EFFECT OF POLYSACCHARIDES AND PROTEINS ON REFRIGERATED DOUGH QUALITY

A Thesis
Submitted to the Graduate Faculty
of the
North Dakota State University
of Agriculture and Applied Science

By

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In Partial Fulfillment of the Requirements
for the Degree of
MASTER OF SCIENCE

Major Program:
Cereal Science

November 2009

Fargo, North Dakota

North Dakota State University
Graduate School

Title

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

ABSTRACT

Zhang, Yu, M.S., Department of Plant Sciences, College of Agriculture, Food Systems, and Natural Resources, North Dakota State University, November 2009. Effect of polyaccharides and proteins on refrigerated dough quality. Major Professor: Dr. Senay Simsek.

Refrigerated dough is considered one of the most popular dough products in the food industry. Consumers appreciate the convenience, storage stability, and good organoleptic properties of refrigerated dough products. However, in practice, the quality of refrigerated dough can deteriorate during the storage as a result of liquid formation in dough, which is called "dough syruping". The objectives of this study were (1) characterization of the structural properties of dough components that affect dough syruping; (2) identification of rheological changes that occur during refrigeration, and as they relate to arabinoxylans (AX), starch and protein solubility.

The data showed that AX solubilization and degradation occurred simultaneously with dough syruping. Nuclear magnetic resonance (NMR) spectroscopy analysis and the viscosity analysis of AX aqueous solution confirmed that AX solubilization and degradation resulted in the increase of low molecular weight fraction and the decrease of high molecular weight fraction in water extractable AX (WEAX). The pasting properties and thermal properties of starch changed during the storage: peak viscosity decreased up to 23.1% compared to flour samples during 34 days refrigerated storage. Variation in starch granular morphology was detected. These results showed that physicochemical properties of starch changed during refrigerated storage. The rheological properties of dough changed dramatically during refrigerated storage, which may have significant impacts on end-product quality. Both, the elastic modulus (G') and viscous modulus (G") decreased. Dough

exhibited the major decrease on the moduli on day 3 and day 16. By comparing the viscoelastic properties of dough samples on day 0 and day 16, 50% decrease on the elastic modulus and a roughly 30% decrease in the loss modulus were observed. Changes in the protein fractions of dough samples were related to their rheological properties. Therefore, the physicochemical properties of polysaccharides and proteins appear to be directly correlated to dough syruping, which may result in the diminishment of rheological and organoleptic properties of refrigerated doughs.

ACKNOWLEDGMENTS

To my adviser, Dr. Senay Simsek, I want to express my appreciation for her valuable advice, thoughtful assistance, and continuous guidance throughout this research project and in the preparation of this thesis. It was an honor being her first graduate student.

My sincere appreciation is also extended to:

Dr. Frank Manthey, Dr. Jae Ohm, Dr. Osvaldo Campanella, Dr. Paul Schwarz, and Dr. Jacob Glower. I thank all of them for the assistance, advice, and time contribution to this committee, and efforts for reading and correcting the thesis.

I must thank Kristin Whitney, Angela Ostenson, Hector Chang, Angel Ugrinov, and Daniel Wanner for the lab and equipment help within this project.

I would like to thank Dr. Deland Myers for the valuable suggestions and encouragement. I want to thank the entire professional and support staff of the Cereal Science Program and Department of Plant Sciences for their cooperation and helpfulness.

To the graduate students and my friends in the department, thank you for listening and supporting me. Your friendship has been an encouragement for me.

For all of those who were directly involved through their teachings and hands-on experience in the completion of this thesis and my Master degree, I appreciate all the support.

Special thanks must be given to my parents, Xiuguo Zhang and Hongyun Guan, for their love, sincere support, and understanding that made the fulfillment of this work possible.

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THESIS ORGANIZATION

This thesis has an overall abstract, general introduction, and literature review. The references cited in the general introduction and literature review are given at the end of each section. The thesis is comprised of three separate papers. Each of the papers follows the manuscript style for Carbohydrate Polymers. Each of them has the following sections: abstract, introduction, materials and methods, results and discussion, conclusion, and references cited. Following the three papers, there is a general conclusion.

GENERAL INTRODUCTION

Today's consumers prefer commercial products that are convenient to use, stable during storage and have good organoleptic properties, such as aroma, color, texture, and taste. Refrigerated dough is one of consumer dough products and is designed to accommodate those preferences. The refrigerated dough market is a non-traditional dough product market. Refrigerated dough systems contain typical dough ingredients (flour, water, salt, and chemical leavening agent) and optional flavor agents or other additives (McDilda et al., 1994).

Refrigerated doughs should maintain a stable and fresh appearance during storage. However, it is still a challenge to produce doughs that can be refrigerated for extended periods and can maintain the quality that is similar to doughs that have not been stored at refrigeration conditions. Dough constituents are known to degrade over time, resulting in the loss of textural properties. There is also a particular problem existing in the refrigerated dough products, which is called dough syruping (McDilda & Rice, 1992). Liquid may separate from the dough matrix and form a syrup on the surface of the dough or even can leak out of the package. The consumers are especially concerned about syrup leakage.

In published research, dough syruping is often linked to degradation of arabinoxylans (AX) in the flour and can result in the diminishment of rheological and organoleptic properties, or even complete product failure (Gys et al., 2003). During storage of refrigerated dough, wheat flour endoxylanases hydrolyze water unextractable AX (WUAX) and degrade water extractable AX (WEAX); thus decreasing water-holding capacity of AX, which results in dough syruping (Gys et al., 2004).

There has been limited research conducted in this area, especially about the roles of flour fractions on the quality of refrigerated dough products. There appears to be no clear understanding of which flour components and what changes are primarily responsible for the loss of refrigerated dough quality. Although the functionalities of starch and protein in flour and dough are recognized and dough rheological properties are important attributes for dough quality, there has been no research in terms of their effects on the refrigerated dough quality. Thus, an ongoing need exists for in-depth understanding of the quality changes in refrigerated dough and the structural basis that controls these changes.

The present study was conducted to investigate the following areas:

(1) Characterization of the structural basis of the arabinoxylan component related to dough syruping.

(2) Determination of physicochemical changes in starch components of dough during refrigeration storage.

(3) Identification of rheological changes occurring during refrigeration and their correlation with protein chemistry.

Glenn and Parshall, two Hard Red Spring Wheat (HRSW) varieties, were used in this study. They were selected based on differences in their starch composition.

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LITERATURE REVIEW

Refrigerated Dough and Dough Syruping

Refrigerated dough is defined as a flour-based, unbaked product that is stored between 2-7°C (Allenson, 1982). The refrigerated dough industry was originated from a small bakery in Louisville, Kentucky by a master baker named Lively Willoughby in 1937. The first refrigerated dough product was a chemically-leavened biscuit with a shelf life about three weeks (Allenson, 1982). Today, the refrigerated dough market is one of the fastest growing segments of the ready-to-use, grain-based industry, encompassing a wide range of products available in the United States as well as the international market, including Western Europe and Canada. The items in this category account for more than $1.6 billion / year in sales in the US in 2005 (Ashman & Beckley, 2005).

Refrigerated dough can be prepared by mixing flour, salt, sugar, shortening, water or milk, and leaveners. Refrigerated dough products include doughs for breakfast rolls, dinner rolls, fruit rolls, pizza crusts, and croissants. Traditionally, chemical leavening is used in the refrigerated dough industry to cause the release of carbon dioxide in the dough. Chemically leavened doughs have up to 90 days of shelf life while yeast leavened doughs have about 30 days (Drummond et al., 1994).

Commercially, the dough is prepared in large amounts, then cut into pieces and packed in the containers (Perry et al., 1997). Cylindrical shaped fiberboard and flexible elongated containers are the two most commonly used packages for refrigerated doughs (McDilda et al., 1992). Since the dough is fully prepared, the consumers are left only with the task of baking the dough in order to attain freshness from the oven rolls, biscuits, bread, and pastries.

For both bake-on-site bakeries and food service, there are preferences for developing refrigerated bread dough. Refrigerated bread dough has advantages over existing alternatives, such as in-house batch processing, frozen dough, and par-bake. In-house dough batching is the most costly way to produce dough because it requires dough making skills, a long processing time, and specialized equipment (Lu, 1997). Also, most of the work must be done at night. Frozen dough can be made economically in a high speed bakery, but the quality deteriorates under freeze and thaw cycles that yield inferior flavor, moisture, and shelf life (Wolt & D'Appolonia, 1984; Inoue & Bushuk, 1991). Additional storage cost plus a one day refrigeration thaw cycle requirement are the other drawbacks of frozen dough. Although frozen par-bake has recently made significant gains in flavor and no pre-thawing requirement, it is tough and dry with probably the shortest shelf life of all alternatives. Also, it is pre-shaped and fixed in size; it limits the creativity of the on-site baker. Refrigerated dough would be an alternative to provide lower cost, and better quality, and reduce the use of artificial dough strengtheners while maintaining flavor and shelf life.

The end products made with refrigerated dough are expected to have a texture, appearance, and taste comparable to those of their fresh equivalents. The refrigerated storage chain from production through distribution to the consumer's refrigerator can be lengthy. Therefore, the maintenance of dough quality during refrigeration storage is critical.

In the industry, there has been no concern regarding microbial spoilage in refrigerated dough yet, as it can be efficiently prevented by reduced water activity, carbon dioxide atmosphere in the dough containers, and the maintenance of refrigeration temperature (Allenson, 1982). However, the organoleptic problems have drawn

considerable attention. Under some conditions, a dark-yellow liquid can separate from the dough and form a syrup, which in turn can leak out of the package. This specific problem is called "dough syruping" (Atwell, 1982). The dough suffers discoloration because of the formation of yellowish dough syruping. Also, dough pieces become moist and tacky, and difficult to separate from each other when removed from the can. Both physical texture and handling properties deteriorate. Syruping reduces the shelf life of the packaged dough.

Dough syruping is believed to originate from the natural constituents of the flour and contains water and water-soluble components of the dough. The loss of water implies that the water absorption and retention ability of dough is reduced during storage (Courtin et al., 2006). Lack of water holding capacity as a function of time causes the separation of liquid on the surface of the dough.

A number of methods were reported to control the degree of dough syruping (DDS). Syruping has generally been controlled by changing dough formulation, using absorptive agents, or selecting flours based on their performance (Murphy et al., 1992; Seewi et al., 1993). Less water in the dough formula was used to control the syruping. However, it negatively affected the dough during processing. Food gums were used to act as water binding agents in dough and baking products (Schiraldi et al., 1996). All these methods can result in an adverse effect on dough taste, texture, and rheology. So far, the selection of appropriate wheat flours is the method which is used most (Courtin et al., 2006). The nature and quality of the flour used in refrigerated dough can be crucial for the prevention of dough syruping. Therefore, the type of flour used in these products is carefully monitored by the food industry. The special types of flour with stable water

binding capacity are preferred, but many unpredictable and uncontrolled factors dictate the final quality of the flour, e.g., growing seasons, and environment.

Other methods were claimed to control syruping at the theoretical level without affecting organoleptic characteristics of the dough. They can be categorized into three groups, with the principles depending on the relationship between arabinoxylans (AX), xylanases and the xylanase inhibitors in the dough system. The onset of the dough syruping would be delayed by debranning of wheat kernels before milling. Since the wheat bran contains most of the endoxylanase, debranning of wheat kernels prior to milling yields flour with apparent endoxylanase levels three times lower than those found in flour obtained from non-debranned wheat (Gys et al., 2004). Addition of xylan to the dough recipe can reduce syrup development. According to Atwell (1998), xylan from commercial birch wood or oat spelt increases the water absorption capacity of dough and is a preferable substrate for endoxylanase in the flour than the native AX. Syruping can be delayed by the competition between the substrates and the inhibition of added xylan. The endoxylanase inhibitor added into the dough recipe is capable of inhibiting the microbial endoxylanase present in the flour. Endoxylanase inhibitors on a commercial scale could be used, such as *Triticum aestivum* endoxylanase inhibitor (TAXI) I and II (Poulsen & Sorensen, 2001).

In spite of extensive patent applications (Matz, 1968; McDilda & Rice, 1992; Drummond et al., 1994; Atwell, 1998; Poulsen & Sorensen, 2001), there has been very limited number of peer-reviewed research articles about syruping in refrigerated doughs (Courtin & Delcour, 2001; Gys et al., 2003; Gys et al., 2004). These articles focused on DDS in relation to the arabinoxylan population, reduction of xylanase activity to retard syruping and the involvement of arabinoxylan and endoxylanases. Further research is

needed to investigate the dough syruping problem at the micro-structural level. The main ingredient in the manufacture of refrigerated dough is wheat flour. Therefore, a short review is provided on the structure, chemical composition and functionality of some components in wheat flour.

Arabinoxylans in Refrigerated Dough Quality

Characteristics of Arabinoxylans

Arabinoxylans (AX) are cereal cell wall non-starch polysaccharides (NSP) consisting of a linear backbone of β-(1→4)-linked D-xylopyranose attached with α-L-arabinofuranose residues. The substitution can be either a single C(O)-2 or C(O)-3 attachment or C(O)-2 and C(O)-3 di-substituted. Mono-substitution on C(O)-2 is rare in wheat (Gruppen et al., 1993). The main structural features of wheat flour AX are described in Figure 1. The molecular weight of AX is in the range between 22,000-5,000,000 Da. They are found in the tissues of many kinds of cereals, such as wheat, oat, rice, barley, sorghum, and rye, as well as in other members of the grass family – Gramineae.

In wheat AX, 25-30% is water-extractable AX (WEAX) while the remainder is water-unextractable AX (WUAX) (Courtin & Delcour, 2002). The water extractability and solubility differentiate the WEAX from WUAX and can be explained by differences in chemical and/or physical interactions between AX and other cell wall constituents. WEAX are only loosely bound at the cell wall surface while WUAX are strongly embedded in the cell wall network by interactions with other AX through ferulic acid (FA) based cross-linking or with proteins, lignin, cellulose, β-glucans and glucomannans in the cell wall (Courtin & Delcour, 2002; Frederix et al., 2004). In wheat flour, AX originates from the

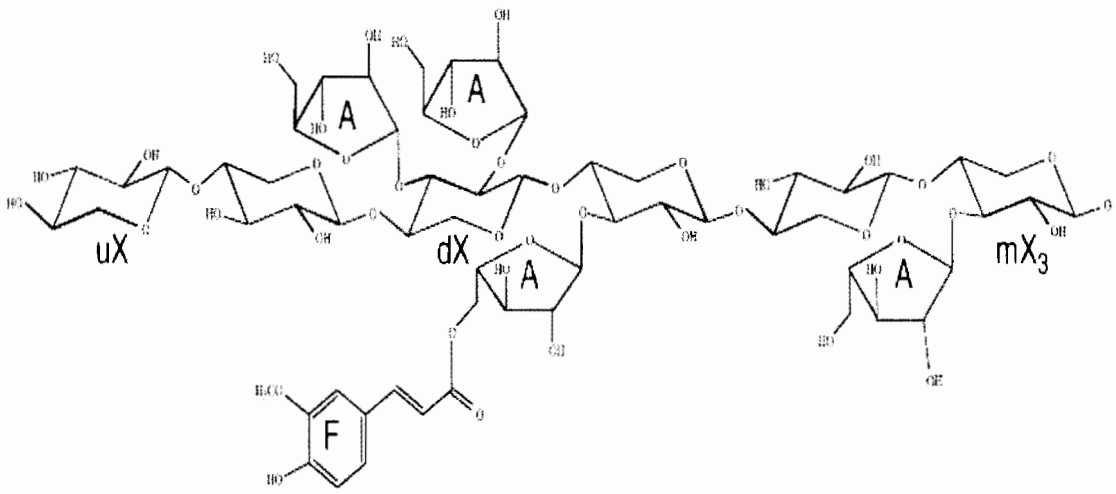

Figure 1. Structural unit of AX from wheat endosperm. A: arabinose; X: xylose; uX: unsubstituted xylose; dX: di-substituted xylose; mX3: O-3 mono-substituted xylose. Source: Saulnier et al. (2007).

endosperm cell wall. The AX level increases with the extraction rate of the flour due to the contamination with bran and aleurone fragments.

In wheat, ferulic (4-hydroxy-3-methoxycinnamic) acid (FA) is a natural component of WEAX and WUAX. It is esterified to C(O)-5 of the arabinose residue, and occurs in high concentrations in aleurone, pericarp and embryo cell walls, but only in trace amounts in the starchy endosperm of ripe kernels. FA plays a key role in cross-linking of AX with other macromolecules. The formation of the covalently cross-linked gel is called oxidative gelation, which is believed to be unique to the WEAX fraction (Hoseney & Faubion, 1981; Biliaderis et al., 1995).

The physicochemical properties of AX are closely related to their structure as these properties depend on the size and shape of AX molecules. WEAX and WUAX are of utmost importance for AX functional properties Native WEAX has a high molecular weight which leads to a highly viscous solution when dissolved in aqueous media. WUAX has a strong water holding capacity (Meuser & Suckow, 1986). The varying ratio of arabinose over xylose (A/X) in AX molecules is also an important parameter characterizing AX. Clear differences in A/X ratios (0.49-0.71) were found between AX in endosperm of different wheat classes and varieties (Izydorczyk et al., 1991).

