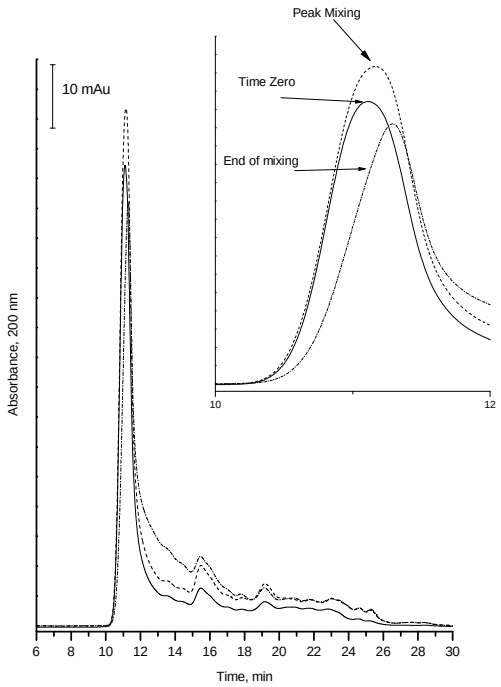

Figure 4. Size exclusion chromatograms of insoluble proteins extracted from carob germ based dough taken from different stages of mixing in the farinogram. Inset shows the region from 10-12 min enlarged.

Baking analysis

General Description:

Breads made from sorghum were brown-grey in color with a thin brittle crust. These loaves of bread showed little signs of rounding and generally had splits that transversed the top of the loaf in a lengthwise fashion. Overall batter based breads from sorghum had little resemblance to wheat bread or carob bread, but specific volumes of carob germ based breads and sorghum based batter breads were determined to be similar (Table 1). Unlike batter breads, the breads produced from carob germ flour bore a strong resemblance to wheat bread, with exception of the slight yellow tint and lower loaf volume. The crust was thicker than that

of wheat but was both flexible and resistant to tearing when manipulated by hand. Wheat breads were typical of bread made in accordance to method 10-10B of the AACC methods.

Crumb Structure:

Analysis via C-Cell showed that the internal structure of the three types of bread was quite different (Table 1). All factors were determined to be significantly different. The number of cells per cm^2 was the greatest for the carob germ based bread. However this is not indicative of lighter loafs and greater specific volumes as demonstrated in Table 1. The carob germ based bread had the smallest cell diameter and cell wall thickness close to that of wheat. For this reason the amount of cell wall per cell area was greatest for the carob germ based bread. Overall carob appeared to be the most uniform of the three bread types and contained cells of similar size and shape throughout a slice. This is different than the batter based bread and wheat breads which had a coarser crumb structure (Fig. 5).

Table 1. Post baking results of specific volume and C-Cell analysis of sorghum based batter bread, carob germ based bread, and wheat based bread*

Parameter	Sorghum Batter Bread	Carob Germ Bread	Wheat Bread
Specific volume (mL/g)	2.57 ± 0.03^{B}	2.47 ± 0.15^{B}	5.57 ± 0.36^{A}
Cell wall thickness (mm)	0.548 ± 0.02^{A}	0.425 ± 0.01^{C}	0.476 ± 0.01^{B}
Cell diameter (mm)	3.46 ± 0.30^{A}	1.48 ± 0.16^{C}	2.44 ± 0.17^{B}
Cells/cm^2	41.2 ± 3.2^{C}	82.2 ± 8.2^{A}	52.6 ± 2.3^{B}

*Values with a common upper case letter within the same parameter are not significantly different ($P<0.05$).

