ENZYMATIC HYDROLYSIS OF WHOLE GRAIN AMARANTH

by

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B.S., University of Wisconsin-Stout, 2008

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Food Science

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2014

Approved by:
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Abstract

There is evolving evidence that intake of whole grains protects against development of chronic diseases. Increasing the appeal of whole grain products proves difficult as they often have poor organoleptic properties attributed to the high water holding capacity, viscosity, and insolubility of components of the bran. Amaranth is a promising grain that is naturally gluten-free and has received much attention in recent years because of its excellent nutritional profile. Studies have shown encouraging approaches to modify the molecular makeup of amaranth by enzymatic hydrolysis. This approach suggests an increase in processability and incorporation of whole grain amaranth into processed foods by ameliorating the challenges inherent in the use of whole grain flours, thereby expanding consumer acceptance and intake of whole grains. This research investigates the effects of a two-level factorial design on the enzymatic hydrolysis of whole grain amaranth with enzymes alpha-amylase, cellulase, xylanase, and protease maintaining constant pH of 6 and temperature, 50°C. The main effects show decreased viscosity and water holding capacity, and increased solubility of whole grain amaranth. The most notable findings show enzymatic treatment decreases viscosity, with alpha-amylase having the most significant impact ($P < 0.0001$) 21,363-59± 244 cPs. Protease was eliminated from further testing as bitterness was generated with its hydrolysate. To solubilize insoluble components, a second two-level factorial design was employed analyzing pH (4.5 and 7.5) and temperature (50°C-70°C) with the same enzyme dosage as the first design. The main effects of the second design revealed alkaline conditions significantly increase soluble fiber ($P < 0.0001$) 3.01-5.05% ± 0.3%. Subsequent investigation proposes a response surface design with alpha-amylase in optimizing the effects of reaction time and alkaline conditions.

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Acknowledgements

I am fortunate to have many extremely knowledgeable individuals share their expertise and guide me throughout this research. I have gained many valuable insights while doing this study. This could not have occurred without the support of those individuals.

First, I thank Dr. Jon Faubion for his guidance as my major professor and for his continued assistance throughout this research study. His supportive suggestions were invaluable to me.

I also I owe much gratitude to Dr. Laura Hansen, who has served as my mentor through my Master's studies at KSU along with being my mentor at General Mills. Laura has helped me direct the research with an academic focus, while influencing my research to be relevant in the real-world food industry.

My family has continually supported me, thanks to them for encouraging me to do the best I can. I love all of them very much. I particularly want to thank my mother, who has personally experienced the demands of full-time work and school. She inspired me to continue my education.

Also, I would like to thank my husband, Mike, who has been by my side through this entire process. He has advocated for me every day for the past several years and has given me support whenever I needed it. I owe much of my success to him.

Finally, I would like to acknowledge General Mills, my past and current managers/mentors, and my peer support system. I have been provided many opportunities to learn at work while obtaining this degree. General Mills has supported my career development while advancing my academic knowledge. I sincerely thank you!

Chapter 1 - Literature Review

1.1 Introduction

There is evolving evidence that intake of whole grains protects against development of chronic diseases. The consumption of bran and germ affords increased health benefits because they both contain nutrients not present in refined grains. Improving the appeal of whole grain products is complicated as these products often have poor organoleptic properties attributed to the high water holding capacity, high viscosity, and insolubility of components of the bran (Fardet, 2010).

Amaranth has received much attention in recent years as it is naturally gluten-free and it has an excellent nutritional profile that includes good-quality protein, dietary fiber, and lipids rich in unsaturated fats. Little research has been done on the development of a novel ingredient from amaranth by enzymatic hydrolysis; however, incorporation of whole grain in processed foods can be improved by modification of the grain components themselves (i.e., decreasing viscosity, increasing solubility, and/or improving the grain's sensory qualities) (Roger, Schaffer-Lequart, & Wavreille, 2012).

This thesis explores the modification of the molecular makeup of amaranth by use of enzymes to catalyze reactions of hydrolysis in the whole grain. A compositional evaluation will assess the nutritional, organoleptic, and physical characteristics of the innovative ingredient produced by enzymatic hydrolysis of amaranth (amaranth hydrolysate). The amaranth hydrolysate has potential to improve the process-ability and potential for incorporating whole grain amaranth into processed foods. Thereby, it may increase consumer acceptance and intake of whole grains and. This modification of amaranth can potentially increase the bioavailability of nutrients found in whole grains. Also, comparative effects of hydrolysis by the enzymes alpha-amylase, cellulase, xylanase, and protease on whole grain amaranth are examined.

Finally, this study examines the combination of enzymes and their potential to provide synergistic, unique effects on whole grain amaranth. By decreasing whole grain amaranth's viscosity, it is believed that its water holding capacity will decrease; therefore, its components may be solubilized, releasing favorable phytochemicals with potential beneficial health effects.

1.2 Research Objectives

In order to improve the ability of incorporating whole grain amaranth in processed foods, these objectives were created.

1. Determine main effects and potential synergies in the degradation of the grain.
2. Determine an optimized method to improve processing of the grain.

The following chapters describe the background, experimental approach, results, and discussion of the research employed to complete these objectives.

1.3 Whole Grains and Health

According to the American Association of Cereal Chemists (AACC, 1999), "A whole grain consists of the intact, ground, cracked, or flaked caryopsis, whose principal anatomical components—the starchy endosperm, germ, and bran—are present in the same relative proportions as they exist in the intact caryopsis." Several studies have shown wide-ranging evidence that the intake of whole grain products is associated with various health benefits. Some of these studies report a consistent inverse association between dietary whole grain intake and the incidence of cardiovascular disease (Mellen, Walsh, & Herrington, 2008) and type-2 diabetes (Fung et al., 2002). Most of the available data also shows the ability of whole grains to prevent body weight gain and obesity (Koh-Banerjee et al., 2004) as well as some types of cancers (Jacobs, Marquart, Slavin, & Kushi, 1998). Another study also found an association between the consumption of whole grains and decreased inflammatory markers (Lefevre & Jonnalagadda, 2012).

In addition, whole grains contain phytochemicals which help to fight oxidative stress in the body by assisting in the maintenance of a balance between oxidants and antioxidants. Oxidative damage increases the threat of degenerative diseases such as cancer and cardiovascular diseases (Adom & Liu, 2002).

Products made with refined flour (flour without the bran and germ) contain fewer biologically active compounds then do whole grain products and they are often supplemented or fortified. However, fortified foods have been found to possess weaker health-promoting activities compared to components found naturally in grain (Barlomiej, Rosicka-Kacsmarek, & Nebesny, 2012). Many of the epidemiological studies showing the health benefits of consuming whole grains have shown an independent association only for the bran component, demonstrating that bran is one of the key factors in determining whole grains' health benefits (Adom & Liu, 2002). That said, the use of whole grain is limited in food applications because of challenges in sensory properties of the bran fraction. The consumption of whole grains leads to a greater sense of satiety (Sjoberg & Marquart, 2012); however, incorporation of whole grains in food products leads to negative taste and texture issues (Vitaglione, Napolitano, Fogliano, 2008).

1.4 Gluten-Free Products

There has recently been an increase in the awareness of celiac disease (CD), an autoimmune disorder exhibited in predisposed individuals with a sensitivity triggered by ingesting gluten, a protein prevalent in wheat. The disease can present at any age and when gluten is ingested it affects the intestinal mucosa by causing constant distresses, resulting in nutrient malabsorption. However, the mucosa can recover (at least partially) when gluten is eliminated from the diet (Arentz-Hansen, et al., 2000).

1.4.1 Market research reveals an increased demand for gluten-free foods

CD patients and consumers of gluten-free products have limited food choices since many food products are based on wheat. The use of alternative gluten-free grains in foods has been a growing interest for this reason; however, using wheat in grain-based foods is customary and dominantly used in the United States.

CD is prevalent in the United States, affecting 0.71% or 1 in 141 (Rubio-Tapia, Ludvigsson, Brantner, Murray, & Everhart, 2012). It has also been estimated that 15% of American consumers say they have eliminated gluten from their diet (even though only a small amount have CD) and nearly half (47%) of all consumers believe gluten is bad for you (Mintel, 2013). Despite the broadened appeal and consumer interest in gluten-free products, their creation is challenging and their quality has not kept up with the increasing demand. Many commercial gluten-free products on the market today are nutritionally inadequate and lack the superior flavors and texture that wheat products deliver (Engleson & Atwell, 2008).

Gluten replacement in foods is difficult for several reasons. Many gluten-free bakery products are nutritionally inferior as they are made predominantly from starches and are lacking minerals, vitamins, fiber, and protein (Berti, Riso, Monti, & Porrini, 2004; Engleson & Atwell, 2008). Gluten is a structure-building protein, and eliminating it in baked goods is a complicated technological task; so, the removal of gluten from baked products and pasta creates foods with very poor shelf-life and sensory qualities (Alvarez-Jubete, Arendt, & Gallagher, 2010). While research is ongoing to address some of these concerns, the demand still prevails to create gluten-free ingredients that are rich in nutrients. Amaranth hydrolysate may further expand the use of whole grain amaranth by improving the adverse sensory qualities, processing, and technological issues associated with manufacturing gluten-free foods.

1.5 Amaranth

Amaranth is an emerging gluten-free grain, or pseudocereal, that is highly nutritious and has numerous health benefits for individuals with unique diets (Venskutonis & Kraujalis, 2013). Pseudocereals are not true cereals, but are dicotyledous plants, unlike most cereals that are monocotyledonous. Except for their different taxonomic classification, they have composition and functions similar to most cereals (Alvarez-Jubete et al., 2010). Although the use of amaranth has gathered much interest recently, it is has been consumed for centuries. The grain is native to the New World and was relied on by Aztecs as a dietary staple. It was believed to have magical properties, imparting strength and was used in religious ceremonies and gatherings. Consumption of amaranth declined after the arrival of the Spanish; some speculate, for reasons that with the collapse of Indian cultures following the Spanish conquest, the Spanish conquistadors prohibited growing the grain and suppressed the Aztec culture, making amaranth consumption scarce (Pedersen, Kalinowski, & Eggum, 1987).

Although there was a decline in amaranth consumption in the Aztec region, its adoption and cultivation in other parts of the world increased. The grain grows in subtropical, temperate, and tropical climate zones (Rastogi & Shukla, 2013) and is cultivated in small areas of Mexico, the Himalayan foothills of India and Nepal, and the Andean highlands (Ruskin, 1984). Most recently amaranth genotypes have been adapted to grow in northern USA and eastern Canada (Mustafa, Seguin, & Gélinas, 2011).

Amaranth has great potential for use in the food industry as it has dietary quality that is higher in comparison to common cereals (Burisova, Tomaskova, Sasinkova, & Ebringerova, 2001). Amaranth is also gluten-free; therefore, patients suffering from celiac disease can eat it without suffering from side-effects associated with the disease. Research has suggested that this grain can be used in the development of food products that are non-allergenic and that have potential to fight allergies (Hibi, Hachimura, Hasizume, Obata, & Kaminogawa, 2003). Components have been found in the grain that can prevent the cascade of allergenicity for specific antigens (Hibi et al., 2003). Health benefits linked with consuming the grain include anti-allergic and antioxidant activities, lowering plasma cholesterol levels, exerting an anti-tumor activity, stimulating the immune system, decreasing blood glucose levels, and improving conditions of hypertension and anemia (Caselato-Sousa & Amaya-Farfán, 2012).

1.5.1 Protein

The quality of protein in amaranth has prompted a renewed interest in amaranth grain (Pederson, Bach Knudsen, & Eggum, 1990). The grain's essential amino acids are more balanced compared to common grains wheat, rye, oats, and barley (Correa, Jokl, & Carlsson, 1986).

Amaranth has high protein content at about 14% protein (Table 1). Amaranth protein has nutritional quality close to that of casein, comprised of high levels of lysine and methionine totaling about 0.78% and 0.226% of the total protein content, respectively (Mendoza & Bressani, 1987). Sixty-five percent of the protein is concentrated in the embryo (Figure 1).

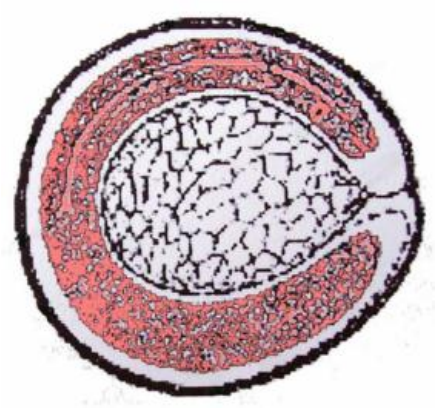

Figure 1: The internal structure of amaranth grain. Protein is contained in the stained embryo (Vaskova, 2008).

1.5.2 Lipids

Whole grain amaranth contains about 6% fat (Rodas & Bressani, 2009) from which amaranth oil is extracted. Found in the germ, the oil is rich in unsaturated fatty acids, containing mainly linoleic acid (Betschart, Irving, Sheperd, & Saunders, 1981; Lorenz & Hwang, 1985). Although linoleic acid is necessary for our existence, humans are unable to produce it.

The phytosterol content in whole grain amaranth has been shown to be much higher than that found in other plants (vegetables, nuts, and vegetable oils) (Marcone, Kakuda, & Yada, 2004). Consumption of phytosterols or plant sterols has been shown to reduce plasma cholesterol levels. Amaranth oil is also a significant source of

squalene at 2.4-8% of the oil, Table 1. Squalene has antioxidant properties and can strengthen the immune system (Vaskova & Kolomaznik, 2014).