Wheat flour contains approximately 1.5-2.5% AX (Cleemput et al.,1993). Although AX represents only small percentage of wheat endosperm flour, it has been the subject of extensive research. Wheat AX are important functional ingredients in baked products, affecting water binding, rheology, and starch retrogradation. They also protect the gas retention in dough due to the viscous influence on gluten-starch films.

Water solubility

The delicate balance between chain-chain and chain-solvent interactions determines the water solubility of AX (Saulnier et al., 2007). Generally, a higher degree of substitution favors water solubility. Arabinosyl substitution stiffens the β-D-xylan backbone, resulting in a more extended conformation and limiting aggregation of AX molecules (Ragaee, 2001). For AX, water solubility is not only related to structural features of the polymer chain, but also to the covalent linkage to other cell-wall polymers. Andersson (1993) reported that partial removal of substituents by hydrolysis decreases solubility of AX because the adjacent xylan residues can form inter-chain associations more easily and consequently produce more insoluble aggregate.

Viscosity

AX is usually assumed to have an extended wormlike conformation in aqueous solution. The behavior of AX in aqueous solution is characteristic of semi-flexible polysaccharides (Izydorczyk & Biliaderis, 1995). Intrinsic viscosity is the measurement of the hydrodynamic volume of the isolated polymer in the solution, which is different from the viscosity of the solution (Saulnier et al. 2007). Intrinsic viscosity is the ratio between specific viscosity and concentration, with the units of inverse density. Intrinsic viscosity of AX is affected by its structure, such as A/X ratio, the substitution pattern, and the chain length of the molecules. Usually, higher arabinose substitution is related with increased asymmetry of AX and thus with higher intrinsic viscosity. A chain with a high degree of substitution has a rigid rod-like conformation, which is partly responsible for the high viscosity of AX solution (Andrewartha et al., 1979). In contrast, it was also found that the wheat endosperm AX polymers isolated from several cultivars have low A/X ratios, but

high intrinsic viscosity value. However, those polymers had high feruloyl residue content and a low content of double-substituted Xyl*p* which can also contribute to the high intrinsic viscosity. The behavior of AX in solution is not solely influenced by the overall asymmetrical conformation, but also by the specific arrangement of arabinose residues along the xylan backbone. The relative amount of single-substituted Xyl*p* at O-2 vs. O-3 increased with decreasing intrinsic viscosity, and short Ara*f* side chains were more common in fractions having low limiting viscosity (Izydorczyk & Biliaderis, 1992). The length of the chain backbone is another determinant of the size of these polysaccharides (Izydorczyk & Biliaderis, 1995).

Apparent viscosity is the resistance of a substance to deformation under a particular set of conditions. The apparent viscosity of AX aqueous solutions was affected by the shear rate due to the disruption of the intermolecular association between polymers (Ragaee 2001). The relative molecular weight of AX determines the magnitude of shear thinning, as indicated by a low non-Newtonian index of the power law model. The difference in the chain length and fine structure was reflected in the difference in critical concentration among different AX fractions. However, an opposite opinion against the above generally accepted assumption held that the presence of arabinose side-chains had no significant influence on the conformation (rigidity) of the xylan backbone as indicated by molecular modeling (Ordaz-Ortiz et al., 2004). Saulnier et al. (2007) also reported that the conformation of the AX chain is not affected by the degree of branching of the xylan backbone. They concluded that structural features, such as A/X ratio, probably have very limited effects on the viscosity of the AX solution. The viscosity of the AX solution is

therefore mainly dependant on changes in the concentration and molecular size of the polymer.

Water holding capacity

When AX are added into wheat flour, they compete with other constituents of dough for water. Bushuk (1966) calculated that about 225 mg of AX is associated with 1.0 g of water in the dough. WUAX have a strong tendency to absorb water and swell, and have been reported to hold 6.7 times their weight in water. WEAX are said to have water holding capacity with retention of 6.3 times their weight in water (Jelaca & Hlynca, 1971). The amount and molecular weight of AX are important determinants of the extent of these effects (Izydorczyk & Biliaderis, 1995). Oxidative gelation can greatly increase the hydration capacity of AX (Izydorczyk et al., 1990).

Overall, it is a generally accepted assumption that the behavior of AX in solution is determined by the overall asymmetrical conformation, the degree of polymerization, the specific arrangement of arabinose residues along the xylose backbone, and the backbone chain length. The main reason that AX influence refrigerated dough syruping is probably because of their water-holding capacity. The water holding capacity of the flour WUAX was radically reduced when hydrolysed by endoxylanases (Courtin et al., 2006).

Characteristics of Endoxylanases

Xylanolytic enzymes include Endo-(1,4)-β-D-xylanase (EC 3.2.1.8), α-L arabinofuranosidase (EC 3.2.1.55), (1,4)-β-D-xylosidase (EC 3.2.1.37), and feruloyl esterases (EC 3.1.1.73). The most important ones are endoxylanases in terms of the effects on AX functionality. Endoxylanases have a strong influence on AX structure and functionality. They attack the AX backbone internally and result in a decrease in the degree

of polymerization of the substrate and the molecular weight of AX (Courtin et al., 2006). They also work with the other xylanolytic enzymes synergistically to achieve further and complete hydrolysis of the AX molecules. For example, endoxylanases attack the AX backbone internally, then α-L-arabinofuranosidase releases arabinose side chains from AX.

Endoxylanases occur in most plants and are also found in bacteria and fungi (Dekker & Richards, 1976). Cereal grains have endogenously low endoxylanase activity, such as wheat, barley, rye, and the different tissues of wheat. Expression of the genes encoding endoxylanases in germinated grain appears to be confined largely to the aleurone layer, and no mRNA transcripts could be detected in young vegetative tissues (Banik et al., 1996). During germination, the aleurone tissue secretes endoxylanases into the endosperm storage tissue. Endoxylanases hydrolyze the endosperm cell wall, which makes the starch and protein more accessible to amylases and proteases secreted from the aleurone or scutellum (Corder & Henry, 1989).

Endoxylanases can be mainly classified into glycosyl hydrolase families 10 and 11 based on genetic information and structural analysis (Henrissat, 1998). Family 10 endoxylanases have a more complex structure and relatively higher molecular weight than family 11 endoxylanases. Also, family 10 endoxylanases have less substrate specificity and produce oligosaccharides with a low degree of polymerization, while family 11 endoxylanases tend to be more specific for xylan and produce larger oligosacchardies (Moers et al., 2003). Fungi and bacteria produce endoxylanases from both family 10 and 11, whereas all plant endoxylanases so far identified belong to family 10 (Simpson et al., 2003). Therefore, all endogenous wheat endoxylanases most probably belong to family 10. Endoxylanases differ in substrate selectivity, which means that some of them preferably

degrade WEAX while other endoxylanases have preference for WUAX solubilization. The family 10 endoxylanase of *Aspergillus aculeatus* preferentially degrades WEAX, while the family 11 endoxylanase of *Bacillus subtilis* more readily solubilizes WUAX (Courtin & Delcour, 2001). Depending on the substrate selectivity of the endoxylanases, the degradation of WEAX or the solubilization of WUAX will initially prevail.

Endoxylanases degrade native WEAX to a lower molecular weight, thereby lowering viscosity. They render WUAX soluble, resulting in a loss of water holding capacity. The solubilization of WUAX would initially increase the viscosity because of the resulting solublised high molecular weight AX segments. However, further degradation of enzymically solublised AX into smaller molecules will decrease the viscosity.

Endoxylanases from microbial sources are commonly used in the food industry for the process of separating wheat gluten and starch where their main function is to break down WEAX. The lower viscosity of the batter results in better processing conditions, and higher gluten and starch yields. In bread-making, endoxylanases are routinely used to improve the dough handling, oven spring, and loaf volume. Using an endoxylanase at a proper dosage can result in redistribution of the water in the dough and a rise in viscosity.

Impact of AX and Endoxylanases on Dough Syruping

Dough syruping is believed to be caused partially by the enzymatic degradation of AX (Courtin et al., 2006). Endoxylanases are primarily responsible for syruping because the substrate for their action is the unsubstituted xylan compound. This compound is relatively less hygroscopic compared to the native AX, thus resulting in the loss of water holding capacity (Atwell, 1998). AX act as water binding agents in the dough. Water absorption capacity of the dough is reduced during storage. Thus, the water which was

originally bound by AX is freed and migrates to the dough surface. The loss of water holding capacity caused by hydrolysis of AX is proposed to be the main cause of dough syrup formation (Gys et al., 2003). In the meantime, the degradation induces changes in the physicochemical properties of AX. As a function of time, wheat flour associated endoxylanases solubilize WUAX, leading to the decrease of the water-holding capacity of the dough by diminishing WUAX content. Further degradation of enzymatically solubilized AX and WEAX into low molecules weight residues has a negative impact upon the viscosity of the dough aqueous phase.

Atwell (1998) added commercial birch wood and oat spelt xylan to the dough and investigated their functions on the dough syruping development. According to this work, the added xylan is a competitive substrate for endoxylanases. Thus, the endogenous endoxylanases preferentially degrade the added xylan rather than native AX in wheat flour, which reduces syruping. The addition of AX degrading enzymes to the wheat flour in buffer increased the liquid development (Beldman et al., 1995), which explained the work of Atwell in a more fundamental way.

Further evidence for the role of endoxylanase is suggested by the existence of proteinaceous endoxylanase inhibitors. An endoxylanase inhibitor which is present in wheat flour can reduce or prevent the enzymatic degradation of AX (Rouau & Surge, 1998). This inhibitor is a di-peptide with molecular weight of about 29 kDa, and it has a pI of about 8 to 9.5 (McLauchlan et al., 1999). The involvement of AX, endoxylanase, and endoxylanase inhibitor in dough syruping was illustrated by the work of Poulsen and Sorensen (2001). Variable levels of endoxylanase inhibitor were added to dough, and

syruping was decreased as the doughs were stored for 10 days. They hypothesized that the endoxylanases inhibitor can reduce or prevent enzymatic degradation of AX in the dough.

Starch in Refrigerated Dough Quality

The Structure and Physicochemical Properties of Starch

Wheat flour consists of 70-80% starch on a dry matter basis. Therefore, the role of starch is likely important in refrigerated dough quality. Wheat starch granules show a bimodal size distribution of A and B type granules: the large, lenticular (A type) with a diameter of more than 10 μm, and small, spherical (B type) with a diameter of 1-5 μm) (Jane et al., 1994). The starch granules consist of a mixture of two polymers: a basically linear polysaccharide (α-1→4 linked glucose) named amylose, and a highly branched (α-1→6 branches) polysaccharide named amylopectin.

Cereal starches produce an X-ray diffraction pattern (A-type pattern) that is indicative of parallel, double helices separated by interstitial water. Granules, with molecules arranged radially, contain both polycrystalline and noncrystalline regions in alternating layers. The clustered branches of amylopectin occur as packed double helices. Those double-helical structures form many small crystalline regions in the dense layers of starch granules that alternate with the less-dense regions, generally called amorphous layers. Amylose molecules reside in amorphous regions and can diffuse from water-swollen granules. Because the crystallinity is produced by ordering of the amylopectin chains, waxy starch (the starch which does not have amylose) has about the same amount of crystallinity as does normal starch. The starch crystalline structure is related to the branch chain length of amylopectin (Hanashiro & Hizukuri, 1996).

The granule size has been correlated to different rheological properties and baking characteristics. The relative proportion of the types of starch granules is thought to influence gelatinization and pasting properties (Peng et al., 1999). The inner structure of the starch granules also determines its functionality. The structure of amylose and amylopectin and their relative contents in starch granules determine pasting, gelation, and retrogradation properties of starch and hence, product quality and stability. Amylopectin is the major component of most starch; it exists in all the known starches. Its fine structure plays a critical role in the characteristics of starch. The branch chain length affects the gelatinization, retrogradation and pasting properties (Jane & Chen, 1992).

Low amylose starches are caused by mutations at the waxy (Wx) locus, which encodes the granule-bound starch synthase (GBSS) protein, also called Wx protein. This enzyme is involved in amylose synthesis and its entire or partial loss results in no or low amylose content starches. When starch is primarily composed of amylopectin, it is defined as waxy starch. Hexaploid partial waxy wheats are characterized by slight reduction (2-3% lower) in amylose content due to the absence of GBSS, alleles at one or two of the three Wx loci (Nakamura et al., 1995).

Partial waxy wheat seems to have intermediate properties between normal and full waxy wheats. The potential use of wheat with reduced amylose content is a current focus of interest among wheat breeders, geneticists and cereal scientists (Dong et al., 2007; Qin et al., 2009). Partial waxy wheats are sources of flours with optimal quality characteristics in certain Asian wet noodle products. They are preferred for the production of Asian *Udon noodle* because of their high swelling volumes and high peak viscosities (Crosbie, 1991).

Partial waxy wheats were shown to enhance *Udon noodle* quality, which generally decreases with an increase in flour amylose content (Oda et al., 1980).

Impact of Starch on the Quality of Dough Products

There is no report that explains the role of the starch component in refrigerated dough systems; however, this has been studied in frozen dough. It is believed that understanding the role of starch in refrigerated dough can be enhanced by understanding the role of starch in frozen dough. Lu & Grant (1999b) used fractionation and reconstitution of wheat flour components to investigate the roles of starch and other components in bread-making quality of frozen dough. They found that the starch fraction contributed significantly to frozen dough quality. Also, they found that starch isolated from frozen dough made with different wheat flour varied greatly in enthalpy (ΔH), and all the samples showed a significant increase of ΔH with increased storage time (Lu & Grant, 1999a). This observation could be explained by the way that water interacts with the starch during the freezing process and the rate of water migration in the frozen dough during frozen storage (Kulp, 1995). Low-temperature Scanning Electron Microscopy (SEM) was used to examine the effects of storage on the structure of frozen bread doughs. Starch granules were no longer associated with gluten fibrils in the dough structure (Kulp, 1995). The onset temperature of starch gelatinization measured by differential scanning calorimetry (DSC) increased during frozen storage (Autio & Sinda, 1992). The increase of onset temperature may be attributed to the delay in the rate of diffusion of water into the starch granules or the increased crystallinity of the granules. In frozen dough, the amylose-amylopectin ratio was negatively correlated with frozen storage time (Wolt & D' Appolonia, 1984). It was uncertain whether this relationship reveals any fundamental

functional effect or whether it simply reflected a higher degree of retrogradation of the soluble starch polymers in frozen dough. Understanding the changes in the physical and functional properties of starch during storage would be helpful to predict the quality of the end product.

The Funtion of Alpha-amylase

Alpha-(1→4)-Glucan 4-glucanohydrolase (EC 3.2.1.1), also called α-amylases, are endoenzymes that catalyzed the hydrolysis of α-(1→4) glycosidic linkages in the starch polysaccharides, amylose and amylopectin, and their degradation products. The enzymes can be produced by plants, fungi, bacteria, and animals and differ in physical and chemical properties.

Both the α-amylase source and the nature of the substrate determine the mode of starch degradation. Amylases are endogenously present in wheat flour, but their activity can vary considerably. In general, α-amylase activity is low in unmalted flour. It can hydrolyze the damaged starch particles produced from milling during the dough stage and generate low molecular weight dextrins (Drapron & Godon, 1987). The α-amylolysis of amylose and amylopectin differs due to the linear and branched nature of these polymers. Amylopectin is hydrolyzed at a slower rate than the linear amylose because the branches impose sterical constraints (Greenwood & Milne, 1968).

Protein and Dough Rheology

The Role of the Protein Component in the Flour and Dough

Proteins are one of the primary factors responsible for the variation in wheat quality. High protein content is normally associated with increased dough strength and

improved baking quality of the bread wheat (Johansson et al., 2001). From a chemical point of view, wheat proteins can be divided into two primary groups: the soluble proteins and the insoluble gluten proteins. The soluble groups, which can dissolve in the natural aqueous mediums, consist of albumins, globulins, and peptides. The insoluble proteins are gliadins and glutenins which constitute 80-85% of the wheat storage protein. During mixing, they can form gluten, which provides the unique viscoelastic and gas-retaining characteristics in dough. Gluten plays a key role in determining the quality of wheat flour and its derived end products (Veraverbeke & Delcour, 2002).

From a functional point of view, wheat endosperm proteins can be classified into polymeric proteins (mostly glutenin) held together by interchain disulphide bonds, and monomeric proteins (albumins, globulins, and gliadins) (MacRitchie, 1992), in which the only disulphide bonds are intrachain. Gliadin is soluble in 60-70% aqueous ethanol and provides viscosity and extensibility. The proteins in the gliadin fraction have a molecular weight range from 25 to 100 kDa when analyzed by gel filtration. They have little or no resistance to extension and appears to be responsible for dough's cohesiveness (Hoseney, 1998). Glutenin proteins vary in molecular weight from about 100,000 to several million Da with an average of about 3 million Da. These proteins provide dough with its resistance to extension, and are considered to be the most important wheat flour proteins in giving strength and elasticity to the dough.

High Performance Size Exclusion Chromatography (HP-SEC) for the proteomic analysis of wheat protein was developed in the mid-1980s (Bietz, 1986) and is widely used now. This technique does not require protein reduction, and results obtained are highly correlated with the bread making quality of flours, particularly when considering the first

peak (polymeric protein) of the chromatogram, and when determining the molecular size distribution within the polymeric fraction (Gupta et al., 1993).