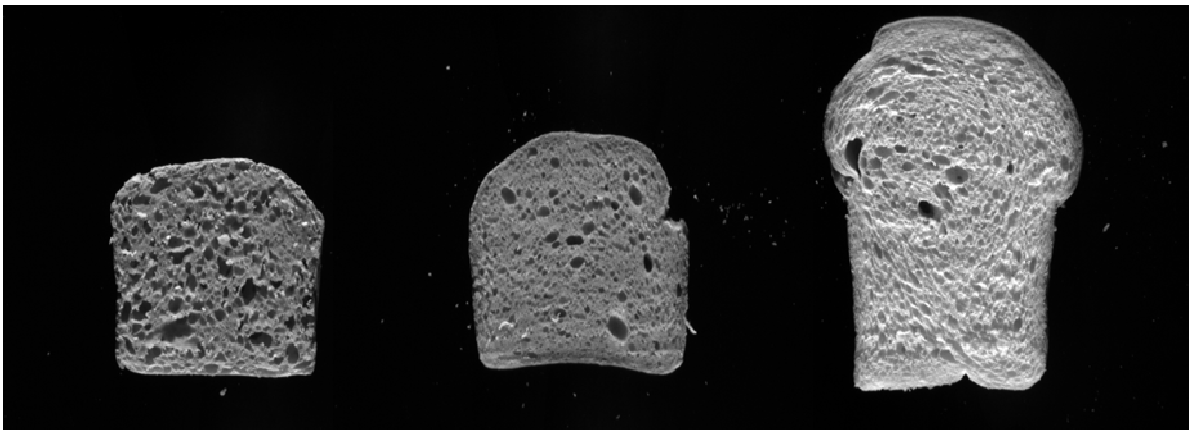

Figure 5. C-Cell images of sorghum batter bread (left), carob bread (middle), and wheat bread (right).

TPA:

The TPA results demonstrated that changes in the three types of bread over the three testing periods were significantly different (Fig 6, 7, & 8). The change in springiness over time was significantly less for carob based bread than for the batter based bread. Wheat was shown to perform significantly better than both carob and batter based bread with no significant changes occurring over time. Carob and batter based breads had significantly decreased springiness from time 2 to time 50. However, for both breads there was no significant change from time 50 to time 122 (Fig 6). Wheat again out performed carob and batter based breads by having higher values of cohesiveness. Changes occurred over all three reading times for both wheat and batter based breads. Carob based bread was shown to outperform batter based bread and only showed significant changes in cohesiveness from time 2 to time 50. There was no significant change in cohesiveness from times 50 to time 122 (Fig 7). Carob based bread had increased levels of hardness when compared to both wheat and batter based breads. This confirms work done by Bienenstock et al (1935) in that they described breads made from carob

germ meal as having a strong resemblance to European rye bread that was more dense and firm than a typical wheat bread. Wheat bread showed much less hardness and no changes over time while both batter and carob based breads displayed significant changes from all three data collection times (Fig 8). Fracturing of crumb did occur only in batter and carob based breads. This fracturing was random and occurred at both testing times 50 and 122 (data not shown). Fracturing in the two types of bread displayed similar TPA peaks. However, when fractured, batter based bread would shatter resulting in a slice broken into two or more large pieces. Fracturing in carob based bread resulted in slight separation in crumb producing a small indentation where the probe compressed the bread.

Although hardness was much greater in carob bread than batter based bread, the changes which occurred over time show that the batter based bread staled greater than that of carob based bread. Both non gluten types of bread were greatly outperformed by wheat, which showed little changes over time when compared to its gluten-free counterparts. It has been hypothesized that protein network formation in wheat helps to prevent staling in bread. There is a plethora of different systems which function together in a bread to determine its properties. Although all three bread types contained very different functional ingredients, this research does seem to support the hypothesis that protein network formation can help in preventing staling in bread. This is evident by the results discussed above. If this is true, one reason why carob might be drastically outperformed by wheat bread is that it has far less high molecular weight polymeric proteins than wheat. These proteins have been shown to play a major role in dough formation and functionality.

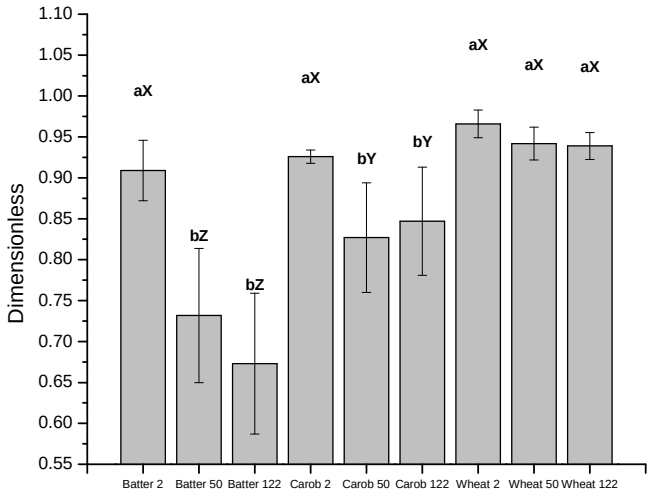

Fig 6. Springiness data for batter, carob, and wheat breads at 2, 50, and 122 hours post baking. Values with a common lower case letter within the same bread type are not significantly different. Values for a given storage time with a common upper case letter are not significantly different (P<0.05).