1.5.3 Carbohydrate

About seventy-two percent of amaranth is carbohydrates, comprised of 63.1% starch. Starch plays an important role in technological processing, bioavailability and physiochemical and rheological properties in grains. Amaranth starch is comprised of very small granules, measuring 1 to 3 mm in diameter, contributing to the grain's distinctive gel characteristics and high swelling power (Hoover, Sinnott, Perera, John, & Canada, 1998). These very small granules are round or polygonal in shape, are found in the endosperm, and are comprised of roughly 3.9-5.7% amylose and 96.1-94.3% amylopectin (Tomita, Sugimoto, Sakamoto, & Fuwa, 1981). Amylose levels are much higher in other grains: wheat (27.3%), corn (26%), and oat (22.7%) (Guzman-Maldonado & Paredes-Lopez, 1993). As compared to the starch in wheat and corn, amaranth starch has lower solubility and higher water absorbance and swelling power (Rastogi & Shukla, 2013).

The amaranth that was used in this thesis research contained 13.8% dietary fiber comprised of 11.1% insoluble and 2.7% soluble fiber, which is concentrated in the bran (Table 1). Dietary fiber is defined as "Carbohydrate polymers with 3 or more monomeric units which are not hydrolyzed by the endogenous enzymes in the small intestine" (CODEX Alimentarius (CODEX), 2010). This includes non-starch polysaccharides, the remains of plant cell walls resistant to human digestion comprised of: cellulose, hemicelluloses, pectin, arabinoxylan, beta-glucan, glucomannans, plant gums and hydrocolloids. Dietary fiber is classified into two categories according to its solubility in water: soluble and insoluble fibers.

1.5.4 Macro nutrients, vitamins, minerals and antioxidants

About 66% of minerals in amaranth remain concentrated in the bran and germ fractions (Saunders & Becker, 1984). Amaranth contains adequate levels of micronutrients such as vitamins and minerals and significant amounts of bioactive components such as polyphenols, saponins, squalene, and phytosterols (Alvarez-Jubete et al., 2010). The grain contains high contents of calcium, zinc, iron, magnesium and potassium (Rastogi & Shukla, 2013).

Saponins, the strongly bitter tasting compound mainly found on the surface of quinoa grain, are present at trace quantities not hazardous to consume in some amaranth species analyzed (Banerji & Chakravarti, 1973; Banerji, 1980; Junkuszew, Olezek, Jurzysta, Piancente, & Pizza, 1998).

Table 1: Nutritional content of whole grain amaranth (USDA, 2014).

Nutrient	Value per 100 g	Unit
Water[a]	6.89	g
Energy[a]	390.00	kcal
Protein[a]	14.40	g
Total lipid (fat)[a]	5.82	g
Ash[a]	2.88	g
Carbohydrate, by difference[a]	70.00	g
Fiber, total dietary[a]	13.80	g
Insoluble Fiber[a]	11.10	g
Soluble Fiber[a]	2.70	g
Sugars, total[a]	1.20	g
Starch[a]	63.10	g
Calcium, Ca	159.00	mg
Iron, Fe	7.61	mg
Magnesium, Mg	248.00	mg
Phosphorus, P	557.00	mg
Potassium, K	508.00	mg
Zinc, Zn	2.87	mg
Manganese, Mn	3.33	mg
Vitamin C	4.20	mg
Thiamin	0.12	mg
Riboflavin	0.20	mg
Niacin	0.92	mg
Folate	82.00	μg
Vitamin E	1.19	mg
Vitamin B6	0.59	mg
Fatty Acids, total saturated	1.46	g
Fatty Acids, total monounsaturated	1.69	g
Fatty Acids, total poly unsaturated	2.78	g
Phytosterols	24.00	mg
Squalene in amaranth oil	2.40-8.00	%

[a] Determined analytically from Conagra whole grain amaranth flour in this study

1.6 Amaranth Hydrolysis by Enzymes

Increasing the appeal of products made with whole grains is difficult as those products possess many complexities in their formulation and can have poor organoleptic properties (Sjoberg & Marquart, 2012). Enzymatic hydrolysis of amaranth may potentially allow for a wider application of the grain in processed food applications.

1.6.1 Water absorption and viscosity

Native starch granules are highly resistant to hydrolysis by enzymes; however, when starch is gelatinized it becomes highly susceptible to degradation by amylases. Gelatinization causes starch to solubilize, imbibe water, and swell. This results in a significant increase in viscosity, creating technical difficulties in processing and decreases ability to incorporate high amounts of the grain into processed foods. Converting starch with alpha- amylase enzymatic hydrolysis to low molecular weight polymers will allow food applications requiring high whole grain content without excessive thickening (Dokic, Jakovljevic, & Dokic, 2004).

Protein, pentosans, starch (Morgan, Dexter, & Preston, 2000) and fiber (Mudgil & Barak, 2013) have been shown to be involved in water absorption. The amount of water absorbed is based on the swelling behavior of polymer networks in the presence of water (Kweon et al., 2009). Bread baking relies on water absorption capacity in influencing texture and yield (Morgan et al., 2000). Although this research did not explore use of the product in food formulations, it examined how water absorption and viscosity had a significant effect in determining how the product may respond in processed foods.

1.7 Enzymatic hydrolysis

Enzymes are proteins and biological catalysts. They are highly effective in catalyzing chemical reactions by bringing substrates together in optimal orientations to break chemical bonds in a very selective manner (Whitehurst & van Oort, 2010). In this thesis, the substrate is amaranth. Enzymes used binds to the substrate by the active site, a cleft or pocket in the enzyme, and breaks very specific chemical bonds to create the product. The active site contains residues, called catalytic groups, which directly participate in the making and breaking of bonds during the reaction. Enzymes bind substrates by weak non-covalent attractions called ES complexes comprised of electrostatic bonds, hydrogen bonds, van der Waals forces, and hydrophobic interactions (Damodaran, Parkin, & Fennema, 2008).

Chemical kinetics allows the interpretation of kinetic processes by studying the reaction rates for chemical conversion of substrates into products. For many enzymes, the rate of catalysis varies with the concentration of the substrate. By use of the Michaelis-Menten Model, 1913, the kinetic properties of many enzymes can be calculated to form their products (Harris & Keshwani, 2009).

Although the kinetic properties of the enzymatic reaction can theoretically be calculated, the reaction is dependent on conditions, leaving much area for interpretation (Harris & Keshwani, 2009). There are complexities in expected products because of interferences such as the substrate (may contain molecules that inhibit the enzyme), pH, temperature, and ionic strength. Inhibition is the effect of specific molecules present in the substrate that compete for the active site of the enzyme and prevent the substrate from binding to the same active site, reducing the effect the enzyme has in the reaction.

1.8 Effects of Enzymatic Hydrolysis

Amylolytic enzymes (amylases) are enzymes that hydrolyze the glycosidic bonds of starch. Xylanases break down arabinoxylanoses contained in plant cell walls, catalyzing the hydrolysis of arabinoxylan, the fibrous portion of cereals. Cellulases hydrolyze cellulosic materials, the major component of plant materials, typically to produce fermentable sugars for use in various biotechnological processes and in the paper and textile industry. Proteases hydrolyze peptide bonds in proteins releasing smaller peptides and free amino acids (Whitehurst & van Oort, 2010).

Starch, fiber, and protein all greatly contribute to the high viscosity of whole grains in solution. The use of amaranth hydrolysate materials have been studied to increase the concentration of protein in products made with them (Vaskova & Kolomaznik, 2014). These materials have converted grain components into less viscous forms by use of enzymes.

1.8.1 Alpha-Amylase

Amylases have a number of applications in the pharmaceutical, fermentation, and food industries. Yeasts, bacteria, plants, animals, and fungi are the most common sources of naturally occurring amylases capable of converting starch into a large variety of specific products. The alpha-amylase enzyme family has about 30 enzymes that differ somewhat with respect to what they produce.

Amaranth kernels contain mostly starch, which can be hydrolyzed via liquefaction into maltodextrins, by use of alpha-amylase. Provided the starch has been gelatinized, liquefaction functions to decrease the viscosity of a slurry in which the starch has been hydrolyzed so that large amounts of water are not necessary to make it pumpable (Roger et al., 2012).

Alpha-amylases are endo-amylases, creating low molecular weight oligosaccharides and alpha-limit dextrins, or maltodextrins, by randomly cleaving alpha, 1-4 glycosidic bonds present in the inner part (endo-) of the amylose or amylopectin chain (Figure 2). These hydrolysis products are termed maltodextrin, with a Dextrose Equivalence (DE) lower than 20 (DE 3-20).

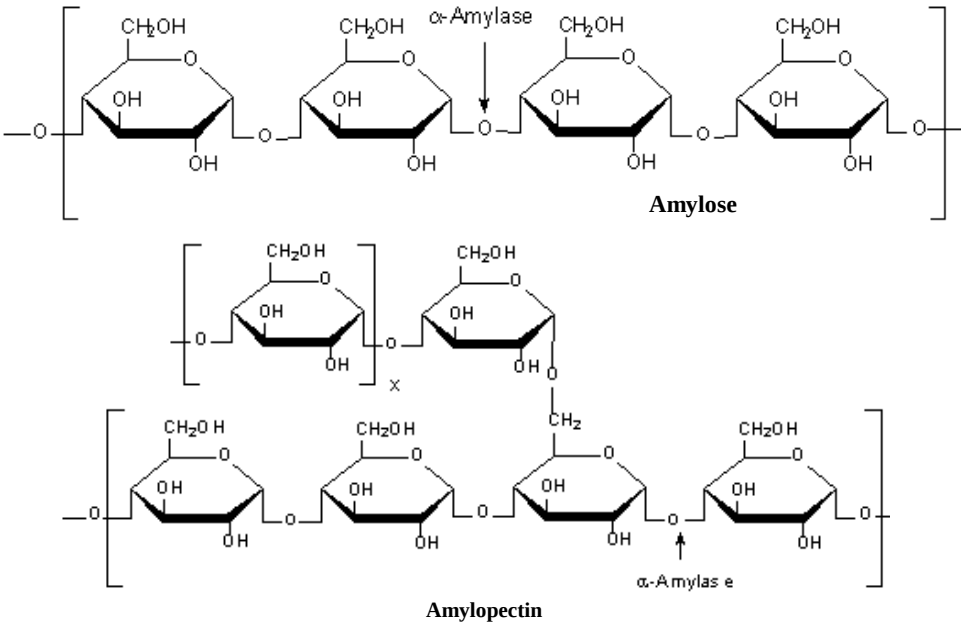

Figure 2: Alpha-amylase specificity on starch (Larner, Boyer, Lardy, & Myrback, 1960).

Dextrose equivalent (DE) expresses the percentage of glycosidic bonds that are hydrolyzed. Figure 3 shows the action of alpha-amylase on starch, which increases the DE but decreases the viscosity of the starch slurry. One monomer with reducing end is freed with each hydrolytic cleavage of an alpha 1-4 bond. This reaction produces an increase in the percentage of bonds broken, thus an increase in the measurement of DE.

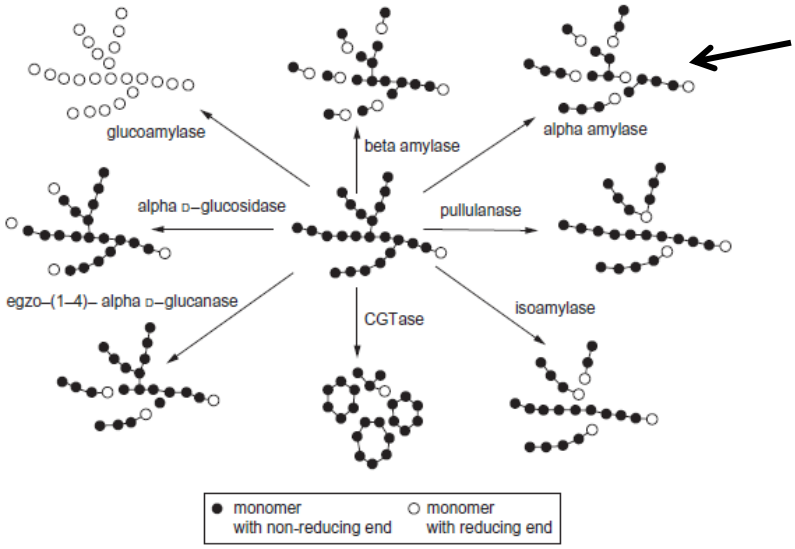

Figure 3: Pattern of hydrolysis of hydrolases digesting starch (Tomasik & Horton, 2012).

Whole grains, including amaranth, can undergo enzymatic hydrolysis by using alpha-amylase and still keep the dietary fibers intact, as described by Roger et al., 2012. It is not clarified if other nutrients will remain undamaged during the hydrolysis process. This raises the question of whether the other beneficial nutrients present in whole grain amaranth will be affected adversely by hydrolysis. The study describes the use of an alpha-amylase and alternatively a protease, which would create a less viscous product, and proposes that use in a food product—thereby increasing the whole grain content in the product. Unlike this thesis, this study does not consider the use of a xylanase or cellulase to lower the viscosity of the product even further.

1.8.2 Xylanase

Hemicellulose consists of different polymers like hexoses (mannose glucose, and galactose), pentoses (xylose and arabinose) and sugar acids. Cellulose is difficult to degrade, but hemicellulose has a lower molecular weight, and has easy-to-hydrolyze polymers consisting of branches with short lateral chains of various sugars. Amaranth bran is mostly cell wall polysaccharides (hemicelluloses, a form of arabinoxylan) and cellulose, which is the most common hemicellulose found in grasses, herbs, and cereals (Ebringerova & Heinze, 2000).