Wheat endosperm proteins have been extensively studied due to their viscoelastic properties that give wheat flours unique baking characteristics (MacRitchie, 1987; MacRitchie et al., 1991). Wheat gluten proteins largely affect the rheological properties of dough (Janssen et al., 1996). Although starch granules affect rheological properties of dough by working as a supporting material of the protein phase and interact with protein molecules, the complicated viscoelasticity of dough is mainly due to its unique protein, gluten.

Dough Rheology

Rheology is the study of the deformation and flow of matter under the influence of an applied shear stress or extensional stress. Rheological properties are the mechanical properties of materials that flow (liquid) and deform (solid) and are expressed in terms of the effects of stress, strain, and time (Steffe, 1992a).

In a Hookean solid, the deformation (strain) is directly proportional to the applied force (stress), independent of the strain rate:

$$\sigma = E\gamma$$

σ is the stress and γ is the strain. E is called the elastic modulus or Young's modulus, which is the ratio of stress to strain in a material.

In an ideal liquid, even a small force can cause the liquid to flow, and the flow is completely irrecoverable when the stress is removed. This type of liquid is called Newtonian liquid. In a Newtonian liquid, the rate of flow is directly proportional to the applied force.

$$\sigma = \eta \frac{d\gamma}{dt}$$

η is called the coefficient of viscosity, which is the applied shear stress divided by the resulting shear rate.

Materials can be divided into three groups: elastic, viscous and viscoelastic (Hoseney, 1998). Most food materials exhibit viscoelastic properties. Dough is a typical example of a viscoelastic material which is complicated in its rheological behavior, and shows a combination of viscous and elastic properties. The structures of the material and interactions between macromolecules forming the dough have a strong influence on its behavior. Thus, viscoelastic properties are a macroscopic reflection of the structural and chemical characteristics of the materials.

The rheological properties of the dough are not only important to cereal chemists, but also to the bakers and the baking industry. Scientists can observe correlations between the quality of the flour and the physicochemical properties of the dough. For the baking industry, it is important to measure the rheology of dough from the mechanical point of view with the increasing trend on mechanization and automation of these processes.

The most common utilized instruments to study the rheology of dough are farinograph, mixograph, alveograph, and extensigraph, which are referred to as "empirical rheological measurements". These provide information on the quality and performance of cereal products, such as consistency, hardness, texture, and viscosity (Hoseney, 1998). Although they are good quality control tools, they are highly dependent on the sample size and the type of geometry used for testing. On the other hand, these empirical tests do not describe the macroscopic structure of the material, composition, and potential interactions

between macromolecules as fundamental methods do. From a research point of view, their data can hardly be interpreted in basic terms.

Fundamental rheological methods are applied widely for dough testing and to study dough properties and the interactions among its chemical components, as illustrated by Lu & Grant (1999a). There are two common ways to measure viscoelastic behavior fundamentally: static and dynamic measurements. Static measurement includes creep and stress relaxation, where a sample is placed under a known constant stress or strain and the respective strain or stress is measured for a given time. Dynamic oscillation measurement is one of the most popular and widely used fundamental rheological techniques for measuring cereal doughs and batters (Dobraszczyk, & Morgenstern, 2003). Methods used for dynamic measurement of viscoelastic behavior include small amplitude oscillatory tests where a sample is subjected to a low strain sinusoidally varying at a given frequency (ω).

The small amplitude oscillatory test is a rapid test with practically little effect on the sample chemical or physical changes. The small-strains assure a linear viscoelastic behavior and the application of various frequency and temperature sweeps allows a more complete determination of rheological properties (Steffe, 1992b).

If a sample exhibits linear viscoelastic behavior, the stress will oscillate in response to a sinusoidally oscillating strain at a frequency ω.

$$\sigma=\sigma_0 \sin(\omega t + \delta)$$

$$\sigma=\gamma_0(G' sin\omega t + G'' cos\omega t)$$

G′ is the storage modulus and G″ is the loss modulus (Faridi, & Faubion, 1986). G' is related to the storage of energy as potential energy and its release during deformation. It is an indicator of the elastic characteristic of the material. G″ is related to the dissipation of

energy as heat as the material is deformed and indicates a viscous behavior of the material. The tangent of the angle by which the stress and strain are out of phase, called the loss tangent, is a dimensionless parameter used to characterize viscoelastic materials. The ratio of the moduli is the measure of the relative contributions of the viscous and elastic components to the rheological behavior of the sample.

$$\tan \delta = \frac{G''}{G'}$$

Although many researchers have looked at fundamental rheological methods, they could not find a rheological method that can completely replace the empirical methods due to the complexity of dough systems. However, valuable information has been provided on the relationship between these two rheological methods (Kenny et al., 1999).

Rheological changes of refrigerated dough have been studied by empirical methods (Gys et al., 2003) to investigate the changes that could affect the quality of the dough during refrigerated storage. No one has yet explained dough syruping at the micro-structural level by investigating structural changes in dough-macromolecules and their functionality using fundamental rheological methods.

In summary, during extended periods of dough storage, either under freezing or refrigeration conditions, there is a substantial deterioration in the quality of the dough. The loss of stability of refrigerated dough subjected to an extended refrigeration storage period is still a problem that needs to be solved.

The overall goal of this research was to elucidate the physicochemical changes occurring in samples of refrigerated dough made with different types of Hard Red Spring Wheat (HRSW) which differ in the starch composition. This study will provide a clear and deep understanding of structural changes that occur during dough refrigeration storage.

Hypothesis

The hypothesis of the research are 1) physicochemical properties of polysaccharide components, including arabinoxylans (AX) and starch, in refrigerated dough system change and are correlated to the degree of dough syruping (DDS); 2) There are rheological changes in refrigerated dough during storage and these changes are mainly associated with protein chemistry.

In this study, the phenomenon and origin of dough syruping in refrigerated dough made of two different HRSW flours were investigated. Arabinoxylans and starch were isolated from refrigerated doughs and their structural changes were studied. The rheological changes during refrigeration storage were identified by both empirical and fundamental rheological methods and these changes were directly related to protein chemistry.

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PAPER 1. PHYSICOCHEMICAL CHANGES OF ARABINOXLANS IN REFRIGERATED DOUGH

Abstract

Dough syruping is considered a problem associated to the quality of refrigerated dough products. It was proposed that the degradation of arabinoxylans (AX) by endogenous xylanase is one of causes for the dough syruping development. The objectives of this study were to determine the endoxylanase activity and monitor the physicochemical changes in AX chemistry during dough refrigerated storage. Arabinoxylans were extracted from the refrigerated dough samples. The changes in relative molecular weight and the monosaccharide composition were investigated and the viscosity of AX solutions was measured. Results showed that the structural features of AX change dramatically during storage, resulting in changes in the physicochemical properties. Based on the High Performance Size Exclusion Chromatography (HP-SEC) profiles, the high molecular weight fraction (HMWF) of Glenn samples were decreased by 2% and HMWF of Parshall samples were decreased by 17% after 34 days of refrigerated storage. The viscosity of water extractable AX (WEAX) isolated from Glenn samples was reduced by 18% and the viscosity of WEAX from Parshall samples were declined by as much as 43% during the storage. These observations were directly correlated with the deterioration in the refrigerated dough quality.

Introduction

Arabinoxylans (AX) are the major non-starch polysaccharides found in wheat flour. They are heteropolysaccharides and consist of a backbone structure of β-1,4-linked-D-xylopyranosyl, which can be unsubsitituted and variously substituted with arabinose residues at the C-3 or the C-2 and C-3 positions of xylose backbone (Gruppen et al., 1993). Wheat flour contains approximately 1.5-2.5% AX of which 25-30% is water extractable AX (WEAX), while 70-75 % is water unextractable AX (WUAX). WEAX and WUAX differ in physicochemical and functional properties, including viscosity-forming potential, water-binding capacity, solubility, and gel-forming capacity (Courtin & Delcour, 2002). The variable ratio of arabinose to xylose (A/X) in AX molecules is an important parameter characterizing AX. Differences in A/X ratios (0.49–0.71) were found between AX in endosperms of different wheat classes and varieties (Rattan et al., 1994). Furthermore, A/X ratio plays a role in the physicochemical properties of AX (Izydorczyk & Biliaderis, 1995).

Although the amount of AX in flour is relatively low compared to other components of flour, there has been tremendous interest due to the various functionalities of AX. AX have significant influences on the behavior of the grain during milling, the quality of the flour, the rheological properties of the dough and the quality of the baked products (Courtin & Delcour, 2002; Delcour et al., 1991). Most of the AX from wheat endosperm are water extractable and result in highly viscous aqueous solutions. The hydration properties of native AX have direct influence on the ability of flours to absorb water. The high water-holding capacity of the cross-linked polymers might affect the distribution of moisture among the dough constituents, thereby altering the rheological properties of dough system. WEAX positively affect the dough and bread quality due to

their water-binding capacity and viscosity. Addition of, WUAX decreases the loaf volume (Courtin et al., 1999).

Wheat flour contains several functionally significant enzymes, such as xylanases and amylases. These enzymes can play critical roles in determining the functional characteristics of the flour when they become active as water added (Rani et al., 2001). Endoxylanases (EC 3.2.1.8), the most important enzyme in the group of xylanolytic enzymes, hydrolyze the AX, thereby decreasing their water holding capacity. Xylanases differ in their relative capabilities to degrade the unsubstituted or arabinose-substituted parts of the xylan backbone (de Vries et al., 2000). Xylanases degrade high molecular weight WEAX to low molecular weight WEAX. An optimal dose of xylanases results in a redistribution of the water in the dough and a rise in viscosity (Courtin & Delcour, 2002).

The natural constituents of the flour, AX and the endogenously present xylanases are believed to be involoved in dough syruping development (Gys et al., 2003). Arabinoxylans degradation by xylanases results in a loss in water-holding capacity of the dough and would be responsible for dough syruping formation (Atwell, 1998). Dough syruping can be reduced by the addition of xylanase inhibitor (Poulsen & Sorensen, 2001), which is also an indication of the importance of flour endoxylanases on dough syruping.

Arabinoxylans are usually assumed to have an extended wormlike conformation in aqueous solution. The behavior of AX in aqueous solution is characteristic of semi-flexible polysaccharides (Izydorczyk & Biliaderis, 1995). The apparent viscosity of AX solution is mainly dependant on changes in the concentration and molecular weight of the polymer (Saulnier et al., 2007). The molecular weight of AX determines the magnitude of shear thinning, as indicated by a low non-Newtonian index of the power law model. It is also

affected by shear rate due to the disruption of the intermolecular association between polymers (Ragaee, 2001).

The objectives of this research were to characterize the structure and rheological properties of AX from refrigerated dough at various storage points, and to determine the correlation between the physicochemical properties of AX and the changes in refrigerated dough quality during storage.

Experimental

Materials

Glenn and Parshall, two wheat varieties of Hard Red Spring Wheat (HRSW), were obtained from North Dakota State University (NDSU) Casselton Research Extension Center in 2007. Glenn is one of the most commonly grown varieties in North Dakota, which is the main HRSW growing area of the United States. It is normal hexaploid wheat. Parshall is a partial waxy hexaploid HRSW cultivar, developed by North Dakota State University. Differences in the starch composition of the two varieties is shown in Table 1.

The grain was milled using a Buhler MLU-202 laboratory mill (Buhler Inc., Plymouth, MN) in the NDSU HRSW quality laboratories. Test weight, wheat protein, falling number, flour ash, flour protein, wet gluten, farinograph absorption and total starch were determined using standard AACC methods 55-10, 46-30, 56-81B, 08-01, 46-30, 38-12A, 54-21, and 76-13, respectively (Table 1).

Table 1. Properties[a] of HRSW varieties, Glenn and Parshall[b], used in this study.

Variety	Test weight (kg/hl)	Wheat protein (%)[c]	Falling Number (sec)	Flour ash (%)[d]	Flour protein (%)[d]	Wet Gluten(%)[d]	Farinograph absorption (%)[e]	Amy/AP ratio[f]
Glenn	83.0	14.3	429	0.49	13.6	34.8	66.2	0.32
Parshall	80.8	13.5	465	0.51	12.8	32.3	64.2	0.28

[a] Values are are means of duplicates.

[b] The samples were grown in North Dakota State University (NDSU) Casselton Research Extension Center in 2007.

[c] The analysis was expressed on 12% moisture basis.

[d] The analysis was expressed on 14% moisture basis.

[e] Water absorption to reach 500 farinograph units (FU) line and based on 14% moisture basis.

[f] Amylose/Amylopectin ratio

Preparation of Refrigerated Dough

In order to avoid confounding factors arising from the presence of other ingredients, a lean dough formula was used. The dough was prepared using 100 g of flour (14% moisture basis), 1.8 g of salt, and a certain amount of water (containing 0.06% w/v of sodium azide to prevent microbial spoilage), to reach the desired moisture content previously determined according to the specific farinograph absorption test. Dough was mixed in a 100g pin mixer (National Manufacturing, Lincoln, NE) for the optimum mixing time, sheeted, molded, and stored in plastic containers for 34 days at 6°C, during which the

dough syruping was measured. The dough samples from storage days 0 (analysis was done immediately after mixing), 1, 2, 3, 6, 10, 16, and 34 were lyophilized and ground by mortar and pestle (Simsek & Ohm, 2009). These were subsequently referred to as G1, G2, G3, G6, G10, G16, G34 and P1, P2, P3, P6, P10, P16, P34, where G and P denoted the Glenn and Parshall samples, respectively.

Dough Syruping Determination

Dough syruping was measured as the liquid released by the dough after centrifugation (22,000 × g, 20°C, 30 min). The dough was divided in pieces of approximately 10 g and after centrifugation of accurately weighed dough pieces; the liquid inside of the centrifuge tube was removed with a glass pipette. The syrup released was calculated as the difference in weight between the tubes before and after syrup removal and was expressed as a percentage of the initial dough weight.

Xylanase Activity

Xylanase activity in flour and lyophilized dough samples was measured using the Xylazyme-AX method (Megazyme, Bray, Ireland) with modifications of the manufacturers instructions. Flour or lyophilized and ground dough samples (1:5 w/v) were suspended in sodium phosphate buffer (25 mM, pH 6.0) and the slurry was extracted for 1 h at 6 °C. After centrifugation (3,000 × g, 10 min, 6 °C), the supernatant (1.0 mL) was pre-incubated at 40 °C for 10 min before adding an AZCL-AX tablet. After incubation at 40 °C for 17 h, the reaction was stopped with 1.0% Trizma base solution (5 mL). Following filtration, the E_{630} values (extinction at 630 nm) were measured against a control, prepared by incubating the solutions without the substrate tablet for 17 h at 40 °C and addition of the substrate

tablet after adding 1.0% Trizma base solution to the extract. Correction was made for nonenzymatic color release from the AZCL-AX tablets.

Extraction of Water Extractable and Unextractable Solids (WES & WUS)

Extraction of WES and WUS from dough samples was performed as described by by Courtin (et al., 2001) with some modifications. Lyophilized and ground dough samples (4.0 g) were weighed into a 50 mL centrifuge tube. Deionized water (20 mL, 4 °C) was added, and the centrifuge tubes were shaken at 200 rpm (30 min, 4 °C). After centrifugation (10,000 × g, 15 min, 4 °C), the supernatant was transferred to a flask and immediately frozen at -80 °C. The precipitate was washed with water (20 mL, 4 °C) and centrifuged again as above. The supernatant was added to the flask and frozen, and was referred to as WES. The precipitate was also frozen and is referred to as WUS. The percentages of WES and WUS were calculated.

Water Extractable AX Extraction

For the extraction of WEAX, boiling water (150 mL) was poured into the WES, and was then held in a boiling water bath for 30 min to inactivate endogenous enzymes. After freeze-drying, the material was dissolved in 25mM sodium acetate buffer (25 ml, pH = 4.3). Amyloglucosidase (50μl, 3260 U/mL) (Megazyme, Bray, Ireland) was added. After gently shaking for 2 hrs and boiling in water bath for 15 min, the tubes were centrifuged at 10,000 × g for 15 min at 4 °C. The Supernatant was filtered through VWR qualitative filter paper (West Chester, PA), and transfered to a 1000 mwco dialysis membrane (Spectrum Laboratories Inc., Rancho Dominguez, CA). The samples were dialyzed for 3 days at 6 °C, and water was changed 3 times/day.,The dialyzed supernatant was freeze-dried, and the resultant material is referred to as WEAX.

Determination of AX Relative Molecular Weight Using High Performance Size Exclusion Chromatography (HP-SEC)

The relative molecular weight distribution of AX was determined using a Waters Ultrahydrogel Linear 6-13 μm, 7.8 × 300 mm column (Waters, Milford, MA) and ultrahydrogel guard column. An Agilent 1200 series high performance liquid chromatograph (Agilent Technologies, Wilmington, DE), equipped with an auto sampler was used. An Agilent refractive index detector and PC with ChemStation software (HP ChemSation for LC Rev. A.04.01) were used for control and integration.