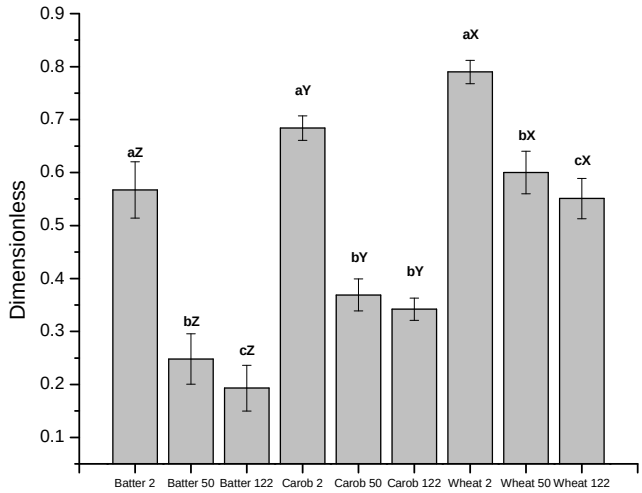

Figure 7. Cohesiveness data for batter, carob, and wheat breads at 2, 50, and 122 hours post baking. Values with a common lower case letter within the same bread type are not significantly different. Values for a given storage time with a common upper case letter are not significantly different (P<0.05).

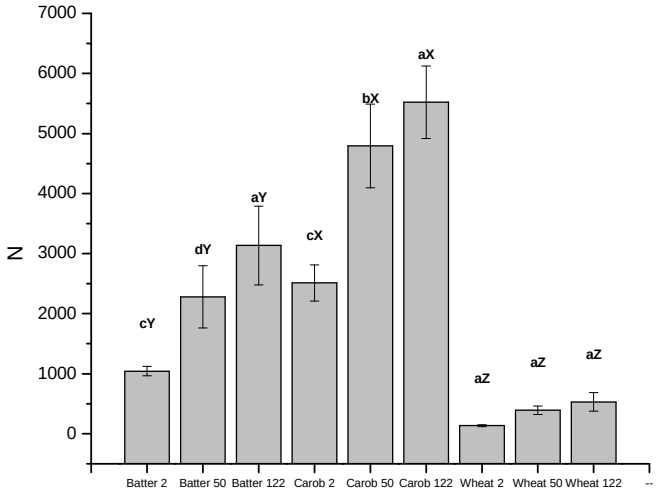

Figure 8. Hardness data for batter, carob, and wheat breads at 2, 50, and 122 hours post baking. Values with a common lower case letter within the same bread type are not significantly different. Values for a given storage time with a common upper case letter are not significantly different ($P<0.05$).

Conclusion:

Carob germ proteins are capable of forming protein networks to produce a wheat-like dough. These protein networks are highly dependent on larger molecular weight proteins dependent on disulfide bonding. Like gluten, the disulfide bonds of these proteins were shown to break down in the presence of a reducing agent or shear in the form of mixing over time. Baked dough made from carob germ protein and maize starch is capable of producing bread similar in appearance to a dense wheat bread. However, this bread is denser than both wheat and batter based sorghum bread. The changes that occur over a period of 122 hrs post baking demonstrated that carob based bread stales less than the batter based bread. Carob germ based bread shows potential for application within the gluten-free market by improving dough

handling and bread staling. However, more research is needed in the characterization of dough rheology, research and development, and other non disulfide chemical interactions taking place during dough formation. More research is also needed for sensory analysis and shelf life testing to help determine its feasibility of entering the gluten-free market.

Literature Cited:

Anonymous. (2006). Gluten-free market set to explode. *Gourmet Retailer.* September 13-30.

Arendt, E. K., Morrissey, A., Moore, M. M., Dal Bello, F. (2008). Gluten-Free Breads. *Gluten-Free Cereal Products and Beverages.* Elsevier Inc. New York. 289-311.

Bean S., Lookhart G. (2001). Factors influencing the characterization of gluten proteins by size-exclusion chromatography and multiangle laser light scattering (SEC-MALLS), *Cereal Chemistry* 78, 608–618.

Bengoechea, C., Romero, A., Villanueva, A., Moreno, G., Alaiz, M., Millan, F., Guerro, A., and

Puppo, M.C. (2008). Composition and structure of carob (*Ceratonia siliqua* L.) germ proteins. *Food chemistry*, 107, 675-683.