Amaranth contains 0.72-0.98% arabinoxylan (Repo-Carrasco-Valencia, Peña, Kallio, & Salminen, 2009). Arabinoxylan (AX) consists of a linear backbone of 1,4-β-D-xylopyranosyl units, substituted with monomeric α-L-arabinofuranosyl units by 1,2 and/or 1,3-α-glycosidic linkages. In addition, highly branched arabinoxylan is present

in the non-starch polysaccharide fractions isolated from amaranth (Burisova et al., 2001). The high level of branching indicates these arabinoxylans are very capable of binding water and increasing viscosity. Figure 4 shows a section of arabinoxylan and Figure 5 shows xylanase specificity on arabinoxylan.

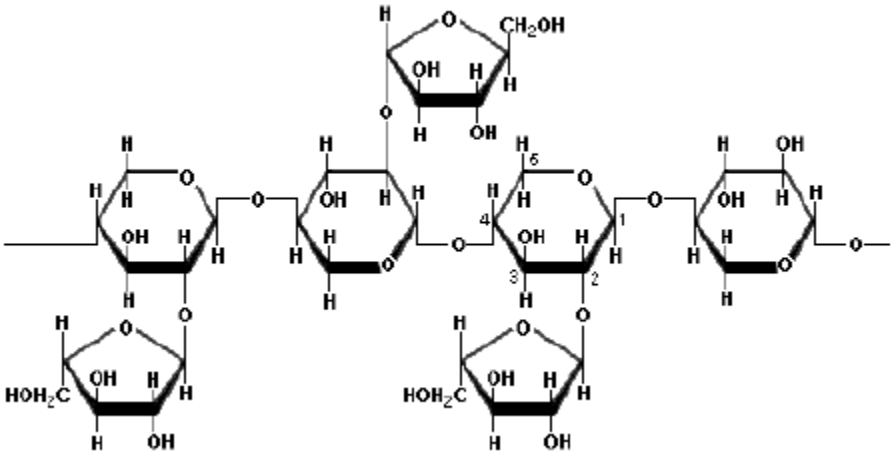

Figure 4: Section of Arabinoxylan showing substituents (Schoovenvel-Bergmans, Hopman, Beldman, & Voragen, 1998).

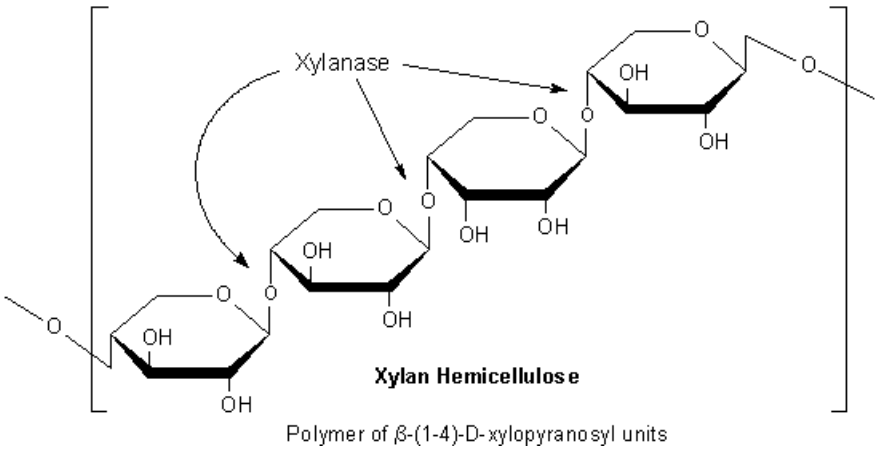

Figure 5: Xylanase specificity on Xylan Hemicellulose (arabinoxylan) (Whistler & Masek, 1955).

Arabinoxylans are either water-extractable (WE-AX) or water-unextractable (WU-AX) and have very different physiochemical properties. WU-AX has a large water-holding capacity, while WE-AX can give a very viscous solution (Meuser, Suckow, Blanshard, Frazier, & Galliard, 1986). The water extractability of these

polysaccharides differ in multiple factors including diferulic acid bridges between adjacent arabinoxylan chains, physical entanglement, and covalent ester bonding with carboxyl groups of uronic acids and hydroxyl groups of arabinoxylan (Izydorczyk & Biliaderis, 1995).

These indigestible polysaccharides function as dietary fiber in human digestion. There is an increasing interest in modifying AX with xylanases in foods in search of unique physiochemical functionality for novel opportunities for amaranth hydrolysate in processed foods.

Xylanases are capable of cleaving the 1,4- β -D-xylopyranosyl linkages of the 1,4- β -D-xylans in arabinoglucuronoxylan, glucuronoxylan, arabino-4-O-methy-D-glucuronoxylan, and arabinoxylan. Various forms of xylanases are available with many mechanisms of action, hydrolytic activities, substrate specificities, and physiochemical characteristics.

1.8.2.1 Amaranth Hydrolysate by Xylanase

The experiments described in this thesis project focus on the benefits of hydrolyzing amaranth by xylanase to create a product with increased functionality. The technological and nutritional functionality of bran can be improved by enzymatically modifying the complex insoluble network structure of the cell walls by the use of Endo-β-1,4-xylanases, or endoxylanases (Melanson, Angelopoulous, T. J. Nguyen, Martini, Sukley, & Londes, 2006). This increase in functionality is generated by prebiotic health benefits, a decrease in molecular weight of AX fragments and in their viscosity-forming properties, and (although this research does not explore this area) release of bound phenolic compounds.

Firstly, endoxylanases randomly cleave AX fractions, producing arabinoxylan oligosaccharides (AXOS). AXOS has been studied extensively, showing unique biological and technological functionality. AXOS have been shown to have many biological benefits, however; this study will focus efforts on the technological functionality of AXOS and the conversion of insoluble fibers into soluble, benefiting processed foods made with xylanase modified amaranth. The prebiotic activity of AXOS has been studied (Broekaert et al., 2011). Prebiotics are generally oligosaccharides that are not digested by enzymes of the upper gastrointestinal tract, but are fermented by certain varieties of bacteria in the large intestine. Lactobacilli and/or bifidobacteria are generally the bacteria that help promote prebiotic activity of AXOS. Health benefits that result from consumption of compounds like AXOS include

suppression of colon cancer activation, increased absorption of minerals, and decreased infection of the gut (Gibson, Probert, Loo, Rastall, & Roberfroid, 2004).

Secondly, water-unextractable AX (WU-AX) can be solubilized into water-extractable AX (WE-AX) and lose their strong water-holding capacity by hydrolysis (Gruppen, Kormelink, & Voragen, 1993). This reaction also is responsible for a decrease in molecular weight of AX fragments and in their viscosity-forming properties (Petit-Benvegnen, Saulnier, & Rouau, 1998; Rouau & Moreau, 1993). These effects impact the conditions of foods made with flours treated with endoxylanases. In bread making, the WE-AX solubilized AX show an increased loaf volume for breads, while WU-AX is detrimental for bread making (Courtin & Delcour, 2001). Similar effects are promising for other processed foods made with endoxylanase treated whole grain amaranth flour. It is advantageous to produce health benefits by understanding the biological impact; however, the following thesis research and methodology focuses on the improved technological functionality of the product and understanding the main effects of viscosity and water holding capacity of amaranth hydrolysate by xylanase. It is encouraging that the hydrolysis products of the high viscosity polymers in grains can potentially create more usable ingredients from the entire grain seed and maintain naturally occurring nutrients.

Lastly, though this thesis does not focus on it, another benefit of hydrolyzing amaranth with xylanase is releasing bound phenolic compounds. Grains have a wide range in the compositions of phytochemicals with various structures, naturally present in the bran and aleurone layers of cell walls, comprised of phenolic compounds in their insoluble bound forms, esterified to the cell wall polymers (Bunzel, Ralph, & Steinhart, 2005). Phenolic compounds are a group of phytochemicals comprising a large variety of compounds, naturally occurring in plants, all of which have one shared structural element, an aromatic phenol ring. Depending on their structure and function, these molecules are commonly categorized as phenolic acids, flavonoids, stilbenes, and lignans (Adom & Liu, 2002). In plants, these phenolic compounds contribute to various roles from protection to structure building. Phenolic compounds display protective antioxidant behaviors and can be beneficial to humans when digested (Robbins, 2003). Antioxidant compounds prevent cellular damage by terminating the otherwise harmful chain reactions that occur during oxidation. If this reaction is not prevented, cellular damage may be created, increasing the risk of chronic diseases such as metabolic disorders, cancer, and cardiovascular disease (Vetrani, Costabile, Di Marino, & Rivellese, 2013).

A distinctive feature of arabinoxylans is the presence of the phenolic compound ferulic acid, which are covalently bound by an ester linkage. This ester linkage allows other polysaccharides to conveniently cross-link, changing the structure and some mechanical properties, decreasing ferulic acid's susceptibility to digestion (Grabber, Ralph, & Hatfield, 2000; Izydorczyk & Biliaderis, 1995). Liberating phenolic compounds from their bound state by enzymatic hydrolysis can make whole grain foods much more beneficial to health. These compounds are unable to be absorbed in the G.I. tract, but typically when they enter the colon, microbial enzymes solubilize and release them so they are available to the consumer. This effect is not always present as there are multiple factors that affect food nutrient availability for absorption in the gut such as the chemical state of the phytonutrients, the release from the matrix the food is in, the possible interactions with other food components in the gut, and food microstructure. Since these compounds are not always able to be digested, modification of the grain by xylanase enzymes prior to ingestion allows the consumer to profit from these health benefits (Parada & Aguilera, 2007). Enzymatic treatment has been shown to increase the amount of soluble dietary fiber while simultaneously releasing phytochemicals linked to polysaccharide chains in grains (Napolitano et al., 2006). This effect increased the concentration of water-soluble antioxidants, freeing bound phenolics and increasing these compounds' bioacessibility.

1.8.3 Cellulase

As previously described, cellulose is a cell wall polysaccharide and a key structural component of all plant materials. Cellulose is insoluble in water and indigestible by human enzymes. It is comprised of a rigid linear chain repeated connection of D-glucospyranose molecules covalently linked together by β-1 -4 bond (Figure 6) (Klemm, Heublein, Fink, & Bohn, 2005). Every other glucose unit is rotated 180° so that the repeating unit is cellobiose (Beguin & Aubert., 1994).

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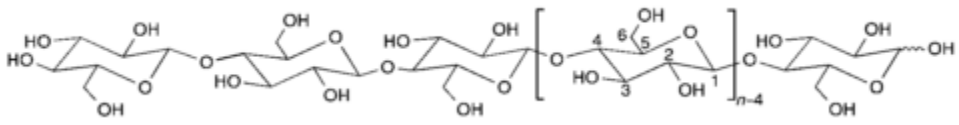

Figure 6: Molecular structure of cellulose (Beguin & Aubert., 1994).

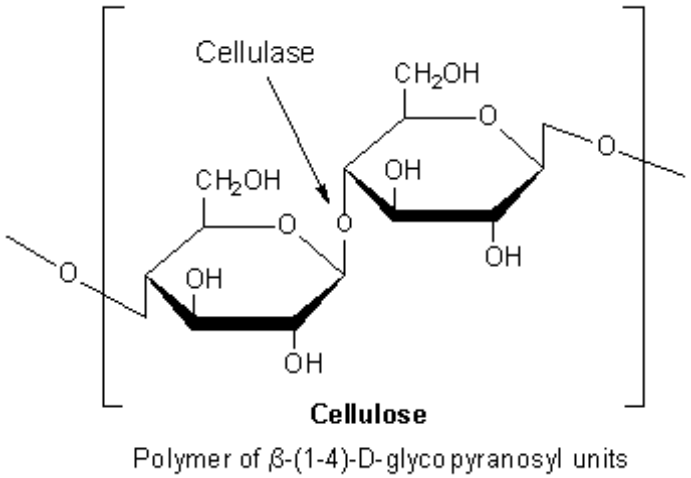

Figure 7: Cellulase specificity on cellulose (Larner et al., 1960)**.**

Amaranth has been shown to contain 1.2% cellulose of the alkali-insoluble residue in amaranth flour, contributing to viscosity-building components in the grain (Burisova et al., 2001). Cellulosic materials have traditionally been decomposed to D-glucose by acid treatment and cellulase enzymes to produce fermentable sugars for a variety of biotechnological processes (Klemm et al., 2005). This thesis research utilizes a cellulase, Celluclast, hydrolyzing β-1,4-D-glucosidic linkages in cellulose and other β-D-glucans to degrading cellulosic fibrous insoluble components into soluble fiber (Figure 7).

1.8.4 Protease

Amaranth protein isolate has low solubility in aqueous solutions, restricting its use in the food industry (Scilingo, Molina Ortiz, Martínez, & Añón, 2002). Enzymatic hydrolysis can change functional properties of proteins by forming smaller size peptide chains and remaining proteins with different structural characteristics. Protein hydrolysate has been created with improved solubility and digestibility without diminishing nutritional value (Ventureira, Martinez, & Anon, 2009). In an attempt to improve functionality in food by decreasing viscosity and water holding capacity, proteases (or peptidases) were used in this thesis research and it can be construed that the protein insolubility is present in the whole grain as well.