The temperature of the column and detector were set to 40° C and the HPLC grade water was pumped at a flow rate of 0.4 mL/min. The system pressure during analysis was 18 Bar. The injection volume for filtered WEAX aquoues solution was 20 μL. Effluent fractions were monitored for total carbohydrates and soluble starch assays (Mccleary & Nurthen, 1986; Wu & Doehlert, 2002). Dextran standards (Sigma-Aldrich, Vallensbaek Strand, Denmark) with molecular weight of 1,400, 670, 410, 270, 150, and 50 kDa were used as molecular weight markers.

Determination of Monosaccharides Using Gas Chromatography (GC)

The monosacchride compositions of flours, WES, and WUS were determined following acid hydrolysis and GC analysis alditol acetates. Flours, lyophilized WES or WUS (7 to 10 mg) were hydrolyzed with 2M Trifluoroacetic acid (TFA) and dried under nitrogen (Gys et al., 2003). The internal standard m-inositol was added to the samples after hydrolysis. The hydrolyzed samples were reduced by adding 1M ammonium hydroxide and sodium borohydride in DMSO. Glacial acetic acid was added to the tubes and then the samples were acetylated. After the addition of 1-methylamidizol and acetic anhydride, the

reaction was stopped with water. Methylene chloride was added to the tubes twice to remove the acetylated monosaccharides. Methylene chloride was evaporated with a stream of nitrogen and the samples were redissolved in acetone and transferred to vials for analysis. The alditol acetate samples were analyzed on a Hewlet Packard 5890 series II GC system with a Flame Ionization Detector (FID) (Agilent Technologies, Inc. Santa Clara, CA). Supelco SP-2380 fused silica capillary column (30m x 0.25mm x 02μm) (Supelco Bellefonte, PA) was used in the GC system. The system parameters were as follows: flow rate of 0.8 ml/min, 82737 Pa flow pressure, oven temperature of 100° C, detector temperature of 250° C, and injector temperature of 230 °C. AX were calculated as the sum of xylose and arabinose monosaccharides.

Proton Nuclear Magnetic Resonance (^{1}H NMR) Spectroscopy Analysis

Proton NMR analyses of AX samples were performed using a Varian Unity Inova 500 MHz NMR spectrometer (Varian Inc., Palo Alto, CA). Samples of WEAX were first dissolved in 1 mL of deuterium oxide (D_2O), left for 2 h at room temperature, and then lyophilized. The lyophilized samples were dissolved in 0.6 mL D_2O again and transferred into NMR tubes. ^{1}H NMR spectra were obtained at 80 °C.

The Apparent Viscosity of the Arabinoxylan

The apparent viscosity of AX (4%, w/v) was measured using an ARG2 Rheometer (TA Instruments, New Castle, DE) at 25 °C. The AX aqueous solution was placed between two parallel plates of 8 mm diameter and the gap was adjusted to 100 μm. Steady state flow step tests were performed to assess viscosity as a function of the shear rate from 1 to 1000 s^{-1}.

Statistical Analysis

All analyses were performed in duplicate. The mean and standard deviations were calculated. The data were analyzed using linear regression, Duncan's multiple range test and Pearson's correlation using the statistical Package for the Social Science (SPSS) 15.0 system. Values with probabilities greater than 0.05 and 0.01 were considered significant and highly significant, respectively.

Results and Discussion

Dough Syruping Measurement

Figure 1 shows measured syruping as a function of storage time. Initially, dough sample exhibited a considerable increase on the degree of dough syruping (DDS) in the first 10 days. After day 10, DDS increased slowly compared to first days of storage. By the end of the storage period (Day 34), DDS was 5.6% in Glenn dough and was 5.3% in Parshall dough. It has been reported that endoxylanases found in wheat hydrolyze water unextractable arabinoxylans (WUAX) and degrade water extractable arabinoxylans (WEAX) during refrigerated storage of dough. Therefore, water-holding capacity of AX decreases and a syrup forms (Courtin et al., 2005). Even though, changes in AX structure were investigated closely (Simsek & Ohm, 2009), protein composition changes and fundamental rheological analysis studies have not been performed on refrigerated dough.

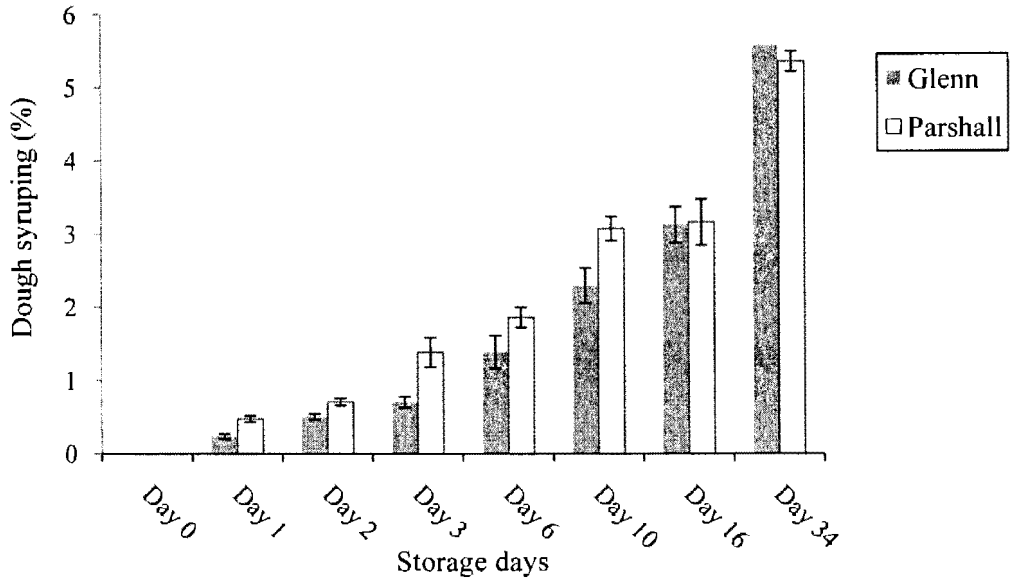

Figure 1. Dough syruping during refrigeration storage (expressed as a % of initial dough weight). Error bars represent standard deviations.

Xylanase Activity

Wheat flour is reported to contain endogenous xylanase inhibitors (Debyser et al., 1997). During aqueous extraction, a variable proportion of xylanases would bind to the inhibitors and thus can not be measured in the available assay. As such, the xylanase activity measured in the current study is referered to as apparent. During milling, wheat bran is partly mixed into the flour, which cause the presence of xylanases and microorganisms in the flour and dough. Therefore, syruping may be induced by xylanases resulting from the field or by the xylanases produced post-harvest by the microbial contamination. The wheat kernel associated xylanses are from both exogenous microbial and endogenous plant origins (Gys et al., 2004). Microbial xylanases Microbial exogenous

xylanases associated with wheat kernels were found to play a role in the syruping phenomenon (De Schryver et al., 2008).

Xylanase activity was detected in the dough samples and was active throughout the entire storage period (Table 2). Among Glenn samples, there was no difference in xylanase activity as storage progressed. Xylanase activity of Parshall samples did not show marked changes. Because of the presence of endoxylanase and its effects on solubilisation and degradation of AX, the structure of AX was expected to change dramatically during storage.

The use of a xylanase inhibitor in dough formulation was reported to be capable of reducing the dough syruping (Poulsen and Sorensen, 2001). Therefore, it was of importance to analyze the flour to be used for production of refrigerated dough for both the AX content and the presence of the xylanase activity. Flours with internally low xylanase activity should be used in order to reduce syruping in refrigerated dough.

Table 2. Endoxylanase activity[a] of flour and dough samples from Glenn and Parshall

Endoxylanase activity (mU/g)[b]	Days of refrigerated storage				
	0	1	6	16	34
Glenn samples	0.56 a ±0.01	0.51 a ±0.04	0.69 a ±0.07	0.72 a ±0.17	0.72 a ±0.04
Parshall samples	0.50 b ±0.02	0.54 ab ±0.01	0.60 a ±0.04	0.56 ab ±0.01	0.51 b ±0.03

[a] Dry basis

[b] Data are mean values and standard deviation of duplicates. Mean values followed by different letters in rows indicate significantly different at $P \leq 0.05$ level by Duncan's new multiple range test.

Changes in AX Relative Molecular Weight Distribution

The relative molecular weight distribution of the WEAX in flour and doughs stored at 1, 6, 16, and 34 days was determined by HP-SEC. Aliquots were analyzed for both total carbohydrate and starch to determine if any impurities were present in the WEAX samples. AX was purified and there was no soluble starch in the samples as the analysis indicated. As such, it is assumed that all the peaks observed on the chromatograph (Figure 2) were derived from AX.

Four distinct peaks (I, II, III, and IV) of different molecular weight ranges were observed in the chromatogram for WEAX extracted from Glenn and Parshall samples (Figure 2). The percentage of peak area for each peak was shown in Table 3. Significant information was obtained about the solubilization and hydrolysis of AX during refrigerated dough storage for both flours.

There were significant changes in relative molecular weight distribution of both Glenn and Parshall samples during refrigerated storage. For peak I (high molecular weight fraction), compared WEAX from flour and from dough on storage day 34, Glenn samples decreased significantly from 35.54% to 33.87% (decreased by 1.67%); Parshall samples decreased significantly from 37.24% to 20.21% (decreased by 17.03%). For peak III (low molecular weight fraction), compared WEAX from flour and from dough 34, Glenn samples increased significantly from 47.06% to 49.69% (increased by 2.63%); Parshall samples increased significantly from 44.16% to 55.61% (increased by 11.45%). Thus, Parshall samples exhibited more changes in molecular weight distribution of WEAX over storage, which indicated there was more degradation in AX of Parshall. In our previous

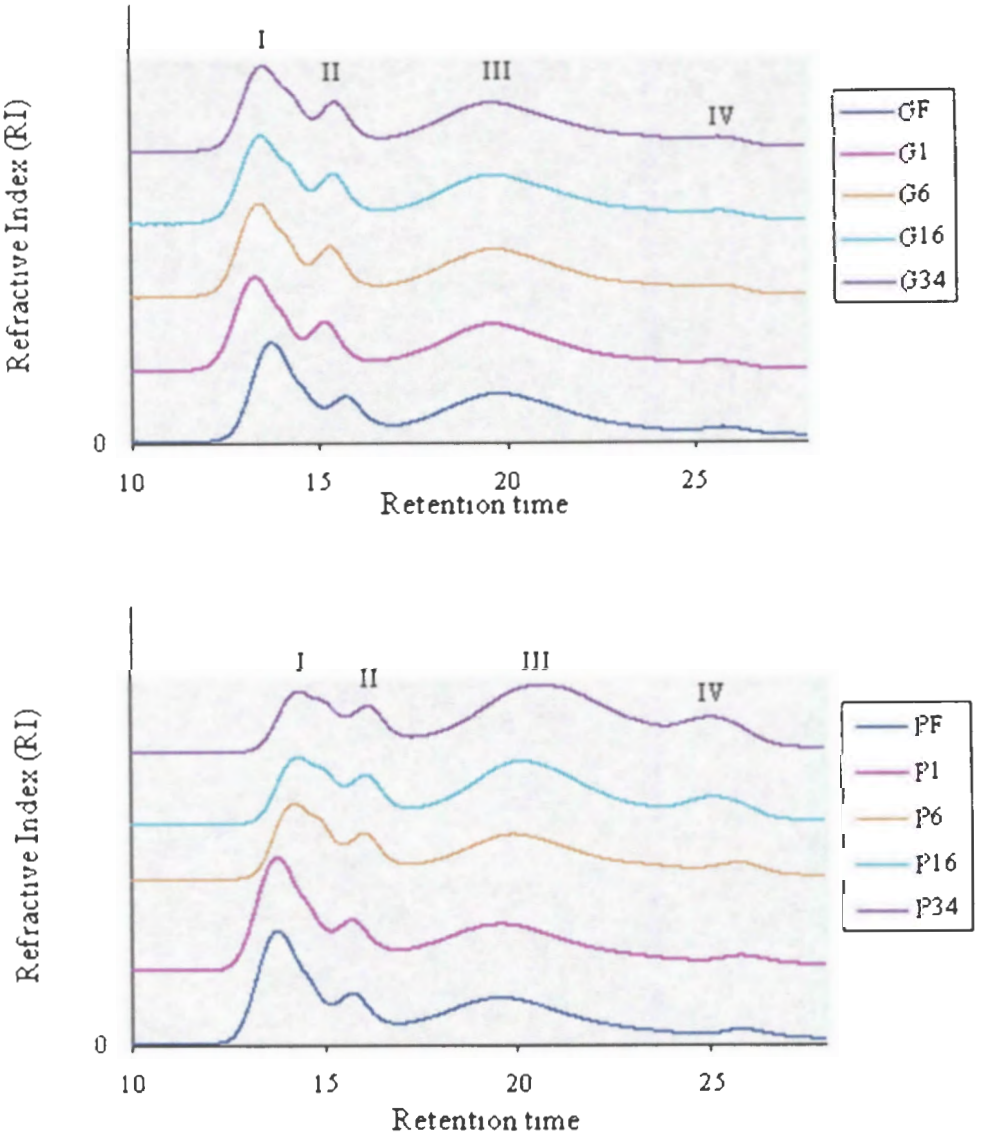

Figure 2. High Performance Size Exclusion Chromatography (HP-SEC) analysis of WEAX extracted from flour and dough samples. Peaks are indicated.

Table 3. Molecular weight distribution for the water extractable AX from Glenn and Parshall [a]

		Flour	Day 1	Day 6	Day 16	Day 34
Peak area (%) in Glenn samples	I	35.5 a ±0.2	34.6 bc ±0.3	34.8 b ±0.1	34.2 cd ±0.2	33.9 d ±0.1
	II	12.6 b ±0.1	13.7 a ±0.2	13.5 a ±0.2	13.4 a ±0.2	13.6 a ±0.2
	III	47.1 c ±0.06	48.7 b ±0.3	48.7 b ±0.0	49.5 a ±0.0	49.7 a ±0.01=
	IV	4.8 a ±0.1	3.0 b ±0.2	3.0 b ±0.1	2.8 b ±0.0	2.9 b ±0.0
Peak area (%) in Parshall samples	I	37.2 a ±0.8	30.8 b ±0.3	31.9 b ±0.2	23.6 c ±0.3	20.2 d ±0.2
	II	13.1 b ±0.3	14.8 a ±0.2	14.7 a ±0.1	12.9 c ±0.0	11.7 d ±0.1
	III	44.2 e ±0.7	49.8 c ±0.1	48.1 d ±0.5	52.5 b ±0.4	55.6 a ±0.4
	IV	5.5 c ±0.5	4.6 c ±0.2	5.3 c ±0.4	11.0 b ±0.7	12.5 a ±0.6

[a] Data are mean values and standard deviation of duplicates. Mean values followed by different letters in rows indicate significantly different at $P \leq 0.05$ level by Duncan's new multiple range test.

study, there was also more degradation in starch of Parshall samples during the refrigeration storage (Zhang & Simsek, 2009).

Monosaccharide Content and Composition

The monosaccharide compositions of hydrolyzed aqueous extracts and the total monosaccharide composition of the lyophilized doughs were determined by GC. Percentages of WES and WUS, as well as AX composition and A/X ratio in WES and WUS, were determined to understand the structure of AX in the samples.

The percentages of WES and WUS in Glenn and Parshall samples are summarized in Table 4. Refrigerated storage time significantly increased the percentage of WES and decreased the percentage of WUS. On day 0, Glenn samples had 9.2% of WES and Parshall had 9.1% of WES. On day 34, there was 13.1% of WES in Glenn sample and 13.7% in Parshall sample. Conversely, the percentage of WUS decreased across the days of storage as: from 90.8% for Glenn and 90.9% for Parshall at day 0 to 86.9% for Glenn and 86.3% for Parshall at day 34 (Table 4).

Chemical analysis of WES and WUS with GC showed that AX exhibited significant changes in structural characteristics in each storage point. The percentage of AX was calculated as the sum of monomeric arabinose and xylose, multiplied by a factor 0.88 for the release of water. The apparent degrees of solubilization of AX in the different doughs showed almost the same trend. Total AX% increased in WES while total AX% decreased in WUS during the storage.

Table 4. WES and WUS composition of whole-lyophilized-ground dough samples during storage [a]

Storage day	Glenn		Parshall	
	WES %	WUS%	WES %	WUS%
Day 0	9.2 d ±0.3	90.8 a ±0.3	9.1 e ±0.1	90.9 a ±0.1
Day 1	10.3 c ±0.0	89.7 b ±0.0	10.8 d ±0.0	89.2 b ±0.0
Day 2	10.8 c ±0.0	89.2 b ±0.0	11.00 d ±0.1	89.0 b ±0.1
Day 3	11.0 bc ±0.1	89.0 bc ±0.1	11.4 cd ±0.7	88.6 bc ±0.7
Day 6	11.2 bc ±0.9	88.8 bc ±0.9	11.8 bc ±0.3	88.2 cd ±0.3
Day 10	12.0 b ±0.0	88.0 c ±0.0	12.3 b ±0.3	87.7 d ±0.3
Day 16	12.7 a ±0.9	87.3 d ±0.7	12.4 b ±0.0	87.6 d ±0.1
Day 34	13.1 a ±0.2	86.9 d ±0.2	13.7 a ±0.1	86.3 e ±0.1
Regression[b]	A=10.040	A=89.585	A=10.646	A=89.354
	B=0.180	B=−0.095	B=0.101	B=−0.101
	sig.[c] ($P<0.01$)	sig. ($P<0.01$)	sig. ($P<0.01$)	sig. ($P<0.01$)

[a] Data are mean values and standard deviation of duplicates. Mean values followed by different letters in columns indicate significantly different at $P\leq0.05$ level by Duncan's new multiple range test.