Bienenstock, M., Csaski, L., Pless, J., Sagi, A., and Sagi, E. (1935). Manufacture of Mill Products for alimentary purposes and of paste foods and bake products from such milled products. U.S. patent 2,025,705.

Bogue, J., and Sorenson, D. (2008). The marketing of gluten-free cereal products. *Gluten-Free Cereal Products and Beverages.* Elsevier Inc. New York. 393-412.

Fasano, A., Catassi, C., (2001). Current approaches to diagnosis and treatment of celiac disease: an evolving spectrum. *Gastroenterology* 120, 636-651.

Feillet, P., and Roulland, T. M. (1998). Caroubin: A gluten-like protein isolate from carob bean germ. *Cereal Chemistry,* 75, 488-492.

Gujrul, H. S., Guardiola, I., Carbonell, J. V., Rosell, C. M. (2003). Effect of Cyclodextrinase on Dough Rheology and Bread Quality from Rice Flour. *Journal of Agricultural and Food Chemistry*, 51, 3814-3818.

Marco, C., Rosell, C. M. (2008). Breadmaking performance of protein enriched, gluten-free breads. European Food Research and Technology, 227, 1205-1213.

Moore, M. M., Schober, T. J., Dockery, P., Arendt, E. K. (2004). Textural Comparisons of Gluten-Free and Wheat-Based Doughs, Batters and Breads. *Cereal Chemistry*, 81, 567-575.

Plaut, M., Zelcbuch, B., and Guggenhem, K. (1953). Nutritive and Baking Properties of Carob Germ Flour. *Bulletin of the Research Council of Isreal*, 3, 129-131.

Rasper, V. E., and Walker, C. E., (2000). Quality Evaluation of Cereals and Cereal Products. *Handbook of Cereal Science and Technology.* Marcel Dekker, Inc. New York. 505 – 537.

Schober, T. J., Messerschmidt, M., Bean, S. R., Park, S. H., Arendt, E. K. (2005). Gluten-Free Bread from Sorghum: Quality Differences Among Hybrids. *Cereal Chemistry,* 82, 394-404.

Schober, T., Bean, S., and Boyle, D. (2007). Gluten-Free Sorghum Bread Improved by Sourdough Fermentation: Biochemical, Rheological, and Microstructural Background. *Journal of Agriculture and Food Chemistry.* 55, 5137-5246.

Schober, T., Bean, S., Boyle, D., Park, A. (2008). Improved Viscoelastic Zein-Starch Doughs for Leavened Gluten-Free Breads: Their Rheaology and Microstructure. Journal of Cereal Science. 48, 755-767.

Van Heel, D. A., West, J. (2005). Recent advances in coeliac disease. *Gut.* 55 (7), 1037-1046.

Wang, Y., Belton, S. B., Bridon, H., Garanger, E., Wellner, N., Parker, M. L., Grant, A., Feillet, P., and Noel, T., (2001). Physicochemical Studies of Caroubin: A gluten like Protein. *Journal of Agriculture and Food Chemistry*, 49, 3414-3419.

Chapter 4:
Recommended Future Work

Future Work

Due to limited amounts of time and resources only a small amount of research could be completed on carob germ proteins. Because carob germ proteins are capable of forming wheat like dough that can be made into yeast leavened bread there needs to be more attention geared towards it. For this reason, more research is needed to determine other chemical and physical properties of caroubin.

While a great deal of characterization was completed within this study, it is only a miniscule amount that could be potentially done. During the formation of wheat dough it is known that there are several non-covalent interactions that occur during dough formation. These interactions can and will affect dough functionality and bread quality. One could hypothesize that this would also be true for caroubin due to the findings of this research in that caroubin, like wheat gluten, requires large molecular weight polymeric proteins that are dependent on disulfide bonding to form a dough. Carob proteins may also need to be modified to function more like wheat including increasing Mw, but also changing hydrophobicity or charge density to be more like wheat gluten polymeric proteins. This has the potential to aid in dough functionality and bread improvement.

No research could be found that identified the rheological properties of carob germ-starch dough. This research should be completed so that the rheological properties of carob can be identified. These values have the potential to aid in product development by identifying certain desirable dough traits.

Finally, more research is needed in research and development. Preliminary research showed a great potential for carob germ flour in the use in gluten-free flat breads, tortillas, and

noodles. To optimize these products quality, additional functional ingredients will need to be added. These ingredients will need to be identified and optimized within a given system.