Unfortunately, protein hydrolysates are known to generate bitter tasting compounds from polypeptide rich fractions, limiting their use in food products. The results of improving solubility without the generation of bitterness depend on the proteolytic enzyme and hydrolysis conditions. Endo-proteases hydrolyze specific peptide bonds

within the polypeptide chain, whereas exo-proteases liberate amino acids by hydrolysis of the N-terminal bond. Endo-proteases have been shown to form bitterness in their hydrolysate; however, their use in combination with exo-proteases helps to selectively hydrolyze bitter peptides and may reduce the formation of bitter peptides. This grouping of proteases minimize the generation of hydrophobic residues including long chain and aromatic amino acids which influence bitterness depending on the type and concentration (Raksakulthai & Haard, 2003). During preliminary experiments in this thesis research, enzyme dosage was optimized to decrease viscosity while reducing the intensity of bitterness and aversion when the product was consumed. This thesis research investigates the use of this category of enzymes with Flavorzyme, consisting of an exo-peptidase (carboxypeptidase and aminopeptidase) and an endo-protease, and it exhibits some alpha-amylase side activity.

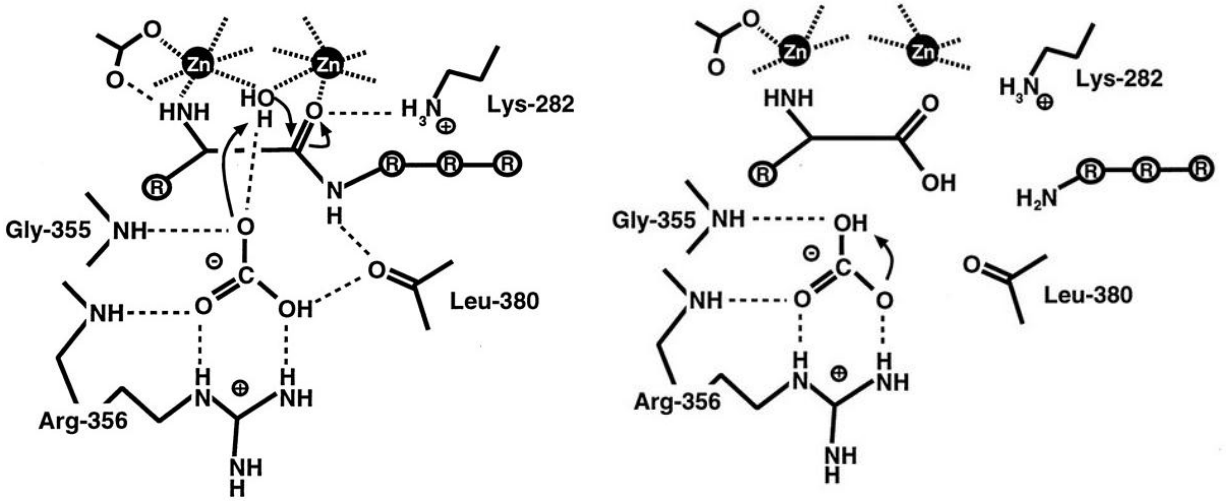

Figure 8: General substrate attack of the peptide bond and its products free amino acid and remaining protein/peptide (Strater, Sun, Kantrowitz, & Lipscomb, 1999)

1.9 Conclusion

All of these individual enzymes present valuable rationale for their use to create a more usable whole grain amaranth ingredient. Using enzymes in combination can dramatically enhance functional properties of the end product even more than using each independently. It is hypothesized that there will be synergistic effects when multiple enzymes are used, and combining these processes may decrease viscosity and water holding capacity, and increase pleasant sensory qualities.

The outstanding nutritional properties of amaranth illustrate the advantages of its use in improving the nutritional value of processed foods made with the whole grain. Enzymatic hydrolysis of amaranth remains to be investigated, supporting the justification of this research in determining new processing parameters for whole grain amaranth. Alpha-amylase, cellulase, and xylanase enzymes can be used to significantly decrease viscosity of whole grains in solution. Although there is the potential to degrade protein and fiber with use of protease, cellulase, and xylanase, their use in experimental research may prove that an even more novel ingredient can be created. Amaranth hydrolysate could be a solution to many technical challenges in formulating processed foods high in whole grain and may assist in creating innovative ingredients, enabling products to meet functional and sensory standards.

Chapter 2 - 2^4 Two-Way Factorial Experimental Design 1

2.1 Introduction

Extensive preliminary testing was done to understand the effect of sample dilution, determine the termination of the enzyme's catalytic reaction, and identify ideal enzyme levels used (dosage).

Many factors affect the kinetic and thermodynamic activity of the enzymes. For each enzyme, there is optimum conditions in which the enzyme performs most effectively including: viscosity of solution (allowing more efficient contact of enzyme with substrate), dosage level based on substrate concentration, active temperature, pH, and time of activity. For the purposes of this research, a variety of enzymes were tested with a variety of optimum conditions; however, homogeneity in reactions was maintained. Enzyme dosage was determined in preliminary work described below (section 2.2.3). These preliminary tests helped to establish optimum conditions for substrate conversion for a two-way factorial experimental design.

2.2 Method Development

2.2.1 Sample preparation

The hydrolysis reaction requires water; therefore, a suspension solution (slurry) was developed. The whole grain amaranth flour was dispersed in water at varying concentrations. It was found that 20% w/v slurry was appropriate for use in this study since its flowable viscosity allowed efficient contact of enzyme to substrate when reaction occurred.

Since alpha-amylase was used in this study, the 20% w/v slurry required the presence of calcium ions (increases the enzymes activity and stability) and gelatinization of the starch in the substrate, allowing more susceptibility to enzyme action (Reeve, 1992). To enable uniformity in conditions, all reactions in this study were performed on a 20% w/v gelatinized whole grain amaranth flour suspension containing 0.1% Calcium Chloride ($CaCl_2$) as recommended by the enzyme supplier.

Finally, it was discovered that the 20% w/v solution was too dilute to quantify sugars and possibly other components of the product. Therefore, hydrolyzed samples were dried using a drum drier to concentrate the product

to ≤ 5% moisture. This also allowed less variation within analysis. Compositional analysis was then performed on samples after drum drying and data was compared on a dry basis (db).

2.2.2 Termination of reaction

Enzyme stability is an intrinsic characteristic that is established by the primary structure of the protein. Protein denaturation can be completely or partially reversible. A method was developed to terminate the reaction and in attempt to establish a most efficient method, experimentation was done to reduce pH and boil. It was discovered that the enzymes are at least partially reversible by pH reduction, thus boiling was determined to be the best method of enzyme denaturation.

2.2.3 Optimized enzyme dosage

The enzymes used are commercially available from Novozymes, each having its own particular enzyme activity and recommended dosage. Dosages were modified to optimize desired results. Therefore, additional testing was done to determine appropriate levels in this application.

Activity profiles of the commercial enzymes BAN 480 LS (Alpha-Amylase), Celluclast (Cellulase), Flavourzyme (Protease), and Shearzyme (Xylanase) were determined by Novozymes and are shown in Table 2. As previously mentioned, uniform conditions of pH, temperature, and time were applied in this two-way factorial experimental design. The ultimate use of the end product would be in commercial food products; therefore, a buffered system was not chosen and distilled water at pH 6 was used in the reaction (which also happens to be within 80% of each enzyme's relative activity) (Figure 9). Analysis of the enzyme activity profiling helped to define the temperature of 50°C for reaction temperature (Figure 8). No significant decrease in viscosity was observed after time points between 30 minutes and 24 hours. Hence, the reaction time of 30 minutes was chosen as an appropriate amount of time allowing sufficient degradation of substrate.

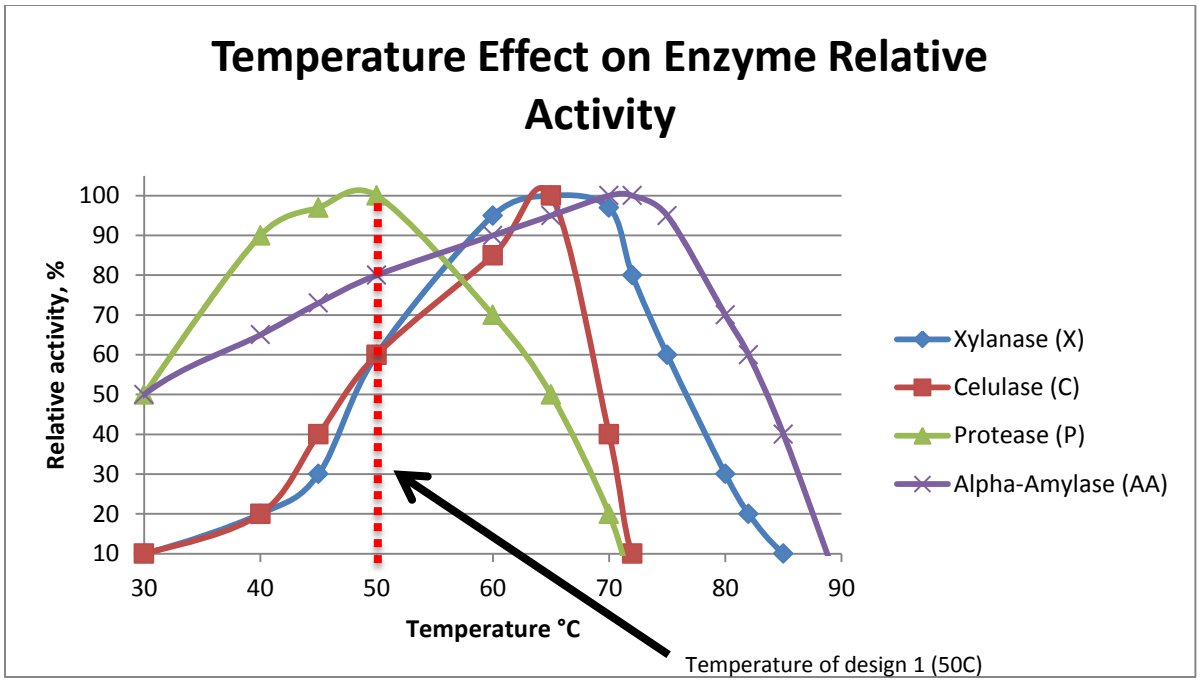

Figure 9: Temperature Effect on Enzyme Relative Activity.

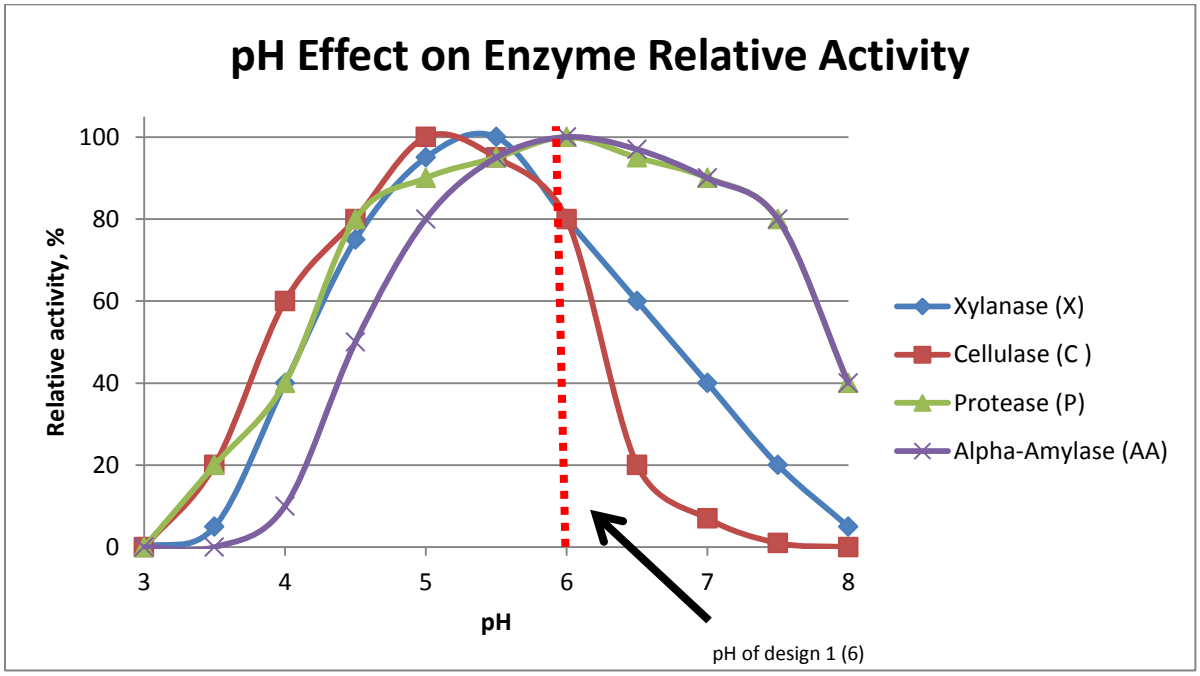

Figure 10: pH Effect on Enzyme Relative Activity.

2.2.4 2^4 Two-Way Factorial Experimental Design

A two-way factorial design was used to explore the main effects of two factors, enzyme type and combination, and their interactions. Two-way factorial designs are beneficial for several reasons: they involve moderately few runs per factor examined, they are easily adapted to form composite designs if more exploration is desired, and they form the foundation for the initial stages of a large study (Box, Hunter, & Hunter, 1978).

In performing this design, there are fixed levels of each number of factors and the experiment is run with all possible combination of these factors (Box et al., 1978). The proposed design enables the evaluation of four variables (i.e., four enzymes) at two different levels (enzyme absent or present at optimum level). A 2 x 2 x 2 x 2 (2^4) factorial design requires 16 runs.

A randomized experiment is shown in Table 7. A total number of 16 runs were generated and experiments were run randomly. Data was collected in triplicates for the responses viscosity and water holding capacity; variations in measurement of other responses are discussed further in results and analysis.