[b] A and B are intercept and slope terms in linear regression model.

[c] sig.= significant

There were also significant changes detected in A/X ratio for both WES and WUS components. Arabinose to xylose ratio in WES was 0.86 for Glenn at day 0 and it decreased to 0.69 at day 34. Arabinose to xylose ratio in WUS from the Glenn had the opposite trend in which the ratio increased slightly from 0.80 to 0.85 at day 0 and 34, respectively (Table 5). In the beginning of the storage (day 0), Parshall had much higher A/X ratio in WUS than Glenn (0.98 for Parshall, 0.80 for Glenn) and had similar A/X ratio in WES compared to Glenn (0.85 for Parshall, 0.86 for Glenn). Over storage time, A/X ratio in Parshall WES decreased from 0.85 to 0.69 on day 0 and day 34, respectively (Table 5). However, A/X ratio in Parshall WUS did not have much change. The different effect of storage on doughs prepared with Glenn and Parshall may have resulted from their different endoxylanase activity.

Correlation between degree of dough syruping (DDS) and AX composition of Glenn and Parshall samples are shown in Table 6. For both Glenn and Parshall, a high negative correlation exists between A/X and dough syruping. The amount of AX in WES was highly and positively correlated to dough syruping in Glenn samples. Oppositely, there is a negative correlation between the amount of AX in WUS of Parshall and its corresponding dough syruping.

Arabinoxylans solubilization and degradation occurred simultaneously with dough syruping (Gys et al., 2003), resulting in increased low molecular weight components (WES) and decreased of high molecular weight components (WUS). Associated with the higher increase of WES, the dough samples could have higher DDS. This can explain why Parshall had both higher increase in WES and higher dough syruping.

Table 5. Arabinoxylans chemical analysis in WES and WUS of dough samples of Glenn and Parshall [a]

	Glenn WES		Parshall WES		Glenn WUS		Parshall WUS	
Storage day	A/X	Total AX%	A/X	Total AX%	A/X	Total AX%	A/X	Total AX%
Day 0	0.86 ab ±0.02	2.53 c ±0.04	0.85 b ±0.02	3.20 b ±0.17	0.80 d ±0.04	0.84 a ±0.02	0.98 a ±0.00	0.72 b ±0.00
Day 1	0.87 ab ±0.02	2.40 d ±0.14	0.82 c ±0.02	3.23 b ±0.08	0.91 ab ±0.01	0.75 a ±0.00	0.96 a ±0.00	0.77 a ±0.01
Day 2	0.84 bc ±0.02	2.80 c ±0.17	0.93 a ±0.01	2.78 c ±0.01	0.89 bc ±0.05	0.73 a ±0.09	0.96 a ±0.02	0.65 cd ±0.02
Day 3	0.89 a ±0.00	2.51 d ±0.04	0.78 d ±0.00	3.25 b ±0.00	0.93 a ±0.03	0.66 a ±0.02	0.97 a ±0.02	0.67 c ±0.01
Day 6	0.84 bc ±0.01	3.00 c ±0.03	0.79 d ±0.01	3.14 b ±0.01	0.88 abc ±0.02	0.86 a ±0.03	0.98 a ±0.01	0.63 d ±0.00
Day 10	0.82 c ±0.00	3.41 b ±0.00	0.77 de ±0.00	2.25 d ±0.09	0.91 ab ±0.01	0.68 ab ±0.02	0.85 b ±0.02	0.64 d ±0.00
Day 16	0.75 d ±0.00	3.30 b ±0.03	0.74 e ±0.01	3.97 a ±0.05	0.83 cd ±0.01	0.72 a ±0.00	0.97 a ±0.00	0.58 e ±0.01
Day 34	0.69 e ±0.00	3.80 a ±0.09	0.69 f ±0.01	3.26 b ±0.00	0.85 d ±0.00	0.53 b ±0.00	0.96 a ±0.03	0.56 f ±0.02

[a] Data are mean values and standard deviation of duplicates. Mean values followed by different letters in rows indicate significantly different at $P \leq 0.05$ level by Duncan's new multiple range test.

Substitution Pattern of AX using ^{1}H NMR

The intensity of the resonance in the range of 4.4 - 4.7 ppm has been assigned to anomeric proton of Xyl*p* (Hoffman et al. 1992). The intensity of the resonance in the range of 5.2 - 5.4 ppm has been assigned to anomeric proton of Ara*f*. Peak 4, 5, and 6 correspond to disubstituted, monosubstituted and nonsubstituted Xyl*p*, respectively. Peak 1, 2, and 3 correspond to single α-L-Ara*f* linked to xylose residues at O-3, α-L-Ara*f* linked to

disubstituted xylose residues at O-3 and α-L-Ara*f* linked to disubstituted xylose residues at O-2, respectively (Andersson, 1993). The unresolved signals between peak 2 and 3 could result from two neighboring disubstituted xylose residues in AX (Figure 3).

Table 6. Correlation coefficients between dough syruping and AX composition changes [a]

	WES		WUS	
	A/X	Total AX%	A/X	Total AX%
Dough syruping (%) in Glenn samples	-0.985**	0.978**	-0.120	-0.874
Dough syruping (%) of Parshall samples	-0.992**	0.309	-0.458	-0.899*

[a] N=5, * and ** = Correlation significant at the $P \leq 0.05$ and at the $P \leq 0.01$ level, respectively.

The chemical shifts of the anomeric protons are predominantely within the range of 4.4-5.5 ppm (Figure 4). Major structural difference among the sample was observed on the peak at δ 4.5 ppm, which corresponded to the unsubstituted anomeric proton of xylose residues. Comparing this peak of WEAX extracted from flour sample (Figure 4A for Glenn, Figure 4B for Parshall) with the WEAX from dough 34 (Figure 4C for Glenn, Figure 4D for Parshall), it increased dramatically. The increase in the peak of unsubstituted xylose backbone indicated that mono- and di-substituted AX have been degraded during the storage. This was in agreement with the data exhibited in HP-SEC part.

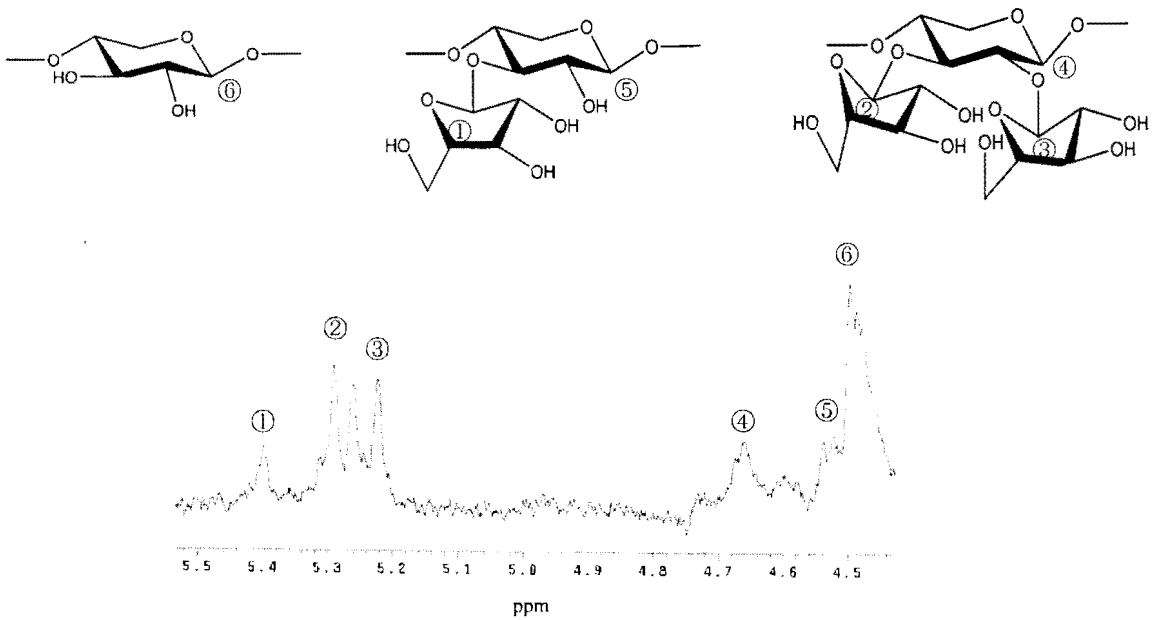

Figure 3. Proton NMR spectrum of the anomeric region of AX fraction from wheat flour. The signals are indicated by the numbers from 1 to 6.

Viscosity of WEAX Aqueous Solution

Arabinoxylans (AX) form highly viscous solutions when hydrated. The apparent viscosity of AX aqueous solution determined by steady shear test. The viscosity vs. shear rate curves for WEAX extracted from the dough samples stored on different days for Glenn and Parshall dissolved in water (4.0% w/v) are compared in Figure 5. Power law equation to describe the flow curve is defined as

$$\sigma = K\gamma^n$$

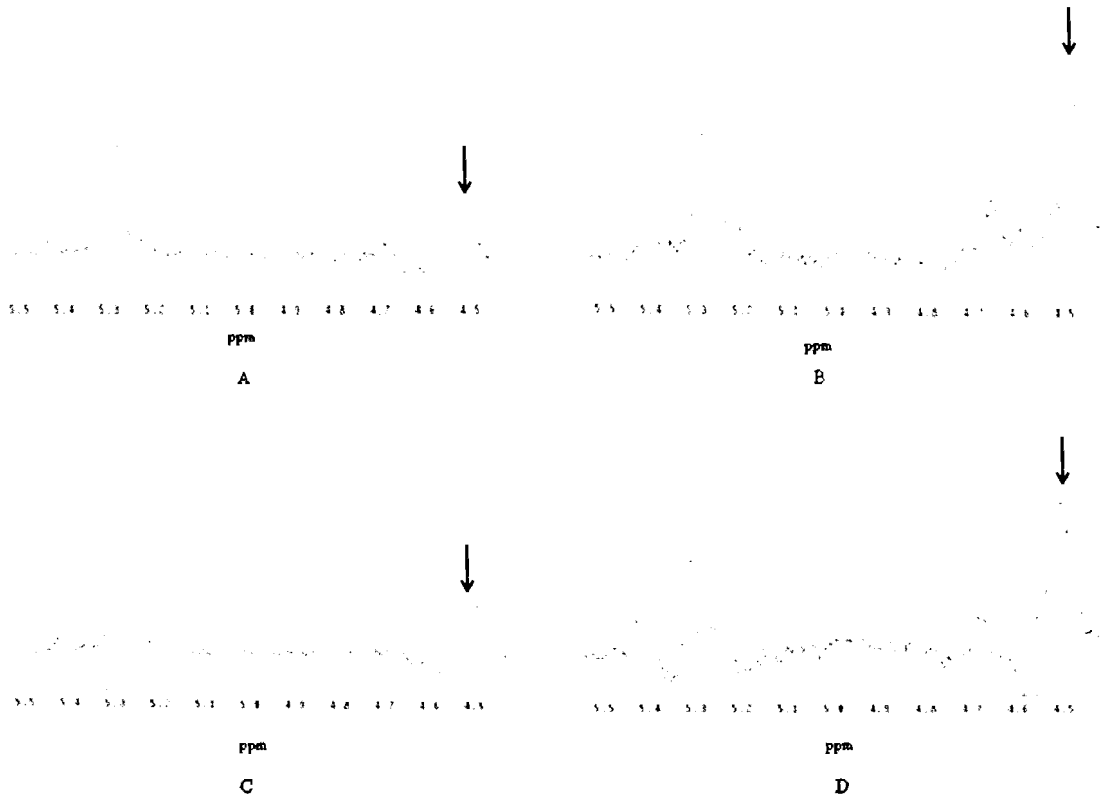

Figure 4. Proton NMR spectrum of the anomeric region of AX fraction from flour and dough sample on storage day 34.

A) GF= AX from the flour of Hard Red Spring Wheat (HRSW) variety Glenn. B) G34= AX from dough prepared using HRSW variety Glenn and stored 34 days. C) PF= AX from the flour of HRSW variety Parshall. D) P34= AX from dough prepared using HRSW variety Parshall and stored 34 days.

σ is viscosity and γ is shear rate. The lower the n, the more shear thinning behavior is more prominent (Andersson et al., 2006). Apparent viscosity equals to shear stress divided by shear rate. At low shear rates (1-10 s^{-1}), the flow behavior of wheat arabinoxylan was not stable. As shear rates increased, a shear thinning behavior was observed in each sample, especially for the samples in the early stage of storage. The shear rate at which the apparent viscosity begins to decrease depends on the molecular weight (Izydorczyk & Biliaderis, 1992). The overall pattern of the flow curves differed substantially. As shear rate reach more than 500 s^{-1}, the shear thinning behavior became less obvious. Newtonian flow characteristics were shown for AX samples from dough 16 and 34 at the shear rate range of 500 – 1000 s^{-1}. For Parshall, Newtonian behavior even started to show at lower shear rate (100 -1000 s^{-1}). Comparing all the samples, the viscosity of AX aqueous solution decreased during storage, which reflected the decrease in the the molecular weight of AX.

The viscosity of a polymer solution is a function of both the size and the basic structure of its molecules and the conformations they adopt in the solvent system. Molecular weight of wheat arabinoxylans is an important determinant of their physical properties in an aqueous environment. The viscosity of water extractable AX decreased during the storage which indicated solubilization and degradation of AX. The arabinosyl substituent has an important effect on the shape of the molecule in aqueous solution. The high intrinsic viscosity of AX correlated with low A/X ratio (Izydorczyk & Biliaderis, 1995).

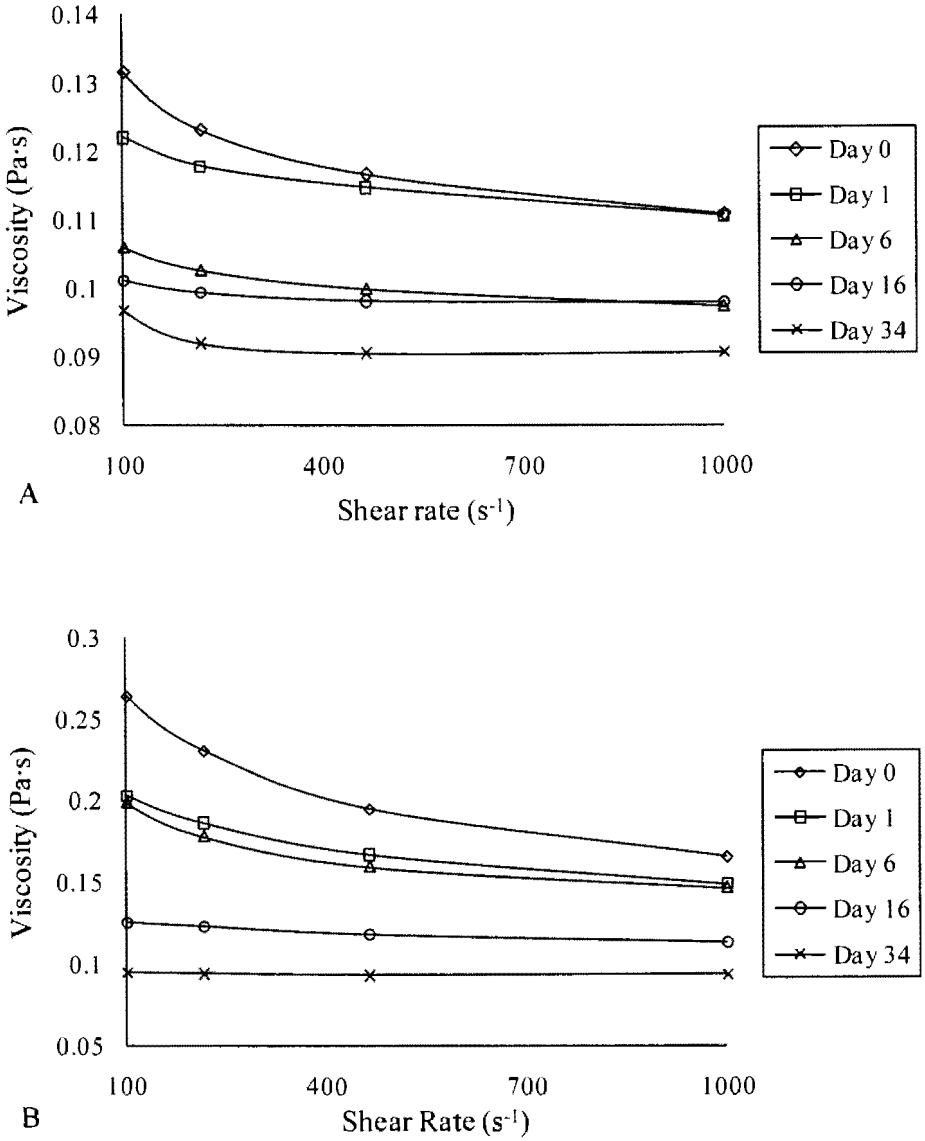

Figure 5. Effect of shear rate on the apparent viscosity of aqueous solutions of the arabinoxylan (4% w/v) at 25 °C. A) Glenn, B) Parshall.