The design assumed constant conditions with time of 30 minutes, temperature of 50°C and pH of 6.0; these conditions are at the approximate center of optimized temperature and pH for enzymes in the study (Figures 7 & 8).

Sources of variation were determined by conducting a multi-way analysis of variance (ANOVA), with enzyme type and combination as the source of variation.

2.3 Materials & Methods

2.3.1 Materials

2.3.1.1 Enzyme Selection

Enzymes, donated by Novozymes A/S (Bagsvaerd, Denmark), with minimal or relatively no side activities were preferred for this study; however, some enzymes used could not be obtained without some side activities present (Table 2).

2.3.1.2 Whole Grain Amaranth Flour Selection

Whole Grain Amaranth flour was obtained from ConAgraMills (Omaha, NE) all of the same lot code.

Table 2: Enzymes tested, including main activities, source and optimal pH and temperature ranges.

Enzyme	Trade name	E.C. number	Active pH range	Active temp range	Side activities	Organism	Functionality	Enzyme Activity (as supplied)
Alpha-Amylase	BAN 480LS	3.2.1.1	5 to7	50 to 80	None	Bacillus Amyloliquefaciens	Endo-amylase hydrolyzes (1,4)-α-D-glucosidic linkages in starch polysaccharides	480 KNU-B/g
Cellulase	Celluclast	3.2.1.4	4 to 6	40 to 65	Hemicellulase side activities such as xylanase and arabinofuranosidase	Trichoderma reesei	Cellulase hydrolyzes (1,4)-β-D-glucosidic linkages in cellulose and other β-D-glucans	700 EGU/g
Protease	Flavorzyme	3.4.11.1	5 to 7	45 to 55	Mixture of proteases: carboxypeptidase, aminopeptidase, and endo-protease and has some amylase side activity	Aspergillus oryzae	Exo-peptidase liberates amino acids by hydrolysis of the N-terminal peptide bond	1000 LAPU/g
Xylanase	Shearzyme	3.2.1.8	4.5 to 5.5	60 to 70	Minor β-glucosidase	Aspergillus oryzae	Endo-xylanase hydrolyzes (1,4)-β-D-xylosidic linkages in xylans	500 FXU-S/g

2.3.2 Methods

2.3.2.1 Amaranth slurry solution

Gelatinized suspension preparation

The whole grain amaranth flour suspension (12000mL) was prepared in distilled water at pH 6 with substrate concentration 20% w/v in a heating and mixing apparatus, Vorwerk Thermomix (Vorwerk & Co. KG, Wuppertal, Germany).

In the Vorwerk Thermomix, 9600mL distilled water was heated to boiling, 12g $CaCl_2$was added for optimal activity of the alpha-amylase enzyme and 2400g whole grain amaranth flour was added. The suspension was boiled for 10 minutes, completely gelatinizing the starch and denaturing any native enzymes in the flour. The suspension was cooled to 50°C and split into sixteen 750mL samples in 1000mL glass beakers.

2.3.2.2 Sample Preparation

Hydrolysis reaction

The four 750 mL samples of 20% w/v gelatinized whole grain amaranth suspension were placed in a 50°C water bath and continuously agitated (300 rpm). Maintaining 50°C, each sample received a corresponding enzyme dosage within the experimental design and the hydrolysis reaction processed for 30 min.

Enzyme deactivation

At the conclusion of the hydrolysis reaction, each sample was boiled for 10 minutes while continuously mixed to deactivate the enzyme and subsequently drum dried for analysis.

2.3.2.3 Total Sugar Measurement

Sugar analysis was done by Medallion Labs following AOAC 977.20. This method directly determines the amount of ribose, fructose, glucose, sucrose, maltose, lactose, galactose, stachyose, raffinose, trehalose, and all polysaccharides up to Degree of Polymerization (DP) 7.

2.3.2.4 Total Starch Measurement

Total starch was determined by Medallion Labs following AOAC 979.10.

2.3.2.5 Dextrose Equivalency Calculation

A Medallion Labs proprietary method was modified from AOAC 977.20 via HPLC and was applied to quantitate percent reducing ends in this study. Dextrose Equivalency (DE) is a measure of the degree to which starch is hydrolytically converted. The DE value is a measure of the total reducing power of all sugars and polysaccharides present in solution on a dry basis. DE is normally applied to starch hydrolysate products such as corn syrup or maltodextrin. At the time of this study, the application of DE to any commercial wholegrain product was not identified. It was concluded that the reducing ends included in the calculation should be from the glucose units included in the starch, and that glucose in the remaining components should not be included.

Typical measurements of DE use redox titration methods; however, these methods have been shown to overestimate dextrose equivalent and they have difficulty recovering and quantifying high DP oligosaccharides and low DE maltodextrins as they may contain some insoluble carbohydrate material (Commerford & Scallet, 1969). From this justification, and as there were limited methods available at the time of this research, it was inferred that the best methods for characterizing maltodextrins are by use of instrumental methods such as HPLC (Charalambous, 1979; D., Sc., & D, 1986; Nikolov, Jakovljević, & Sc, 1984).

For the purposes of this research, a general calculation estimate was used by determining the level of carbohydrates present up to Degree of Polymerization (DP) 7 and calculating DE using the formula below by determining the % reducing sugars present. Each sugar and polysaccharide analyzed has corresponding levels of reducing ends. In quantification of these reducing ends and total starch content, the DE can be calculated using the formula below.

$$Dextrose\ equivalent = \frac{\%\ reducing\ ends \times 100}{\%\ (starch\ content_{db})}$$

2.3.2.6 Viscosity Measurement

A viscosity measurement method modified from AACC 56-11.02 using a Brookfield Viscometer (model DV-III). The enzyme treated flours were dispersed in water reconstituted at 75% (w/v), boiled for 30 seconds, and allowed to cool to50 ^{o}C. Viscosity was immediately measured at 50 ^{o}C using SC4-29 spindle. Since samples exhibit non-Newtownian behavior, a time point was chosen at 3 minutes, and data was collected.

2.3.2.7 Total Dietary, Insoluble, and Soluble Fiber Measurement

Total dietary fiber (TDF) is defined by the CODEX Alimentarius Commission, as non-digestible polysaccharides of DP 3 or higher. This research quantifies soluble, insoluble, and total dietary fiber following method AOACI 2009.01 performed by Medallion Labs.

2.3.2.8 Free Amino Acid Measurement

Samples were submitted to AminoAcids.com for analysis using a proprietary method developed for identifying amino acids in matrices by separating, identifying, and quantifying via Hitachi L-8900 Amino Acids Analyzer Cation Exchange HPLC following methodology developed by Spackman, Stein, & Moore, 1958.

2.3.2.9 Moisture Measurement

The moisture measurement is a "loss-on-drying" type of method, determined using AACC Approved Method 44-16.01 by Medallion Labs. This percent moisture was used in this study to calculate responses on a dry basis.

2.3.2.10 Water Holding Capacity

Modifications to the AACC 56-11.02 were conducted in determination of Water Holding Capacity (WHC) by using 1gram samples of treated flours combined with 30 mL of distilled water in 50 mL centrifuge tubes. Slurries were vortexed and allowed to hydrate for 30 minutes at 25 ºC. Samples were centrifuged for 30 min. at 3000 rpm. Supernatants were decanted off and tubes were allowed to rest in test tube rack inverted for 20 minutes. Samples

were weighed and calculated for WHC. Water holding capacity was expressed as percent water bound per gram flour following AACC 56-11.02 calculations.

2.3.2.11 Statistical Analysis

All statistical analyses were conducted using Design-Expert software (Design-Expert version 7.1.14; Stat-Ease, Inc.; Minneapolis, MN). Several responses used log transformation (i.e., viscosity, water holding capacity, and dextrose equivalence) of their data to attain and examine this information at a more constant variance in the design (Box et al., 1978). This transformation assumed a considerable gain in precision, indicated by the relationship between cell variances and cell averages; the response data described in this research was based on log transformation analysis.

2.4 Results and Discussion

Factors with main effects of each response are labeled with symbols as denoted in Table 3: Alpha-Amylase (A), Cellulase (B), Protease (C), and Xylanase (D). Initially, the values of the main effects, interaction and analysis of variance (ANOVA) were obtained, shown in Table 4. A P-value below 0.05 was considered significant. Raw data values discussed in this section are displayed in Table 6.

2.4.1 Total Dietary, Insoluble and Soluble Fiber

The TDF and soluble fiber raw data did not display any statistically significant differences. Insoluble fiber showed statistically significant differences ($P = 0.0152$) by BC interaction. Although negligible, this interaction shows cellulase (B) and protease (C) degrade insoluble fiber (Figure 10). This interaction is not well understood since it is not anticipated that protease would react with insoluble fiber, but is thought to be a result of side activity of the protease and possibly competition for enzyme sites. The intended mechanism of cellulase is to break down insoluble fiber by degrading cellulose and polysaccharides, converting insoluble into soluble fibers.

2.4.2 Free Amino Acids

A, C, and an AC interaction have shown statistically significant effects on free amino acids content with *P* = 0.0035, <0.001, and = 0.0035, respectively. The contributions of the interaction to the overall ANOVA model were minor compared to the main effects. Overall, protease generates free amino acids; however, when alpha-amylase is used in combination with protease, this effect is suppressed. These unexplained results may be due to the side activity of the protease, as this analysis was excluded from this study. As each enzyme is specific on what it produces, there may be some interaction occurring with the suppression of free amino acid generation in the presence of the alpha-amylase used in this study.

As expected, protease liberated free amino acids and liberated short chain peptides into the system; however, the level of free amino acids generated with protease created unpleasant off flavors and largely contributed to negative sensory qualities of the hydrolysate. It has been shown that specific individual amino acids can be considered as aversive tasting in humans (Akitomi et al., 2013). In some foods bitterness is actually desired. Bitterness perception depends on the compound and concentration when consumed. By their creation, the concentration and type of short-chain peptides or free amino acids determines the acceptability of these foods (Meyerhof, Born, Brockhoff, & Behrens, 2011). Since samples hydrolyzed with Flavorzyme exhibited negative sensory qualities, (unpleasant bitterness) use of this enzyme was not investigated further and the distribution of specific amino acids was not analyzed.

2.4.3 Total Sugars

A and C both *P* <0.001, had statistically significant effects on total sugar content. The data shows the use of alpha-amylase and protease most significantly increased total sugars. As anticipated, alpha-amylase generated sugars by randomly cleaving α-1, 4 glycosidic bonds present in the inner part (endo-) of the amylose or amylopectin chain. The protease used in this study contained amylase side activity, largely contributing to the generation of sugar.

2.4.2 Total Starch

As expected, A had a statistically significant effect on total starch content (P <0.001) 48.10-71.76% ± 0.6%, indicating alpha-amylase had a large influence in the degradation of starch into smaller chain poly- or oligosaccharides or di- and monosaccharides (contributing to increase in total sugars).

2.4.3 Dextrose Equivalency

The main effects of DE have statistically significant effects of A, C, and AC (P < 0.0001). Alpha-amylase and protease had a much greater effect on DE than any other enzymes in the study. Their interaction behaved additively, therefore the effect of each enzyme increases the overall DE, but alpha-amylase increases DE more significantly than protease does (Figure 11). Although this interaction is not large, it was unexpected, as it was believed the use of these enzymes in combination would futher increase DE.

As expected with a decrease in starch and increase in total sugars, DE would increase correspondingly as its existence depends on the presence of starch and reducing sugars.

2.4.5 Viscosity

The main effects of viscosity display a wide variation in viscosity, in the untreated sample (21,363 cPs) and samples treated with A and C (59-102 cPs). The effects are all statistically significant at P < 0.001, indicating their presence greatly reduced the viscosity. AC has a significant effect on viscosity that is different than when the other enzymes are present. The effect of A and C decreases viscosity. The effect of the two-factor interaction, AC, indicates alpha-amylase and protease will decrease viscosity further when used in combination (Figure 13).

This data follows a similar trend that has been displayed thus far; statiscially significant effects of hydrolysis with alpha-amylase and protease have shown a decrease in viscosity and total starch, which contributed to an increase of DE and total sugars.

A relationship between the degree of starch hydrolytic conversion (including level of total sugars), DE, and viscosity has been construed (Dokic et al., 2004). It has been found that with increasing degree of hydrolysis, or increased DE values, the viscosity decreases. The above listed statistically significant effects confirms the

hypothesis that alpha-amylase is required in finding an approach in significantly decreasing viscosity and water-holding capacity.

2.4.6 Water Holding Capacity

The main effects of water holding capacity are A, C, and AC. A and AC are statistically significant at $P < 0.001$, and C at $P = 0.002$. The AC interaction displayseffects in that alpha-amylase and xylanase function synergystically in decreasing water holding capacity (although these effects are minimal) (Figure 14).

2.5 Conclusions

Protease was eliminated from further testing. The protease used in this study was not pure, causing other reactions that created bitterness and off-flavors. The benefits of its use were not as valuable as its adverse effects; for this reason in the next experiments it was chosen not to focus on this enzyme and it was eliminated from further testing.

This design concludes that alpha-amylase had the strongest effect in decreasing viscosity and water holding capacity. However, additional investigations are warranted in solubilizing insoluble fiber components. One more two-factor design is proposed.

As previously described, the use of cellulase and xylanase may hydrolyze insoluble components. This conversion will make these components lose their water-holding capacity and decrease viscosity; however, these effects in the first two-factor design are not statistically significant ($P > 0.05$). It is believed the effect of cellulase and xylanase on these responses may be suppressed by the temperature and viscosity in which the experiments were performed.

Design 1 used conditions beyond where the enzymes (excluding protease) have shown to be more optimally active. Protease was the outlying factor influencing the center point temperature (50C) and pH (6.0) employed in this design, described in section 2.2 Method Development.

There is much value in use of cellulase and xylanase in degradation of this grain. Since it is soluble dietary fiber that is predominantly shown as physiologically beneficial, and since WU-AX can be partially solubilized if treated with alkaline conditions, additional investigations are warranted (Izydorczyk & Biliaderis, 1995).

Chapter 3 of this study looks at altering pH to modify the ratio of soluble to insoluble dietary fiber in amaranth hydrolysate. This subsequent two-factor experimental design in Chapter 3, hereon designated Design 2, analyzes a range of pH and temperatures that are in a more optimum range for the enzymes Alpha-Amylase, Cellulase, Xylanase.

2.6 Tables and Figures

Table 3: 2^4 two-way design indicating actual values as % based on weight of substrate.

	Alpha-Amylase	Cellulase	Protease	Xylanase		Alpha-Amylase	Cellulase	Protease	Xylanase
Symbol	**(A)**	**(B)**	**(C)**	**(D)**		**(A)**	**(B)**	**(C)**	**(D)**
Test condition #	**Factor actual values % based on weight of substrate**					**Factor coded values**			
1	0.4	0	0	0		+1	-1	-1	-1
2	0	0	0.5	0		-1	-1	+1	-1
3	0	0	0	0.2		-1	-1	-1	+1
4	0	1.2	0	0		-1	+1	-1	-1
5	0.4	0	0.5	0		+1	-1	+1	-1
6	0	0	0.5	0.2		-1	-1	+1	+1
7	0	1.2	0	0.2		-1	+1	-1	+1
8	0.4	1.2	0	0		+1	+1	-1	-1
9	0	1.2	0.5	0		-1	+1	+1	-1
10	0.4	0	0	0.2		+1	-1	-1	+1
11	0.4	0	0.5	0.2		+1	-1	+1	+1
12	0	1.2	0.5	0.2		-1	+1	+1	+1
13	0.4	1.2	0	0.2		+1	+1	-1	+1
14	0.4	1.2	0.5	0		+1	+1	+1	-1
15	0.4	1.2	0.5	0.2		+1	+1	+1	+1
16	0	0	0	0		-1	-1	-1	-1

Table 4: Main effect values of 2^4 two-way design for the principal factors of analytical responses.

Factor	P-value	Effects ± Standard Error
Free Amino Acids		
A	0.0035	-0.068 ± 0.01
C	<0.0001	0.25 ± 0.01
AC	0.0035	-0.067 ± 0.01
Insoluble Fiber		
BC	0.0152	0.37 ± 0.065
Total Sugars		
A	<0.0001	5.15 ± 0.083
C	<0.0001	1.29 ± 0.083
Total Starch		
A	<0.001	-15.78 ± 0.73
Dextrose Equivalence		
A	<0.001	2.53 ± 0.076
C	<0.001	1.42 ± 0.076
AC	<0.001	-1.21 ± 0.076
Viscosity		
A	<0.001	-2.76 ± 0.052
C	<0.001	-2.48 ± 0.052
AC	<0.001	2.48 ± 0.052
Water Holding Capacity		
A	<0.001	-0.76 ± 0.072
C	0.002	-1.09 ± 0.072
AC	<0.001	1.14 ± 0.072

Table 5: ANOVA results for the responses studied in the 2^4 two-way design.

Response	Degrees of freedom	Sum of squares	Mean square	F test	p-value
Viscosity					
Model	3	79.590	26.5300	610.930	<0.0001
A-Alpha Amylase	1	30.360	30.3600	699.220	<0.0001
C-Protease	1	24.650	24.6500	567.600	<0.0001
AC	1	24.580	24.5800	565.970	<0.0001
Residual	12	0.520	0.0490	-	-
Total	15	80.110	-	-	-
Water Holding Capacity					
Model	3	12.200	4.0700	48.680	< 0.0001
A-Alpha Amylase	1	2.320	2.3200	27.720	0.0002
C-Protease	1	4.730	4.7300	56.550	< 0.0001
AC	1	5.170	5.1700	61.780	< 0.0001
Residual	12	1.000	0.0840	-	-
Total	15	13.220	-	-	-
Dextrose Equivalence					
Model	3	39.530	13.1766	143.668	< 0.0001
A-Alpha Amylase	1	25.666	25.6657	279.841	< 0.0001
C-Protease	1	8.053	8.0528	87.802	< 0.0001
AC	1	5.811	5.8113	63.362	< 0.0001
Residual	12	1.101	0.0917	-	-
Total	15	40.630	-	-	-
Free Amino Acids					
Model	3	0.278	0.0928	66.620	< 0.0001
A-Alpha Amylase	1	0.020	0.0203	12.940	0.0037
C-Protease	1	0.238	0.2377	173.980	< 0.0001
AC	1	0.020	0.0203	12.940	0.0037
Residual	12	0.019	0.0010	-	-
Total	15	0.297	-	-	-
Insoluble fiber					
Model	3	0.820	0.2700	4.080	0.0328
B-Cellulase	1	0.010	0.0016	0.024	0.8795
C-Protease	1	0.280	0.2800	4.210	0.0626
BC	1	0.530	0.5300	7.990	0.0152
Residual	12	0.800	0.0670	-	-
Total	15	1.620	-	-	-

Total Sugars					
Model	2	112.760	56.3796	511.601	< 0.0001
A-Alpha Amylase	1	106.140	106.1415	963.151	< 0.0001
C-Protease	1	6.620	6.6178	60.051	< 0.0001
Residual	13	1.430	0.1102	-	-
Total	15	114.190	-	-	-
Total Starch					
Model	1	996.034	996.0336	116.587	< 0.0001
A-Alpha Amylase	1	996.034	996.0336	116.587	< 0.0001
Residual	14	119.606	8.5433	-	-
Total	15	1115.639	-	-	-

Table 6: Raw data values determined for responses in 2^4 two-way design.

Coded Factors					Responses								
	Alpha-Amylase	Cellulase	Protease	Xylanase	Free Amino Acids	Total Dietary Fiber	Insoluble Fiber	Soluble Fiber	Total Sugars	Total Starch	Dextrose Equivalence	Viscosity	Water Holding Capacity
Test condition #	A	B	C	D	%	%	%	%	%	%	DE	cPs	%
1	+1	-1	-1	-1	0.17	11.79	8.38	3.41	6.60	58.35	13.56	76	0.30
2	-1	-1	+1	-1	0.50	10.86	7.79	3.07	2.44	71.19	4.03	97	0.23
3	-1	-1	-1	+1	0.16	12.96	8.74	4.22	1.15	66.31	0.44	10,931	1.15
4	-1	+1	-1	-1	0.17	11.82	8.16	3.66	1.38	67.45	0.61	9486	1.78
5	+1	-1	+1	-1	0.32	10.63	7.92	2.71	7.25	52.61	18.19	68	0.25
6	-1	-1	+1	+1	0.46	10.91	7.38	3.53	3.10	71.76	5.64	72	0.21
7	-1	+1	-1	+1	0.17	12.53	7.83	4.70	1.00	69.29	0.16	13,090	2.16
8	+1	+1	-1	-1	0.16	12.35	8.03	4.32	6.20	52.60	14.20	63	0.33
9	-1	+1	+1	-1	0.53	11.76	8.36	3.40	2.71	67.26	5.11	98	0.13
10	+1	-1	-1	+1	0.17	12.65	8.19	4.46	7.05	48.10	17.73	72	0.32
11	+1	-1	+1	+1	0.44	11.19	7.70	3.49	7.49	55.54	17.78	59	0.24
12	-1	+1	+1	+1	0.44	11.62	8.02	3.60	2.76	66.11	5.22	102	0.26
13	+1	+1	-1	+1	0.17	11.57	7.75	3.82	6.62	56.00	15.31	68	0.19
14	+1	+1	+1	-1	0.30	11.71	8.11	3.60	8.15	49.92	20.68	80	0.33
15	+1	+1	+1	+1	0.33	10.96	7.68	3.28	7.56	50.31	18.36	72	0.37
16	-1	-1	-1	-1	0.17	11.16	8.00	3.16	1.17	70.30	0.39	21,363	2.70

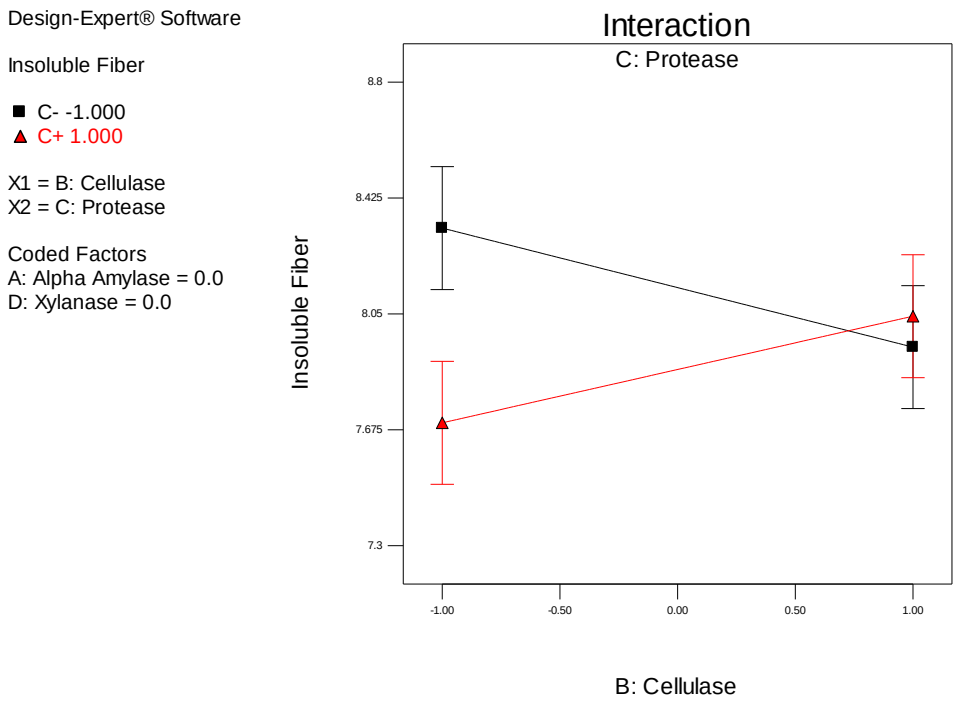

Figure 11: BC interaction on insoluble fiber.

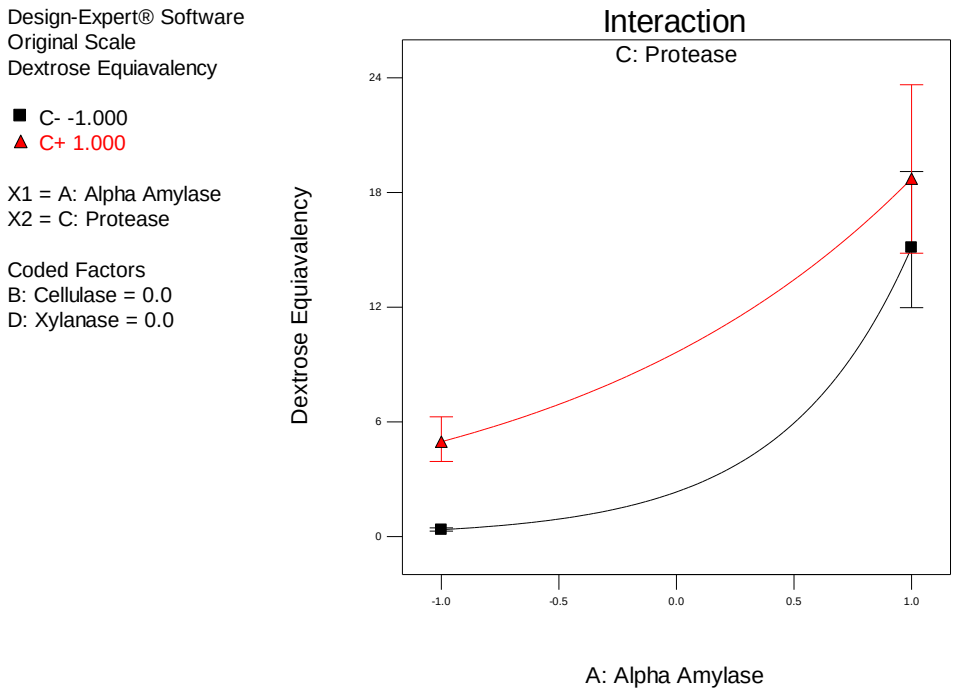

Figure 12: AC interaction on DE.