Conclusions

Arabinoxylans properties changed in many aspects during refrigeration storage in both normal and partial waxy wheat samples. In this study, HP-SEC was used to determine the changes in relative molecular weight, GC to investigate the monosaccharide composition, ^{1}H-NMR to monitor the xylanase enzyme action, and rheometer to analyze the viscosity of the samples.

Changes in the relative molecular weight and arabinose substitution pattern of the AX component changed during storage and were correlated to DDS. The molecular weight of AX decreased while the ratio of unsubstituted xylose in WEAX increased during the storage. The changes in the AX relative molecular weight distribution were more dramatic in the Parshall samples (partial waxy) than the Glenn (normal starch) sample.

The development of dough syruping can be explained by the capacity of AX to hold a large amount of water in dough. When AX are degraded by endoxylanases, they lose the water-holding capacity, leading to free liquid on the dough surface. Therefore, the use of endoxylanase inhibitors can be an effective tool to reduce syrup for the refrigerated dough industry. This work essentially explained how AX chemistry was correlated to DDS and the refrigerated dough quality. It also should be pointed out that although AX solubilisation and degradation play an important role in dough syruping, they are probably not the only determinant. Starch and protein, the major components of flour, should be further investigated.

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PAPER 2. PHYSICOCHEMICAL CHANGES OF STARCH IN REFRIGERATED DOUGH DURING STORAGE

(This paper has already been published. Reference: Zhang, Y., & Simsek, S. (2009). Changes of starch structural and physical properties in refrigerated dough system during storage. *Carbohydrate Polymers*, *78*, 268-274)

Abstract

Refrigerated dough is a flour-based, unbaked product that is stored between 4-7°C. Maintaining the dough quality during storage is very crucial. Starch functionalities have important effects on flour and dough quality. The objective of this research was to determine the physicochemical changes of starch in refrigerated dough during extended storage. Two wheat flours with different amylose/amylopection ratio were used in this study. The relative percentage of amylopectin decreased up to 10.5%. Overall, we observed that the pasting properties and thermal properties of starch were changing during the storage: peak viscosity decreased up to 23.1%, breakdown viscosity decreased up to 57.7%, and setback viscosity decreased up to 41.2% compared to flour samples during 34 days refrigerated storage. Variation in starch granular morphology was detected. These results showed that physicochemical properties of starch changed during refrigerated storage, which may have significant impacts on end-product quality.

Introduction

The refrigerated dough industry is considered one of the fastest growing segments of the ready-to-use grain-based industry. Refrigerated dough products account for more than $1.6 billion of sales per year in the USA. The items in this category include canned refrigerated biscuits, canned refrigerated croissants and sweet rolls (Courtin et al., 2006). In today's market, in-store bakeries use frozen dough or par-bake products, which offer good quality, but to improve further the quality of these products, refrigerated dough is considered a convenient and economical choice for the production of fresh-bread products. The high quality of bakery products made with refrigerated doughs should be characterized by a long shelf life with good organoleptic properties. However, during refrigerated storage, a yellowish liquid separates from the dough forming a syrup that leaks out of the product to produce a deleterious phenomenon known as "dough syruping" (Courtin et al., 2005; Simsek & Ohm, 2009). It has been hypothesized that dough syruping is due to the action of natural enzymes named endoxylanases. These enzymes modify the water holding capacity of Arabinoxylans (AX), which are polysaccharides present in the dough (Gys et al., 2003). Our previous studies also showed that rheological properties and protein fractions of dough also changed during the refrigeration storage.

Starch is the major storage polysaccharide of higher plants, such as wheat. It has unique physical and chemical properties and nutritional quality compared to other carbohydrates. Starch granules are composed of two constituent polymers: a basically linear polysaccharide (α-1→4 linked glucose) named amylose and a highly branched polysaccharide named amylopectin (α-1→4 linked glucose and α-1→6 linked glucose) (Whistler & BeMiller, 1997). Wheat endosperm contains mainly two types of starch

granules: the large, lenticular (A type) and small, spherical (B type). Wheat flour consists of 70%-80% dry matter of starch. Normal wheat starch contains an average of about 25% amylose. High amylose starch has greater than 40% amylose.

When starch is primarily composed of amylopectin, it is defined as waxy starch. Hexaploid partial waxy wheats are characterized by slight reduction in amylose content due to the absence of waxy-protein, granule-bound starch synthase (GBSS), alleles at one or two of the three *Wx* loci (Nakamura et al., 1995). The potential use of wheat with reduced amylose content is a current focus of interest among wheat breeders, geneticists and cereal scientists (Dong et al., 2007; Qin et al., 2009). Partial waxy wheats are sources of flours with optimal quality characteristics in certain Asian wet noodle products. Partial waxy wheats were shown to enhance Japanese Udon noodle quality, which generally decreases with an increase in flour amylose content (Oda et al., 1980). In addition, partial waxy wheats are essential to the development of waxy wheats with acceptable agronomic performance (Graybosch, 1998).

The refrigerated dough market is a relatively new market. There have been limited peer-reviewed research conducted in this area, especially about the role of flour fractions on the quality of refrigerated dough products. In frozen dough, the roles of starch and starch structural and functional properties during storage had been studied. Lu & Grant (1999b) used fractionation and reconstitution of wheat flour components to investigate the roles of starch and others in breadmaking quality of frozen dough. They found that the starch fraction contributed significantly to frozen dough quality. Also, they found that starch isolated from frozen dough made with different wheat flour varied in enthalpy (ΔH) largely, but all the samples showed a significant increase of ΔH with increased frozen

storage time (Lu & Grant, 1999a). This could be explained by the way water interacts with the starch during the freezing process and the rate of water migration in the frozen dough during frozen storage (Kulp, 1995). Low-temperature Scanning Electron Microscopy (SEM) was used to examine the effects of storage on the structure of frozen bread doughs. Starch granules were no longer associated with gluten fibrils in the dough structure (Kulp, 1995). Onset temperature of starch gelatinization measured by Differential Scanning Calorimetry (DSC) increased during frozen storage (Autio & Sinda, 1992). The increase of onset temperature may be attributed to the delay in the rate of diffusion of water into the starch granules or the increased crystallinity of the granules. In frozen dough, the amylose-amylopectin ratios were negatively correlated with frozen storage time (Wolt & D' Appolonia, 1984). It was uncertain whether this relationship revealed any fundamental functional effect or whether it simply reflected a higher degree of retrogradation of the soluble starch polymers in frozen doughs. No studies on the role of starch in refrigerated dough have been done yet. Understanding the changes in the physical and functional properties of starch during storage would be helpful to predict the end product quality.

The objective of this research was the determination of physicochemical properties of starch in refrigerated dough during storage using two different flours, which differed in their amylose-amylopectin ratios.

Experimental

Materials

Glenn and Parshall, two wheat varieties of Hard Red Spring Wheat (HRSW), were obtained from North Dakota State University (NDSU) Casselton Research Extension Center in 2007. Glenn is one of the most commonly grown varieties in North Dakota, the main HRSW growing area in the United States. It is normal hexaploid wheat. Parshall is a partial waxy hexaploid HRSW cultivar, developed by North Dakota State University. Parshall would provide information on the role of amylose reduction in refrigerated dough products.

Preparation of Refrigerated Dough

In order to avoid confounding factors arising from the presence of other ingredients, a lean dough formula was used. The dough was prepared by using 100 g of flour (14% moisture basis), 1.8 g of salt, and a certain amount of water, containing 0.06% w/v of sodium azide (Mallinckrodt Baker Inc. Paris, KY) to prevent microbial spoilage, to reach the desired moisture content previously determined according to the specific farinograph absorption test (Table 1). Dough was mixed in a 100g pin mixer (National Manufacturing, Lincoln, NE) for the optimum mixing time, sheeted, molded, and stored in plastic containers for 34 days at 6°C. The dough samples on storage day 1, 6, 16, and 34 were lyophilized and ground (Simsek & Ohm, 2009). They were named as G1, G6, G16, G34 and P1, P6, P16 and P34, respectively. Glenn and Parshall flour are named as GF and PF.

Alpha-Amylase Activity Measurement

The α-amylase activity (CU/g) of two flour samples and all dough samples was determined using enzymatic digestion assay kits (Megazyme International, Bray,

Ireland).

Starch Isolation and Preparation

Starch was isolated from the flour samples by the dough washing method according to Lu & Grant (1999a). Two percent NaCl solution helped to separate the starch from gluten. The starch was washed with water, centrifuged at 2,000 × g for 15 minutes. The purified starch was freeze-dried, ground using mortar and pestle, and stored in plastic bags.

High Performance Liquid Chromatography (HPLC) Analysis

Percentages of amylose and amylopectin of the starch were determined using a method developed for HPLC by Grant and Ostenson (Grant et al., 2002). The extracted starch was completely dissolved in 1M KOH/urea solution and then analyzed with an Agilent 1200 series HPLC (Agilent Technologies, Wilmington, DE), equipped with an auto sampler. A refractive index detector and PC with ChemStation (HP ChemStation for LC Rev. A.04.01) were used for control and integration. The pencentage of amylose and amylopectin peak area was determined by HPLC analysis using a Waters Ultrahydrogel linear column (Waters Co., Milford, MA).

Rapid Viscosity Analyzer (RVA) Analysis

Pasting properties of the starch samples were evaluated using RVA (Newport Scientific, Narrabeen, Australia) interfaced with a computer equipped with Thermocline and Thermoview software (Newport Scientific). The method used was modeled after that of Chakraborty et al. (2004) with minor modifications. Starch (3 g, 14% moisture basis) was added to pre-weighed deionized distilled water in an RVA canister. The rate of heating and cooling in the Std 1 profile was 12 °C per minute, idle temperature was 50 °C, and the total run time was 13 minutes. Parameters recorded were peak viscosity (PV), hot paste

viscosity (HPV), breakdown (BKD), cold paste (CPV) and setback (STB) viscosity. All viscosity measurements were reported in Rapid Visco Units (RVU).

Differential Scanning Calorimetry (DSC) Analysis

The DSC analysis of the flour and starch was done using a Perkin-Elmer DSC-7 (PerkinElmer Life and Analytical Sciences, Inc., Waltham, MA) with a thermal analysis data station after minor modifications to the method of White et al (1990). The samples (3.5 mg, as is) were weighed into aluminium pans, and deionized water (0.8 μL) was added. The pans were sealed and kept at room temperature overnight. The reference was an aluminium pan with deionized water (0.8 μL). Each sample was heated under nitrogen gas from 10 to 100°C at 10°C per minute. All analyses were carried out in triplicate. Enthalpy of gelatinization (ΔH), onset (To), peak (Tp), and conclusion (Tc) temperatures were computed automatically. The gelatinization temperature range was computed as [(Tc – To)].

X-Ray Powder Diffraction Analysis

Relative degree of crystallinity of starch samples was investigated by X- ray powder diffraction (Philips vertical Multi-Purpose Diffractometer PW3040) operating at 50 KV and 40 mA (Cu K_α radiation of 0.154 nm). The diffracted intensity was measured from 5 to 35 ° as a function of 2 θ. The degree of crystallinity of the sample was defined by the intensity ratio of the diffraction peaks and of the sum of all measured intensity using PANalytical software X'Pert HighScore v. 2.2c. The constant background intensity, arising from imperfections of the sample, the X-ray optics of the instrument, sample fluorescence and scatter, was subtracted from the total intensity. The standard reference material was Respirable Alpha Quartz (NBS 1878, 95.5% crystallinity) which determined the constant

background. All backgrounds were determined by using exactly the same automatic setting (Granularity = 20, Use smoothed input data = Yes, Bending factor = 6).

Scanning Electron Microscopy (SEM) Analysis

Starch samples were sprinkled onto carbon tape attached to aluminum mounts. Loose particles were removed using short bursts of compressed nitrogen gas. The sample then was coated with gold using a Hummer II sputter coater (Technics/Anatech Ltd., Alexandria, Virginia USA). Images were obtained using a JEOL JSM-6490LV Scanning Electron Microscope (SEM) (JEOL, Peabody, Massachusetts, USA). Magnification, accelerating voltages, and micron bars are listed on each photo.

Statistical Analysis

DSC analysis was done in triplicate. All other analyses were done in duplicate. The mean and standard deviations were calculated.

Results and Discussion

Flour Characteristics

The physical and chemical properties of the Glenn and Parshall flours are summarized Table 1 of the previous paper. Glenn had slightly higher protein and wet gluten. Parshall had a higher falling number. Since Glenn has normal starch characteristics and Parshall is partial waxy wheat, the major difference between these two flours was on the amylose content, which was reported further in the part of HPLC analysis (Table 1). These two flours were selected based on their different amylose/amylopectin ratio, which is an important structural characteristic of starches.

Alpha-Amylase Activity and Pasting Properties of Starch from Refrigerated Dough

Both flours showed extremely low α-amylase activity, indicating grain soundness. Alpha-amylase is endogenously present in flour, but at very low level. In a fully mature wheat grain, α-amylase activity is mostly located in the seed coat, aleurone layer and scutellum (Rani et al., 2001). The α-amylase activity of the dough samples was lower than their parental flour samples. Freeze drying could be the reason for the reduction of enzyme activity in the dough samples. The α-amylase activity of all Glenn dough samples was similar to each other, and the same was observed in α-amylase activity of Parshall dough samples (Table 1). These results indicated that refrigeration storage did not have much influence on α-amylase activity.

RVA pasting properties of samples are shown in Table 1. The pasting properties of starch determined by RVA are directly related to its microstructure. Amylose, which contributes to high gel consistency upon cooling, may have caused the initial rigidity to the swollen starch granules (Tsai et al., 1997). Since the amylose content of Glenn-flour was about 2-3% higher than Parshall-flour, peak viscosity (Chakraborty et al., 2004), setback and paste viscosity of starch from Parshall-flour were higher than that from Glenn-flour. Breakdown viscosity was a little lower in starch from Parshall flour than from Glenn. The peak time was the same for starch from Glenn-flour and Parshall-flour. However, the dough samples of these samples did not show the same trend over storage since many factors influenced the starch properties, such as water hydration during dough mixing, water distribution and loss (dough syruping) during storage, and enzymatic degradation of amylopectin.

Table 1. Pasting characteristics of starch samples determined by Rapid Visco Analyzer (RVA) and α -amylase activity of flour and ground dough samples [a]

Sample Name [c]	RVA Pasting characteristics [b] PV [d]	BKD [d]	STB [d]	HPV [d]	CPV [d]	PT (min)	α-amylase activity (CU/g)
GF	270.3 ±5.0	75.3 ±1.2	127.5 ±5.6	194.7 ±3.8	322.2 ±9.5	7.0 ±0.0	0.12 a ±0.00
G1	234.7 ±10.3	72.3 ±0.7	131.5 ±12.4	162.4 ±9.6	293.9 ±22.1	7.0 ±0.0	0.08 b ±0.00
G6	226.6 ±1.6	46.2 ±0.2	55.5 ±1.1	180.4 ±1.8	235.8 ±2.9	7.0 ±0.0	0.08 b ±0.00
G16	225.9 ±0.9	63.9 ±1.2	103.0 ±4.8	162.1 ±2.1	265.1 ±2.6	7.0 ±0.0	0.08 b ±0.01
G34	238.9 ±3.2	41.8 ±2.3	95.5 ±1.9	197.1 ±5.5	292.6 ±7.5	7.0 ±0.0	0.08 b ±0.01
PF	288.3 ±2.9	73.5 ±0.7	134.9 ±0.2	214.9 ±3.6	349.8 ±3.4	7.0 ±0.0	0.12 a ±0.01
P1	251.8 ±9.2	71.8 ±1.0	126.7 ±0.9	179.9 ±10.2	306.7 ±11.1	7.0 ±0.0	0.09 b ±0.00
P6	234.8 ±4.6	54.7 ±1.6	86.2 ±7.0	180.2 ±3.1	266.4 ±10.0	7.0 ±0.0	0.09 b ±0.00
P16	243.9 ±2.5	39.4 ±1.9	102.3 ±1.2	204.6 ±0.6	306.9 ±1.9	6.3 ±0.1	0.09 b ±0.00
P34	221.8 ±3.0	31.1 ±0.2	79.3 ±0.4	190.7 ±3.2	270.0 ±3.6	6.9 ±0.1	0.10 b ±0.00

[a] Data are Mean values and standard deviation of duplicates. For α-amylase activity, mean values followed by different letters within Glenn or Parshall samples indicate significantly different at $P \leq 0.05$ level by Duncan's new multiple range test.

[b] RVA=Rapid Visco Analyzer, PV=peak viscosity, BKD=breakdown, STB=setback, HPV=hot paste viscosity, CPV= cold paste viscosity, PT=peak time.

[c] GF= Flour from Hard Red Spring Wheat (HRSW) variety Glenn; G1=Dough prepared using HRSW Glenn and stored 1 day; G6= Dough stored 6 days; G16= Dough stored 16 days; G34= Dough stored 34 days. PF= Flour from HRSW variety Parshall; P1=Dough prepared using HRSW Parshall and stored 1 day; P6= Dough stored 6 days; P16= Dough stored 16 days; P34= Dough stored 34 days.

[d] The unit is expressed as RVU.

All the RVA parameters changed dramatically during refrigeration storage, except peak time, which indicated that storage had profound effects on starch pasting properties. Viscosities decreased during the storage for both samples. The RVA profile of starch extracted from G34 showed: peak viscosity decreased by 11.5%, breakdown viscosity decreased by 44.5%, and setback viscosity decreased by 25.1% compared with Glenn-flour starch. The RVA profile of starch extracted from P34 showed: peak viscosity decreased by 23.1%, breakdown viscosity decreased by 57.7%, and setback viscosity decreased by 41.2% compared with Parshall-flour starch (Table 1). So, the water binding capacity of starch and starch paste stability decreased during the refrigeration storage.

Changes in Molecular Weight Distribution

Three peaks were shown in the HPLC chromatograms (Figure 1). The first two peaks correspond to amylopectin and the following peak represents amylose (Simsek et al., 2009). Parshall flour exhibited 2.2% lower amylose content than Glenn flour: Glenn had 24.2% amylose while Parshall had 22.0% amylose. The term "partial waxy", characterized by a slight reduction in amylose content, was first defined by Nakamura et al. (1993).

Based on the significant changes in relative molecular weight of starch, we concluded that starch became hydrolyzed during the storage, due to the internal α-amylase activity and formation of free water during storage. Compared the flour starch and the starch from dough on day 34, the percent of amylopectin of G34 decreased by 6.3%, while the percent of amylopectin of P34 decreased by 10.5%. Also, the amylose/amylopectin ratio of Glenn changed from 0.32 to 0.41 and the amylose/amylopectin ratio of Parshall changed from 0.28 to 0.43 during storage. So, the average molecular weight of starch became smaller during the storage. It resulted in that low molecular weight fraction was

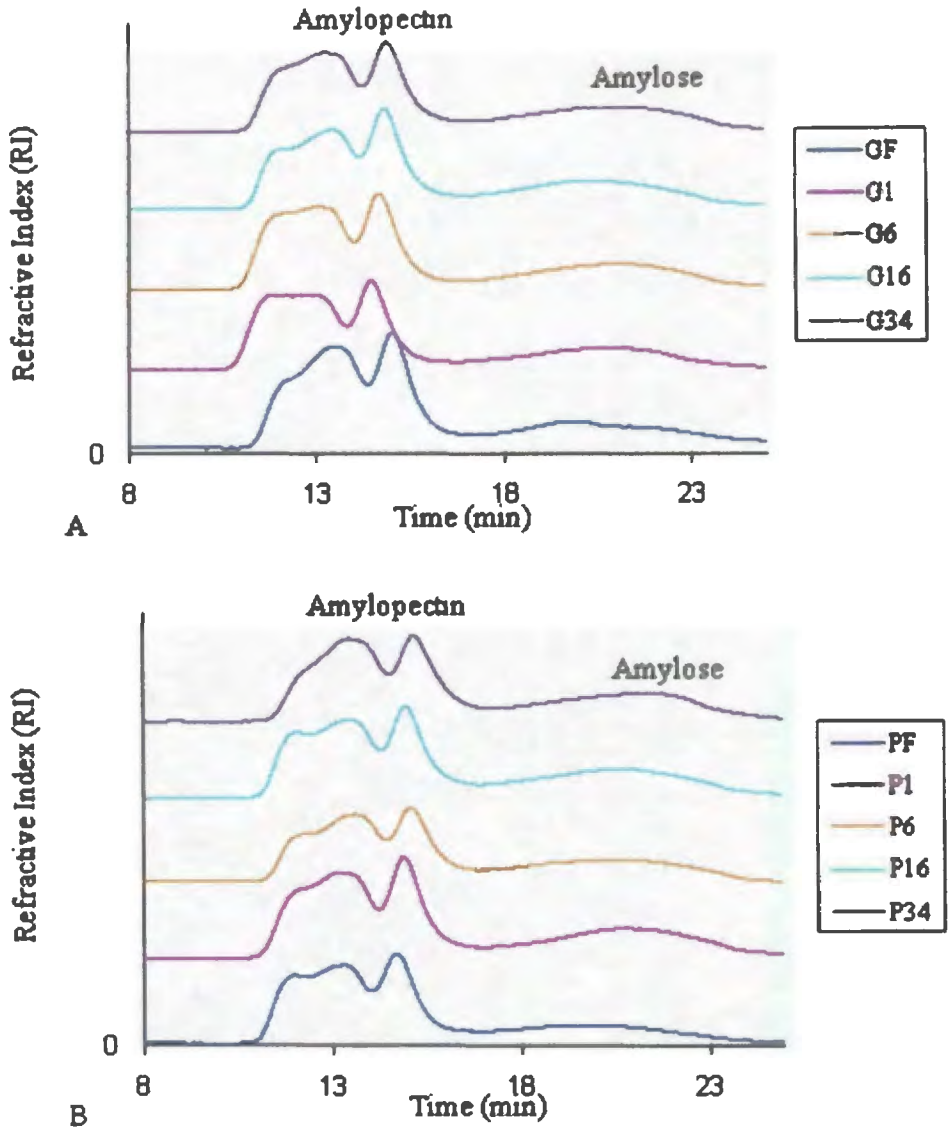

Figure 1. Analysis of starch by High Performance Liquid Chromatography (HPLC)[a]. Chromatograms show elution patterns of starch from flour and dough samples for Hard Red Spring varieties A) Glenn and B) Parshall.

[a] GF= Flour from Hard Red Spring Wheat (HRSW) variety Glenn; G1=Dough prepared using HRSW Glenn and stored 1 day; G6= Dough stored 6 days; G16= Dough stored 16 days; G34= Dough stored 34 days.

PF= Flour from HRSW variety Parshall; P1=Dough prepared using HRSW Parshall and stored 1 day; P6= Dough stored 6 days; P16= Dough stored 16 days; P34= Dough stored 34 days.

higher with Parshall than with Glenn on day16 and day 34 (Table 2).

Thermal Properties and Crystalline Structure

Starch undergoes an irreversible order-disorder transition called gelatinization when heated in the presence of water. Various changes can be observed: granules swelling, absorption of water, loss of cystallinity and leaching amylose. Gelatinization behavior is influenced by starch granule structure, size, molecular alignment, and hydrogen bonding (White et al., 1990).

DSC has been used widely and extensively to study the gelatinization of starch (Donovan et al., 1983; Hayakawa et al., 1997; Yoo & Jane, 2002). In this study, there were not significant changes of *Tc*, *To*, and *Tp* in the storage for flour and dough starch samples (Table 3). However, starch ΔH decreased during storage. A similar result was reported in previous work. ΔH is a result of a breakdown of crystalline order and molecular order (double helices) during gelatinization (Cooke & Gidley, 1992).

For flour starch, Parshall (partial waxy) displayed higher enthalpy of gelatinization compared to Glenn (normal starch). This was in agreement with a previous report (Chakraborty et al., 2004). However, starch extracted from G1 started to have higher enthalpy than starch from P1. That meant the thermal properties changed during refrigeration storage. The dough samples displayed the same trend from day 6 to day 34. Parshall flour and dough starch displayed gelatinization temperatures comparable to Glenn-slightly higher. It was reported that DSC thermal properties were more significantly affected by the degree of crystallinity than by the granule size of starch.

Table 2. Analysis of starch fractions using High Performance Liquid Chromatography (HPLC) [a]

			Flour	Day 1	Day 6	Day 16	Day 34
Glenn [b]	AP (%) [c]	Peak 1	46.7 a ±0.6	47.6 ab ±0.0	45.6 b ±0.2	41.6 c ±1.5	44.7 b ±0.2
		Peak 2	29.1 a ±0.6	26.6 b ±0.1	26.9 b ±0.3	29.6 a ±0.1	26.3 b ±0.1
		Total	75.8 a ±0.1	74.2 b ±0.1	72.5 c ±0.1	71.3 d ±0.5	71.0 d ±0.3
	Amy (%) [c]		24.2 d ±0.1	25.8 c ±0.1	27.5 b ±0.1	28.7 a ±0.5	29.0 a ±0.3
	Amy/AP ratio		0.32 d ±0.0	0.35 c ±0.0	0.38 b ±0.0	0.40 a ±0.0	0.41 a ±0.0
Parshall [b]	AP (%)	Peak 1	47.5 a ±1.7	46.6 b ±1.6	45.5 b ±0.2	43.3 bc ±0.0	43.0 c ±0.9
		Peak 2	30.5 a ±0.9	27.9 b ±0.6	27.4 b ±0.0	26.8 b ±0.0	26.7 b ±0.8
		Total	78.0 a ±0.8	74.6 b ±1.0	73.0 b ±0.1	70.2 c ±0.0	69.8 c ±0.1
	Amy (%)		22.0 c ±0.8	25.4 b ±1.0	27.0 b ±0.1	29.8 a ±0.0	30.2 a ±0.1
	Amy/AP ratio		0.28 c ±0.0	0.34 b ±0.0	0.37 b ±0.0	0.42 a ±0.0	0.43 a ±0.0

[a] GF= Flour from Hard Red Spring Wheat (HRSW) variety Glenn; G1=Dough prepared using HRSW Glenn and stored 1 day; G6= Dough stored 6 days; G16= Dough stored 16 days; G34= Dough stored 34 days. PF= Flour from HRSW variety Parshall; P1=Dough prepared using HRSW Parshall and stored 1 day; P6= Dough stored 6 days; P16= Dough stored 16 days; P34= Dough stored 34 days.

[b] Data are mean values and standard deviation of duplicates. Mean values followed by different letters in rows indicate significantly different at $P \leq 0.05$ level by Duncan's new multiple range test.

[c] AP: amylopectin; Amy: amylose

Table 3. Thermal properties of starch samples determined by Differential Scanning Calorimetry (DSC) [a]

Sample Name [c]	Thermal Properties [b]				
	T_o [°C]	T_p [°C]	T_c [°C]	T_c-T_o [°C]	ΔH [J/g]
GF	56.60 ±0.08	61.53 ±0.00	66.32 ±0.37	9.72 ±0.46	8.92 c ±0.35
G1	56.04 ±0.31	61.12 ±0.12	66.15 ±0.18	10.11 ±0.13	11.11 a ±0.17
G6	56.47 ±0.20	61.70 ±0.17	66.90 ±0.18	10.43 ±0.07	10.93 b ±0.17
G16	56.89 ±0.25	62.12 ±0.14	66.84 ±0.24	9.75 ±0.01	10.93 b ±0.35
G34	56.19 ±0.09	61.02 ±0.49	65.82 ±0.91	9.63 ±0.82	10.74 b ±0.22
PF	55.56 ±0.06	61.03 ±0.00	66.86 ±0.02	11.30 ±0.04	10.20 a ±0.20
P1	55.69 ±0.21	61.19 ±0.15	66.74 ±0.15	11.06 ±0.11	10.23 a ±0.49
P6	55.61 ±0.39	61.13 ±0.09	66.49 ±0.14	10.88 ±0.87	10.18 a ±0.18
P16	56.23 ±0.15	61.27 ±0.09	66.31 ±0.03	10.08 ±0.12	9.74 a ±0.30
P34	56.34 ±0.40	61.29 ±0.26	66.17 ±0.41	9.82 ±0.17	9.00 b ±0.19

[a] Mean and standard deviation of triplicate analysis. For ΔH, mean values followed by different letters within Glenn or Parshall samples indicate significantly different at $P \leq 0.05$ level by Duncan's new multiple range test.

[b] T_o, T_p, T_c = gelatinization onset, peak and conclusion temperatures; T_c-To= gelatinization temperature range, ΔH= gelatinization enthalpy

[c] GF= Flour from Hard Red Spring Wheat (HRSW) variety Glenn; G1=Dough prepared using HRSW Glenn and stored 1 day; G6= Dough stored 6 days; G16= Dough stored 16 days; G34= Dough stored 34 days. PF= Flour from HRSW variety Parshall; P1=Dough prepared using HRSW Parshall and stored 1 day; P6= Dough stored 6 days; P16= Dough stored 16 days; P34= Dough stored 34 days.

X-ray diffraction patterns showed the typical A-type pattern for cereal starch (Paris et al., 1999). It was found that even if the samples were tightly packed, X-rays still can penetrate to the aluminum-well substrate, producing undesired high peaks observed at higher angles (Figure 2). A scan range of 5-35° 2θ was selected to avoid the aluminum peaks. Also, the contribution from the starch material at high angles yielded little valuable information. The peaks in the range between 5-35° 2θ were used to calculate the relative degree of crystallinity.

The crystal structures were derived from studies on amylose (Imberty et al., 1991). In fact, the amylopectin crystallizes within the granule. Its side chain branches interwine to form the double helices which are the basis of the crystals. The observed difference in gelatinization properties is perhaps reflective of the differences in amylopectin structure. For starch from Glenn-flour, G1, G6, G16 and G34, the calculated degree of crystallinity was 8.4%, 10.9%, 9.8%, 9.3%, 8.1% respectively. For starch from PF, P1, P6, P16 and P34, the calculated degree of crystallinity was 8.0%, 8.8%, 9.6%, 8.2%, and 8.5% respectively. Those numbers were comparatively lower than value previously reported (Chakraborty et al., 2004) since the methods were different. Starch samples from Parshall- and Glenn-flour and from dough did not exhibit significantly different polymorph patterns and relative crystallinity, which reaffirms that the mutation for the *Wx*-B1 gene does not significantly reduce amylose content (Kim et al., 2003). d-Spacing at 2θ was a major difference between the waxy and non-waxy starches. It corresponded to the amylose-lipid complex (Zobel, Young & Rocca, 1988). This d-spacing in Parshall was not missing, but the height of the peak was lower than that of Glenn.

ΔH of the starches was consistent with their degree of crystallinity (Yoo & Jane,

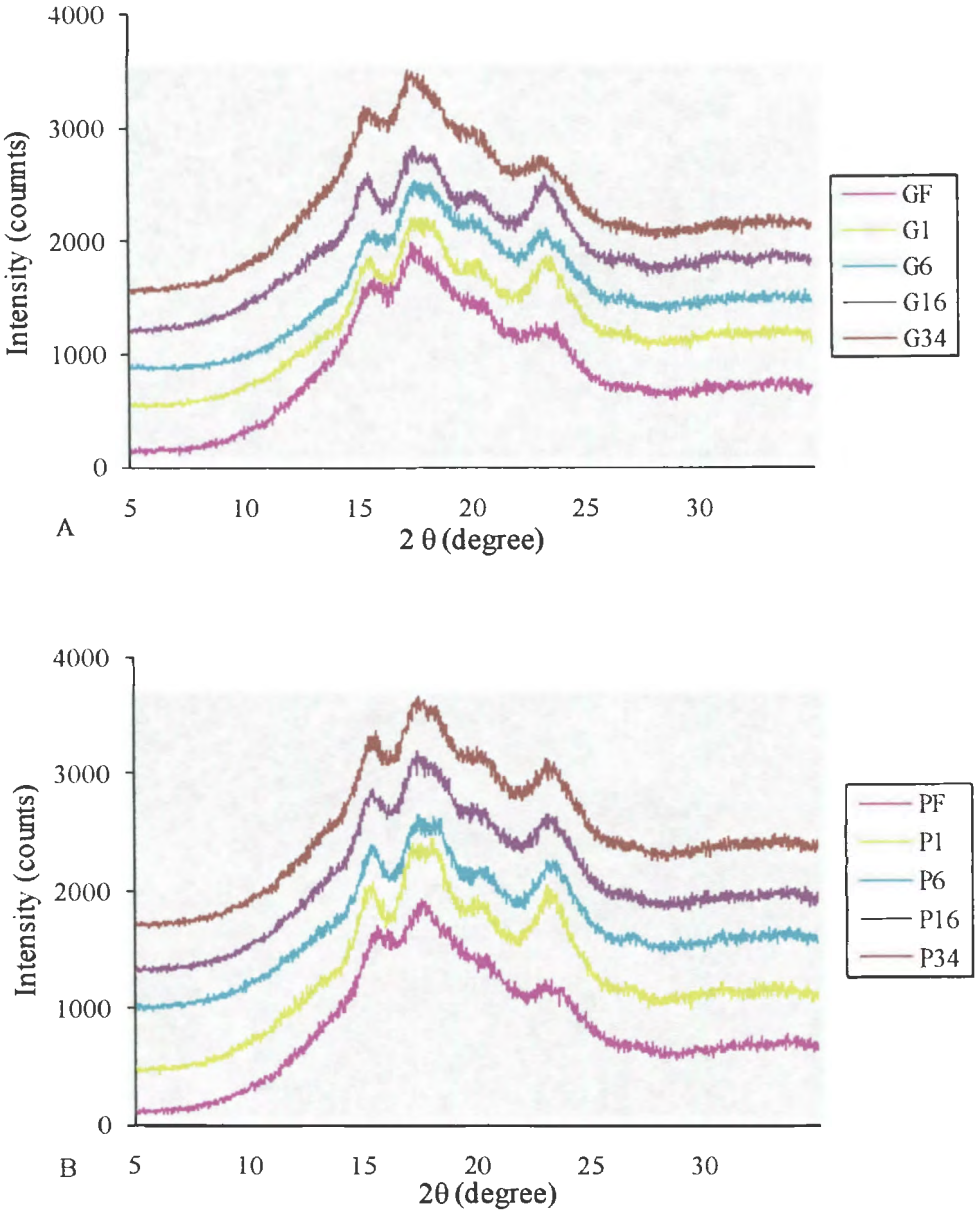

Figure 2. X-ray diffraction patterns of starch from flour and dough samples for Hard Red Spring varieties A) Glenn and B) Parshall [a].