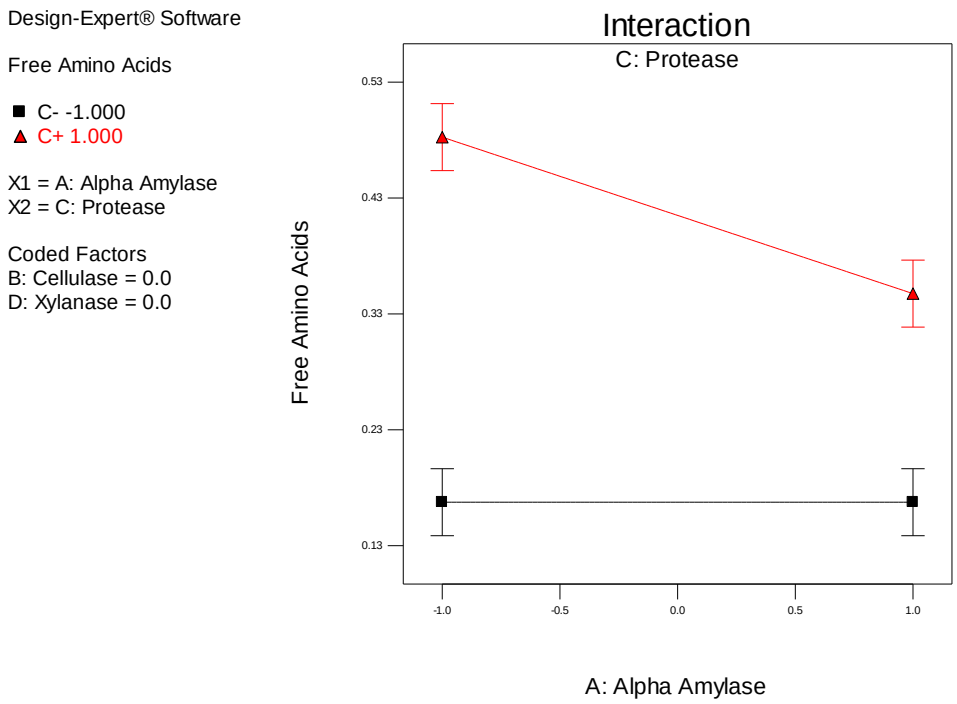

Figure 13: AC interaction on free amino acids.

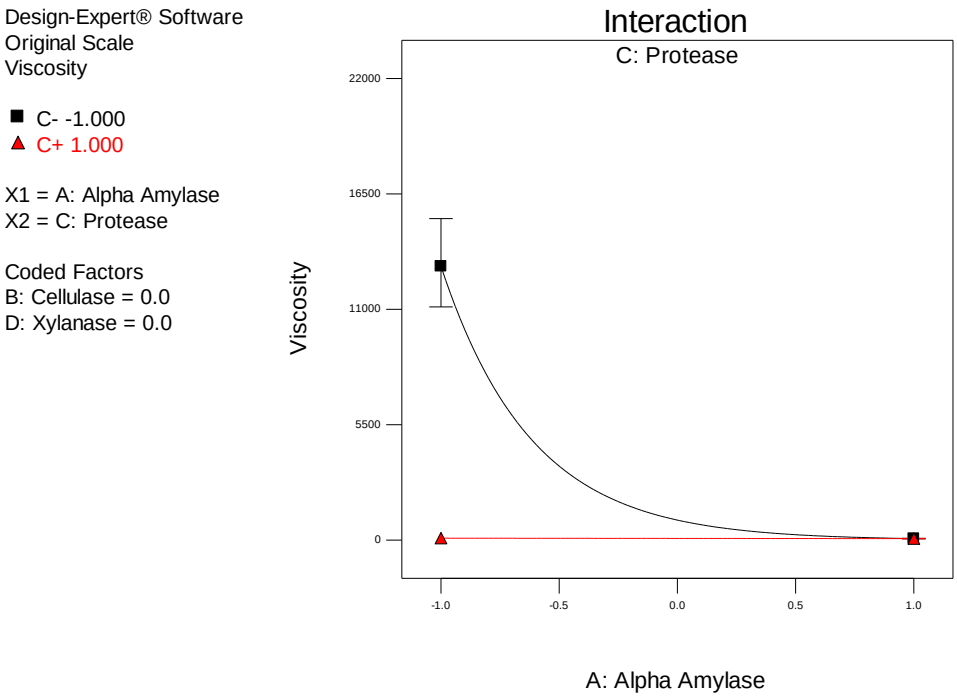

Figure 14: AC interaction on viscosity.

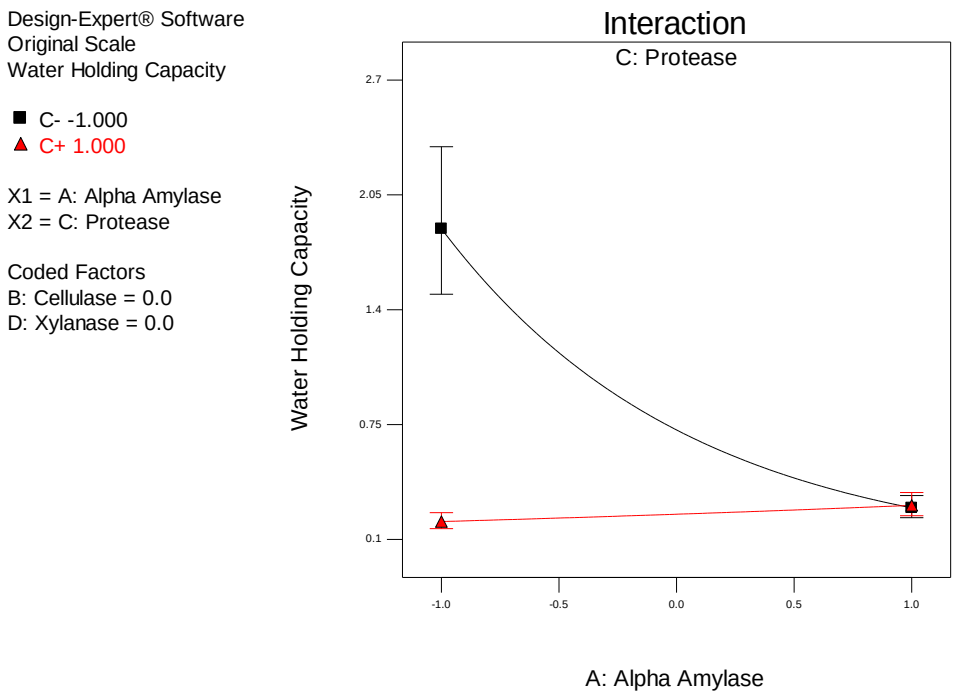

Figure 15: AC interaction on water holding capacity.

Chapter 3- 2^5 Two-Way Fractional Factorial Design 2

3.1 Introduction

The first study showed the use of alpha-amylase is vital in decreasing viscosity and water holding capacity. However, the literature advocates for the biological effects in the creation of AXOS and conversion of insoluble to soluble fibers (as described in section 1.8.2.1). Modifying the processing parameters of temperature and pH will create the optimum conditions for alpha-amylase, cellulase and xylanase in combination. Since a factorial design determines the effect of several factors and interactions between them with minimal runs, another two-factor design was chosen to better understand the effect of pH and temperature.

3.2 2^5 Two-Way Fractional Factorial Experimental Design 2 Development

In efforts to optimize the factors Alpha-Amylase, Cellulase, and Xylanase at low and high pH levels and temperatures (a total of 5 factors), a two-level fractional factorial design was prepared. A fractional factorial design allows minimal runs without replication while maintaining the same amount of confidence in the response analysis. This allows the design to focus on the most important factors and carefully choose a fraction, or half, of the experimental runs of the full two-factor design (fractional factorial). It is a balanced way of reducing the amount of runs in the design.

Design Expert statistical software aided in developing the principal fraction for the design, containing the combinations that are confounded with each other, meaning they cannot be estimated independently of each other. Confounded variables are extraneous and redundant; therefore, they can be excluded from the model. Since the design explores five factors at two levels, 2^5 is 32 runs, we are only looking at half of the design, 2^{5-1}, which is 16 runs (Table 17). This type of design allows the ability to separate main effects and low-order interactions from one another (Box, Hunter, & Hunter, 2005).

3.3 Materials & Methods

3.3.1 Enzyme Selection

Enzymes alpha-amylase, cellulase, and xylanase, donated by Novozymes A/S, were employed in this chapter as previously described (Table 2).

3.3.2 Whole Grain Amaranth Flour Selection

Whole grain amaranth flour was purchased from ConAgraMills (Omaha, NE).

3.3.3 Amaranth slurry solution

Gelatinized suspension preparation

The whole grain amaranth flour suspension (12000mL) was prepared in distilled water at pH 6 with substrate concentration 20% w/v in a heating and mixing apparatus, Vorwerk Thermomix (Vorwerk & Co. KG, Wuppertal, Germany).

In the Vorwerk Thermomix, 9600mL distilled water was heated to boiling, 12g $CaCl_2$was added for optimal activity of the alpha-amylase enzyme and 2400g whole grain amaranth flour was added. The suspension was boiled for 10 minutes, completely gelatinizing the starch and denaturing any native enzymes in the flour. The suspension was cooled to 50°C and split into sixteen 750mL samples in 1000mL glass beakers.

The suspension was cooled to room temperature ~25°C and pH conditions were modified. The pH of half of the samples (8) was adjusted to 4.5 with Hydrochloric Acid (HCl) and the pH of the remaining 8 was adjusted to pH 7.5 with Sodium Hydroxide (NaOH).

3.3.4 Sample Preparation

Hydrolysis reaction and enzyme deactivation

The methodology was followed as previously described in section 2.3.2.2 modified by water bath and amaranth suspension temperatures at corresponding conditions within the experimental design (Table 7).

3.3.5 Analytical Methods

The analytical methodology for: total sugar, starch, viscosity, moisture, water holding capacity, total dietary, insoluble and soluble fiber measurement, dextrose equivalence calculation and statistical analysis was followed as previously described in sections 2.3.2.3-11.

3.4 Results and Discussion

Factors with main effects of each response are labeled with symbols as denoted in Table 7: Alpha-Amylase (A), Cellulase (B), Xylanase (C), pH (D), and Temperature (E). The values of the main effects, interaction and analysis of variance (ANOVA) were obtained, shown in Table 9. A *P*-value below 0.05 was considered significant. Raw data values discussed in this section are displayed in Table 10.

3.4.1 Total Dietary, Insoluble and Soluble Fiber

Alpha-amylase and pH 7.5 have statistically significant effects on soluble fiber ($P < 0.0001$). The interactions between these effects, AD was also significant ($P = 0.017$) indicating increasing pH and alpha-amylase greatly contribute to the generation of soluble fiber (Figure 16).

There are several theories as to why this interaction may occur. First, when starch is degraded by alpha amylase, the change in viscosity may increase the rate of hydrolysis. By this viscosity decrease the configuration of the amaranth slurry changes, possibly exposing more insoluble fiber to the alkaline conditions, which has greater susceptibility of its conversion into soluble fiber. Furthermore, alkaline conditions have shown to increase soluble fiber concentrations. Saulnier & Thibault, (1999), have shown arabinoxylans in corn bran are mostly insoluble due to cross-linking with ferulic acid. Alkaline treatment releases ferulic acid, solubilizing the arabinoxylans.

3.4.2 Total Sugars, Total Starch, Dextrose Equivalence, and Viscosity

Alpha-amylase significantly affected total sugars at $P = 0.0015$. The effects of hydrolysis by alpha-amylase and pH have a statistically significant source of variation of total starch content at $P = 0.0006$ (A) and $P = 0.0250$ (D). As expected, alpha-amylase has a statistically significant effect on DE and viscosity, both at $P < 0.0001$.

Since the data shows significant effects on total starch (decrease) and total sugars (increase), DE will inherently increase as the calculation of DE is dependent on starch and sugar concentration. As previously described in section 2.4.6, it has been found that with increasing degree of hydrolysis, or increased DE values, the viscosity decreases.

3.4.3 Water Holding Capacity

The main effects of water holding capacity are A and AC and are statistically significant at A at $P < 0.0001$, and AC at $P = 0.0235$. Hydrolysis with alpha-amylase follows a similar trend in design 2 that was previously observed in design 1, in that water holding capacity was significantly decreased when alpha-amylase is present. The AC interaction shows water holding capacity of alpha-amylase hydrolysate was very slightly increased when xylanase is present (Figure 17).

3.5 Conclusions

Most significant soluble fiber increase is credited to alkaline conditions (pH 7.5) solubilizing insoluble components of the grain. Total starch reduced most significantly with alpha-amylase and this correlates with an increase in total sugars. Altering temperature did not have much effect on increasing optimum conditions for either cell wall degrading enzymes used in this study (Cellulase and Xylanase). As previously shown in Chapter 2, alpha-amylase can be used independently to control viscosity and water holding capacity since it has a stronger influence in decreasing the effect of these responses as any other enzyme used.

3.6 Next Steps

Research has been limited at the time of this study regarding the identification of heteroxylans present in amaranth. Grain cell walls are fairly resistant to enzymatic degradation, perhaps due to the highly branched nature of

their components; therefore, alternative conditions and cell wall degrading enzymes are recommended in degradation of whole grain amaranth. Several next steps are proposed in continuing research.

A Response Surface Model (RSM) is proposed to further determine the critical and optimum operating conditions for the system. The first step in RSM is finding a suitable approximation for the relationship between the factors and responses, which this research has accomplished with the first and second two-level factorial designs.

In the preliminary work performed, this research found ideal alpha-amylase dosage 0.4% weight of substrate. Although temperature had little effect on the soluble fiber increase seen in design 2, the optimum temperature for alpha-amylase activity is 70C° (Figure 8). Seventy degrees Celsius is ideal for RSM as it is desired to have this enzyme performing as efficiently as possible to decrease viscosity and expose insoluble fiber to the alkaline conditions. The suggested RSM will utilize 0.4% enzyme dosage and will assess and model the effects of three levels of time (30, 60, and 90 minutes) and the effects of increased pH (7.5, 8.5. and 9.5) on viscosity and conversion of insoluble fibers to soluble.

The regression analysis generated by the proposed RSM will estimate the conditional expectation of the dependent variables in this study, and is only believed to be accurate if the same exact conditions that existed during the period of the study would exist for another study. The regression equation for each response can be used to calculate certain conditions required to create a product.

With statistical analysis, data would be assessed for normality and homogeneity of variance by residual plots. The statistical significance of the terms in the regression equation would be examined by ANOVA for each response; however, the non-significant terms found would be excluded from the model. This design uses the basic model regression equation to fit the data of this design:

$$E(y) = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{12} x_1 x_2$$

Where *E(y)* is the expected response, β_0 is a constant, β_1, β_2, β_{11}, β_{22}, and β_{12} are the regression coefficients and x_1, x_2 are the levels of independent variables (Granato, Ribeiro, Castro, & Masson, 2010). The coefficients of correlation (R^2) would be generated along with their equations for the model.

It can be concluded that the mathematical equation generated will predict the effect of each factor on each response. This may be valuable if a specific viscosity, DE, or starch content (etc.) is desired and can theoretically be calculated using the regression equation.

Additionally, it is suggested to further analyze influence of cell wall degrading enzymes for various reasons. To begin with, the association between phenolic compounds and their influence on flavor is of value and interest in future research. Phenolics have been identified as being the most concentrated in outer bran fractions. Since the non-bound (free) phenolic acids have been shown to be the most flavor-active, their degradation may improve flavor of amaranth hydrolysate by degrading bran selectively to release favorable tasting phenolic compounds (Heiniö, Nordlund, Poutanen, & Buchert, 2012). Furthermore, the release of these bound phenolic compounds has potential to increase phenolic (and antioxidant) bioavailability. Additional research is of importance to developing a process to free these compounds in amaranth to become more bioacessible and bioavailable to human digestion.

Next, alternative enzymes are recommended in degradation of whole grain amaranth. Recent research has displayed new insight into the characterization of the dietary fibers of amaranth (Lamothe, Srichuwong, Reuhs, & Hamaker, 2015). Linkage analysis shows insoluble fiber is composed of homogalacturonans and rhamnogalacturonan-I with arabinan side chains (a form of pectic polysaccharides), highly branched xyloglucans, and cellulose. Soluble fiber is composed of arabinose-rich pectic polysaccharides. These latest findings indicate use of pectinase may be valuable in subsequent studies.

In conclusion, it is proposed to utilize amaranth hydrolysate in a food system, establishing its function in food. Application of this ingredient is suggested to identify its potential use in processed foods. Subsequent studies recommend identifying the effect of this ingredient on flavor, nutrition, and shelf life.

3.7 Tables and Figures

Table 7: 2^5 two-way design indicating actual values as % based on weight of substrate.

	Alpha-Amylase	Cellulase	Xylanase	pH	Temperature		Alpha-Amylase	Cellulase	Xylanase	pH	Temperature
Symbol	(A)	(B)	(C)	(D)	(E)		(A)	(B)	(C)	(D)	(E)
Test condition #	Factor actual values % based on weight of substrate						Factor coded values				
1	0	0	0	4.5	70		-1	-1	-1	-1	+1
2	0.4	0	0	4.5	50		+1	-1	-1	-1	-1
3	0	1.2	0	4.5	50		-1	+1	-1	-1	-1
4	0.4	1.2	0	4.5	70		+1	+1	-1	-1	+1
5	0	0	0.2	4.5	50		-1	-1	+1	-1	-1
6	0.4	0	0.2	4.5	70		+1	-1	+1	-1	+1
7	0	1.2	0.2	4.5	70		-1	+1	+1	-1	+1
8	0.4	1.2	0.2	4.5	50		+1	+1	+1	-1	-1
9	0	0	0	7.5	50		-1	-1	-1	+1	-1
10	0.4	0	0	7.5	70		+1	-1	-1	+1	+1
11	0	1.2	0	7.5	70		-1	+1	-1	+1	+1
12	0.4	1.2	0	7.5	50		+1	+1	-1	+1	-1
13	0	0	0.2	7.5	70		-1	-1	+1	+1	+1
14	0.4	0	0.2	7.5	50		+1	-1	+1	+1	-1
15	0	1.2	0.2	7.5	50		-1	+1	+1	+1	-1
16	0.4	1.2	0.2	7.5	70		+1	+1	+1	+1	+1

Table 8: Main effect values of 2^5 design for the principal factors of analytical responses.

Factor	P-value	Effects ± Standard Error
Total Sugars		
A	0.0015	0.89 ± 0.11
Viscosity		
A	<0.0001	-4.99 ± 0.18
Dextrose Equivalence		
A	<0.0001	2.66 ± 0.24
Total Starch		
A	0.0006	-14.79 ± 1.65
D	0.0250	-8.37 ± 1.65
Soluble Fiber		
A	<0.0001	0.83 ± 0.068
D	<0.0001	0.79 ± 0.068
AD	0.017	0.38 ± 0.068
Water Holding Capacity		
A	<0.0001	-1.41 ± 0.11
AC	<0.0235	0.56 ± 0.11

Table 9: ANOVA results for the responses studied in the 2^5 design.

Response	Degrees of freedom	Sum of Squares	Mean square	F test	p-value
Viscosity					
Model	1	99.720	99.7200	193.910	< 0.0001
A-Alpha Amylase	1	99.720	99.7200	193.910	< 0.0001
Residual	14	7.200	0.5100	-	-
Cor Total	15	106.920	-	-	-
Water Holding Capacity					
Model	3	9	3.1000	16.900	0.0001
A-Alpha Amylase	1	8	7.9500	43.310	< 0.0001
AC	1	1	1.2400	6.730	0.0235
Residual	12	2	0.1800	-	-
Cor Total	15	12	-	-	-
Dextrose Equivalence					
Model	1	28.240	28.2400	29.433	< 0.0001
A-Alpha Amylase	1	28.240	28.2400	29.433	< 0.0001
Residual	14	13.432	0.9595	-	-
Cor Total	15	41.672	-	-	-
Total Sugars					
Model	**1**	**3.169**	3.1692	15.459	0.0015
A-Alpha Amylase	1	3.169	3.1692	15.459	0.0015
Residual	14	2.870	0.2050	-	-
Cor Total	15	6.039	-	-	-
Total Starch					
Model	2	1155.400	577.7000	13.230	0.0007
A-Alpha Amylase	1	875.420	875.4200	20.050	0.0006
D-pH	1	279.980	279.9800	6.410	0.0250
Residual	13	567.510	43.6500	-	-
Cor Total	15	1722.910	-	-	-
Soluble Fiber					
Model	3	5.830	1.9400	26.350	< 0.0001
A-Alpha Amylase	1	2.760	2.7600	37.450	< 0.0001
D-pH	1	2.500	2.5000	33.930	< 0.0001
AD	1	0.570	0.5700	7.670	0.0170
Residual	12	0.890	0.0740	-	-
Cor Total	15	6.720	-	-	-

Table 10: Raw data values determined for 2^5 design.

Coded Factors						Responses							
	Alpha-Amylase	Cellulase	Xylanase	pH	Temperature	Total Dietary Fiber	Insoluble Fiber	Soluble Fiber	Total Sugars	Total Starch	DE	Viscosity	Water Holding Capacity
Test condition #	(A)	(B)	(C)	(D)	(E)	%	%	%	%	%	DE	cPs	%
1	-1	-1	-1	-1	+1	12.00	9.10	2.90	1.89	67.76	0.23	13,947	4.08
2	+1	-1	-1	-1	-1	11.57	8.09	3.48	5.02	50.16	14.49	35	0.45
3	-1	+1	-1	-1	-1	11.00	7.81	3.19	2.55	72.79	0.93	9,166	2.27
4	+1	+1	-1	-1	+1	11.93	8.69	3.24	2.87	71.43	3.17	85	0.38
5	-1	-1	+1	-1	-1	12.09	9.17	2.92	1.81	72.51	0.38	751	1.46
6	+1	-1	+1	-1	+1	14.33	10.59	3.74	2.35	72.83	2.39	72	0.48
7	-1	+1	+1	-1	+1	10.74	7.51	3.23	2.18	77.01	0.74	7,598	1.01
8	+1	+1	+1	-1	-1	12.14	8.54	3.60	5.52	51.78	15.36	26	0.43
9	-1	-1	-1	+1	-1	10.88	7.67	3.21	1.79	70.29	0.28	12,302	1.74
10	+1	-1	-1	+1	+1	12.89	8.25	4.64	1.96	47.97	0.64	48	0.36
11	-1	+1	-1	+1	+1	13.86	9.86	4.00	2.09	66.01	0.63	6,894	1.19
12	+1	+1	-1	+1	-1	12.54	7.81	4.73	6.58	49.86	16.98	30	0.12
13	-1	-1	+1	+1	+1	13.85	10.54	3.31	1.84	69.22	0.34	4,886	0.73
14	+1	-1	+1	+1	-1	13.55	9.24	4.31	6.37	49.51	16.83	31	0.47
15	-1	+1	+1	+1	-1	12.60	9.22	3.38	0.78	66.39	0.97	8,234	0.96
16	+1	+1	+1	+1	+1	14.10	9.05	5.05	8.23	50.09	19.34	45	0.35

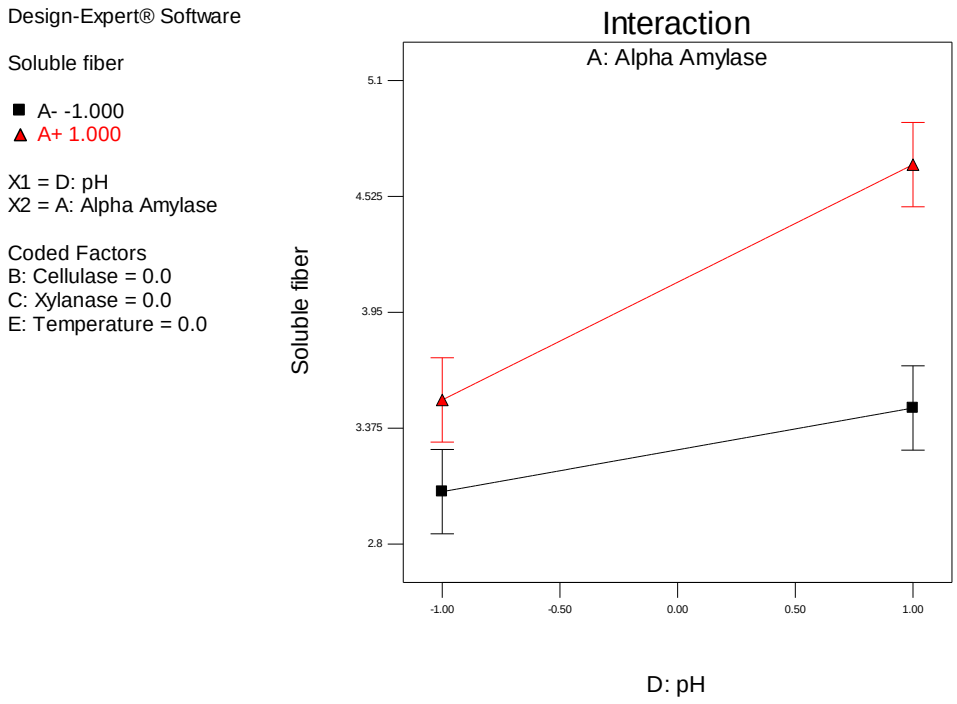

Figure 16: AD interaction on soluble fiber.

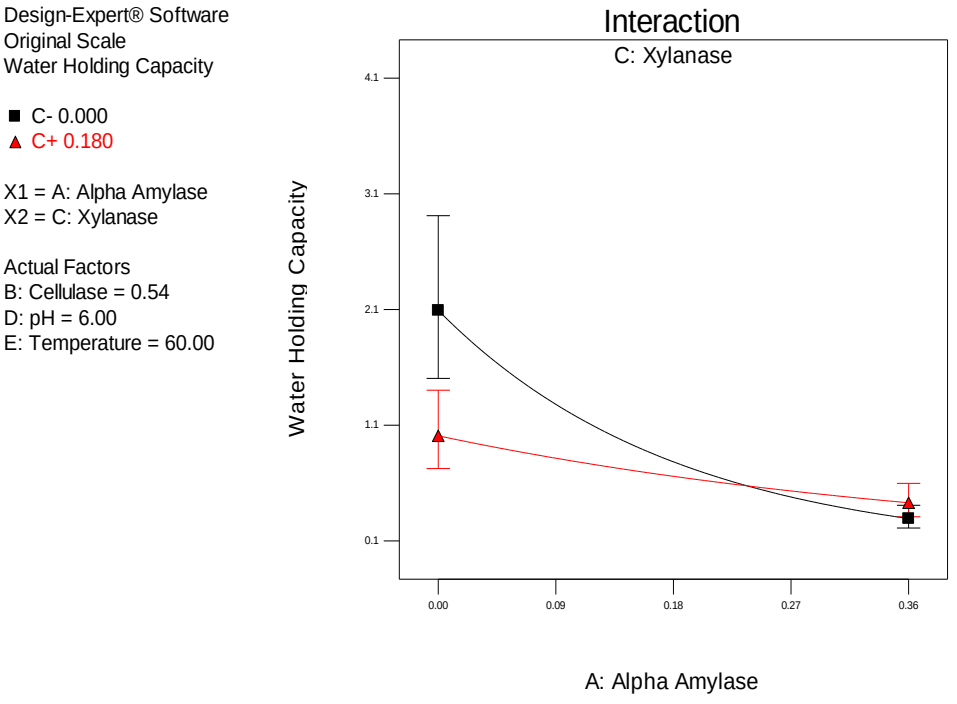

Figure 17: AC interaction on water holding capacity.

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