[a] GF= Flour from Hard Red Spring Wheat (HRSW) variety Glenn; G1=Dough prepared using HRSW Glenn and stored 1 day; G6= Dough stored 6 days; G16= Dough stored 16 days; G34= Dough stored 34 days.
PF= Flour from HRSW variety Parshall; P1=Dough prepared using HRSW Parshall and stored 1 day; P6= Dough stored 6 days; P16= Dough stored 16 days; P34= Dough stored 34 days.

2002). Glenn flour and dough starch samples both had higher ΔH and crystallinity than Parshall. A large amount of energy was required for gelatinization because of the higher crystallinity. However, DSC and X-ray diffraction techniques do not measure the same property of starch. The X-ray diffraction method detects the crystalline part of starch but does not determine the level of molecular order. DSC, on the other hand, determines the breakdown of crystalline order and molecular order (double helices) during gelatinization (Cooke & Gidley, 1992).

Starch Granule Morphology

Starch is seen as discrete granules within the endosperm of the wheat kernel. Wheat endosperm contains mainly two types of granules: a larger type, mostly about 20-35 micrometers (μm) across (A-starch), being lenticular in shape, and a smaller spherical shape, ranging from 2-8 μm in diameter (B-starch) (Cornell, 2004). It was also reported that no detectable differences were found in the granule-size distribution and granule morphology between waxy and other wheat flour (Yoo & Jane, 2002). The ultrastructure of the starch granules in frozen dough was observed as: starch granules were intact after 24 hours of frozen storage, but they were damaged internally and obviously after 24 weeks of storage (Berglund et al., 1991).

The granular sizes of flour and dough starch samples measured in this study were in accordance with the above range (Large granules: 20-30 μm; small granules: 4-8 μm). Glenn and Parshall flour and dough starch did not have difference in granule-size distribution and granule morphology. But, from day 16, the granular surface changed as the cracks showed up, especially in large granules, indicating α-amylase degraded the starch granules, preferentially large granules (Figure 3).

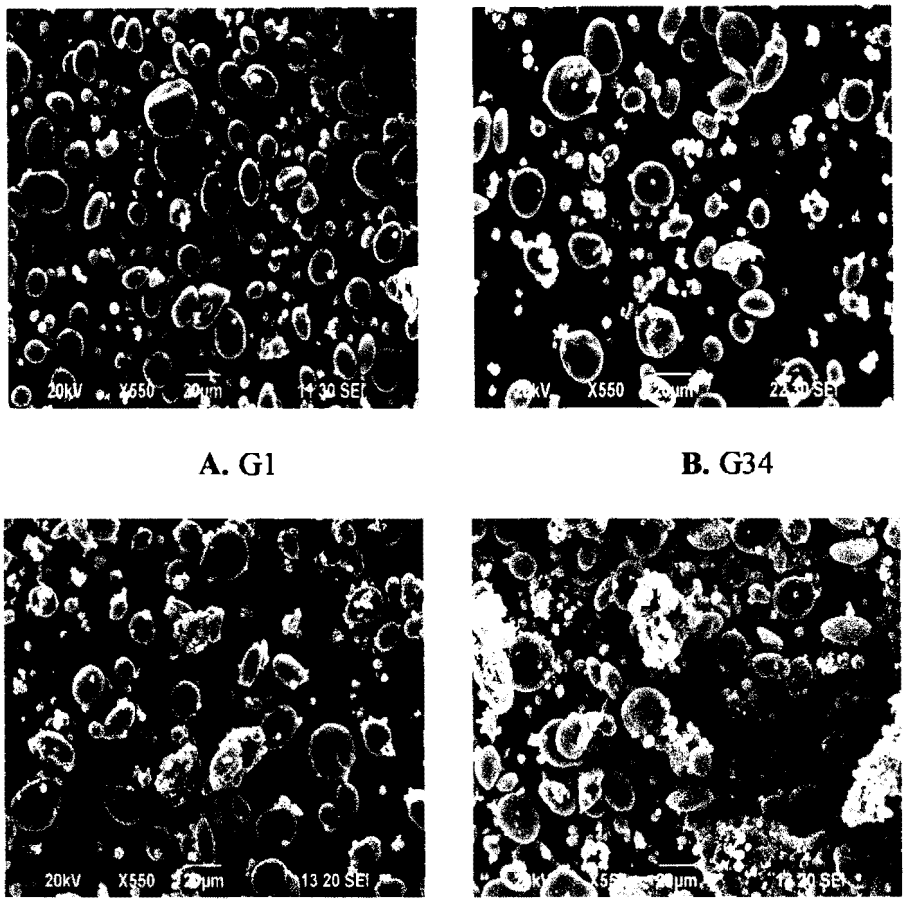

Figure 3. Scanning Electron Microscopy (SEM) images of starch from dough samples.

A: G1= Starch from dough prepared using Hard Red Spring Wheat (HRSW) variety Glenn and stored 1 day; B: G34= Starch from dough prepared using HRSW variety Glenn and stored 34 days. C: P1= Starch from dough prepared using HRSW variety Parshall and stored 1 day D: P34= Starch from dough prepared using HRSW variety Parshall and stored 34 days.

Conclusions

Starch properties changed in many aspects during refrigeration storage in both normal and partial waxy wheat starches. More noticeable changes in starch properties in RVA profiles compared to the X-ray and DSC data were detected. During the refrigerated storage, there was less change in Glenn (normal starch) samples than Parshall (partial waxy) samples in terms of pasting properties and molecular weight distribution of the starch component.

Previously, it had been reported that water holding capacity of dough decreased during refrigerated storage and a dark-yellowish liquid known as syrup was formed (Courtin et al., 2006; Simsek & Ohm, 2009). Therefore, it is believed that formation of this free water is associated with the starch properties. Water has many significant roles in determining the properties of starch. Water below gelatinization (melting + dissolution) temperature acts essentially as a plasticizer (Bizot et al., 1997), while, at higher temperatures, it becomes a solvent (Moates et al., 1997). Water is essential to the crystallinity of starch based glucans as it permits rearrangements by plasticization of amorphous areas and the buildup of crystalline hydrate lattices of different stoichiometries depending on the polymorphic type (Imberty et al., 1991). Lu & Grant (1999a) reported that water in thawed frozen dough separated into pools, causing less free water to be distributed throughout the doughs after 24 weeks of frozen storage, which resulted in significant change of DSC thermal properties.

In the present study, water was also an important factor. In refrigerated dough systems and during their storage, the temperature was below gelatinization temperature. However, as water diffused to the dough surface and dough essentially lost water binding

ability, the properties of starch changed during storage. Also, α-amylase activity, even at low levels, can significantly change the apparent molecular weight of starch during storage (Table 3), resulting in the changes of starch functional properties.

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PAPER 3. RHEOLOGICAL CHANGES IN REFRIGERATED DOUGH DURING STORAGE IN RELATION TO PROTEINS

(This paper is modified from the manuscript which has already been accepted for publication. Reference: Zhang, Y., Simsek, S., Campanella, O. H., Ohm, J. B., Chang, H., Reuhs, B. L., & Mergoum, M. (2009). Rheological changes in refrigerated dough during storage in relation to protein composition. *Journal of Food Process Engineering*. doi: 10.1111/j.1745-4530.2009.00415.x)

Abstract

Refrigerated dough is a flour-based, unbaked product that is stored between 4-7 °C. The aim of this work was to study the rheological properties of refrigerated dough during storage and determine their relationship with dough proteins. Rheological properties were determined using texture analyzer and dynamic oscillatory rheometry during 34 days of storage. The protein analysis was performed by High Performance Size Exclusion Chromatography (HP-SEC). On day-34, R_{max} was 93.8 % higher than day-0. Both, the G' and G" moduli decreased during storage. Dough exhibited major decreases on the moduli on day 3 and day 16. By comparing the viscoelastic properties of day 0 and day 16, a decrease of 50% on the elastic modulus and a roughly 30% in the loss modulus were observed. Changes in the protein fractions of dough samples were related to their rheological properties. The high and low molecular weight polymeric protein and gliadin were positively correlated to dough extensibility ($r > 0.8343$).

Introduction

The refrigerated dough industry is considered one of the fastest growing segments of the ready-to-use grain-based industry. Refrigerated dough products account for more than $1.6 billion of sales per year in the USA. The items in this category include canned refrigerated biscuits, canned refrigerated croissants, and sweet rolls (Courtin et al., 2006).

In today's market, in-store bakeries are using frozen dough or par-bake products, which offer good quality, but to improve further the quality of these products, refrigerated dough is considered a good convenient and economical choice for the production of fresh-bread products. The high quality of bakery products made of refrigerated doughs should be characterized by a long shelf life with good organoleptic properties.

During refrigerated storage, a yellowish liquid separates from the dough forming a syrup that can leak out of the product to produce a deleterious phenomenon known as "dough syruping" (Courtin et al., 2006). It has been reported that dough syruping is the result of natural enzymes found in flour. These enzymes, endoxylanases, modify the water holding capacity of arabinoxylans (AX), which are polysaccharides present in the dough. AX are found in wheat cell wall and they cannot be separated during the milling operation. They have strong water holding capacity, but can be hydrolyzed by the presence of wheat flour endoxylanases during refrigerated storage, thus decreasing AX's water-holding and producing the dough syruping effect (Gys et al., 2004). Associated with the dough syruping phenomenon, there are other changes on the rheology of the dough that have a large impact on the properties of the final bread (Hoseney, 1998). The present research has shown that interactions between macromolecular components present in dough, including polysaccharides and proteins, affect dough syruping and the rheological properties of the

refrigerated dough. It also has been reported that these changes fundamentally affect the overall quality of final bread products (Janssen et al., 1996).

Dough is a complex viscoelastic material system, which is difficult to characterize because of several factors influencing its rheological properties (Weipert, 1990). Among them we can note flour composition, water content, temperature and relevant processing parameters such as mixing time and resting time. Farinograph, mixograph, alveograph, and extensograph are empirical tests used in the industry to measure rheological parameters of dough such as "consistency, hardness, texture, and viscosity" (Hoseney, 1998). As these methods are highly dependent on the sample size and type of geometry utilized during the testing, the result is a single-point measurement. For instance, the force needed to destroy the dough structure is considered to be the dough's "strength" and is often correlated with the flour's baking behavior. These methods often provide good correlations with breadmaking performance, but the results cannot be interpreted in terms of fundamental material properties (Menjivar, 1990). In contrast, fundamental rheometry is capable of describing the physical properties of a material over a wide range of strains and strain rates. Fundamental rheological properties may be adequate to test dough systems as they may reflect the polymeric nature of these materials. Dynamic oscillatory rheometry is simultaneously measures the elastic and the viscous components of viscoelastic materials, such as dough, at different frequencies (Lu & Grant, 1999).

Albumins, globulins, gliadins, and glutenins are the components of protein in the wheat flour. The most important ones are gliadins and glutenins which are 80-85% of the wheat storage protein. In dough, they can form gluten providing its unique viscoelastic and gas-retaining properties, which have a key role in determining the quality of wheat flour

and its derived end products (Veraverbeke & Delcour, 2002). The rheological properties of dough determined by empirical methods are essential for breadmaking evaluation and they are largely affected by the wheat gluten proteins (Janssen et al., 1996). MacRitchie et al. (1987, and 1991) have conducted research aimed to fractionate and characterize glutens from different wheat cultivars that vary in baking quality.

There are no studies reporting changes on the viscoelastic properties of refrigerated dough in relation to changes in the protein composition. Thus, the aim of the present work was to study how the rheological properties, specifically viscoelasticity, of refrigerated dough change during refrigeration storage and how those changes are correlated to the structural and compositional changes of dough proteins. Rheological tests were performed by measuring the properties of dough under a large deformation using texture analyzer and under a small deformation using dynamic oscillatory rheometry. The protein analysis was performed by Size-Exclusion High Performance Liquid Chromatography (SE-HPLC).

Materials and Methods

Materials

Glenn, a common wheat variety of Hard Red Spring Wheat (HRSW), was obtained from NDSU Casselton Research Extension Center in 2007. Glenn is one of the most commonly grown varieties in North Dakota, the main HRSW growing area in the United States. It was milled into flour using a Buhler laboratory mill in the NDSU HRSW quality laboratory. The main psychochemical characteristics of Glenn are reported in Table 1.

Preparation of Refrigerated Dough

In order to avoid confounding factors arising from the presence of other ingredients, a lean dough formula was used. The dough was prepared by using 100 g of flour (14% moisture basis), 1.8 g of salt, and a certain amount of water, containing 0.06% w/v of sodium azide (Mallinckrodt baker Inc. Paris, KY) to prevent microbial spoilage, to reach the desired moisture content previously determined according to the specific farinograph absorption test (Table 1). Dough was mixed in a 100 g pin mixer (National Manufacturing, Lincoln, NE) for the previously determined optimum mixing time (3 min 45 sec), sheeted, molded, and stored in plastic containers for 0 (analysis was done immediately after mixing), 1, 2, 3, 6, 10, 16 and 34 days at 6°C.

Empirical and Fundamental Rheological Methods

Micro-extension test (large deformation rheology)

Micro-extension test was used to investigate changes in the dough extensibility and resistance. A Texture Analyzer (TA-XT2 from Texture Technologies Corp., Scarsdale, NY) was used to perform extension tests on small scale (0.8 g) dough strips at room temperature (Kieffer et al., 1998). The maximum resistance (R_{max}) and the extensibility of refrigerated doughs were determined from the test.

Fundamental viscoelastic analysis (small deformation rheology)

To complement the empirical rheological tests, in which a large deformation was applied, and to be able to characterize macromolecular interactions between the main components of the dough samples, fundamental rheology methods under small deformations were performed.

Table 1. Properties of the hard red spring variety, Glenn [a]

Variety	Test weight (kg/hL)	Wheat protein (%)[b]	Falling Number(sec)	Flour ash (%)[c]	Flour protein (%)[c]	Wet Gluten(%)[c]	Farinograph absorption (%)[d]
Glenn	82.9 ±0.02	14.3 ±0.01	429 ±2.00	0.49 ±0.02	13.6 ±0.03	34.8 ±1.20	66.2 ±0.01

[a] Data are mean values and standard deviation of duplicates.
[b] The analysis was expressed on 12% moisture basis.
[c] The analysis was expressed on 14% moisture basis.
[d] Water absorption to reach 500 farinograph units (FU) line and based on 14% moisture basis.

Dynamic rheological analysis to determine its viscoelastic properties of dough was performed using a controlled stress rheometer and parallel plates geometry (ARG2 Rheometer, TA Instruments, New Castle, DE). The dough samples were placed between two parallel plates of 4 cm diameter) and the gap was adjusted to 1 mm. Strain sweep tests were performed to assess the linear viscoelastic region: dynamic moduli were collected and plotted as a function of the applied strain. Oscillatory tests with a frequency sweep from 0.01 to 100 Hz were conducted at 25 °C for all the samples under strain of 0.5%, which was within the linear viscoelastic behavior as previously determined by a strain sweep test. The dynamic rheological properties of samples measured were the storage modulus G' (elastic modulus) and the loss modulus G" (viscous modulus) (Vansteenkiste et al., 2004). The tests were replicated three times to account for the variability of the data. Average and corresponding standard errors were calculated and reported in the graphs that show the viscoelastic properties of refrigerated dough stored at different storage times.

Protein Composition and Molecular Weight Determination

Flour proteins were extracted as described by Gupta *et al.* (1993) with minor modification (Ohm et al., 2006). Sodium Dodecyl Sulphate (SDS) extractable and unextractable proteins were obtained following the procedure of Gupta et al. (1993). The extracts of total, extractable and unextractable protein fractions were analyzed by High Performance Size Exclusion Chromatography (HP-SEC) (Agilent Technologies, Wilmington, DE) as described by Batey et al. (1991) and Larroque et al. (2000). The modification consisted in using a new separation column such as the Phenomenex BIOSEP SEC S4000 narrow bore column (300 x 4.5 mm, Phenomenex, Torrance, CA) with a guard cartridge. Filtered supernatant (10 μL) was injected and eluted by 50 % acetonitrile in water with 0.1 % trifluroacetic acid for 10 min with a flow rate of 0.5 mL/min. Solutes were detected at a wavelength of 214 nm using Agilent 1200 Photodiode Array Detector (Agilent Technologies, Waldbroann, Germany).

Statistical Analysis

All of the experiments and HP-SEC analysis were performed in triplicate and statistical analyses were performed, using SAS System for Windows (V. 9.1, SAS Institute, Cary, NC). Absorbance Area (AA) was calculated as the mean absorbance multiplied by the time interval, which was 0.002 min. The sum of AA for each retention time interval of 0.01 min between 3.6 and 7.7 min of run time were used for data analysis. Simple linear correlation coefficients (r) were calculated between AA and rheological parameters for each 0.01 min retention interval, and shown as a continuous spectrum over retention time.

Results and Discussions

Micro-extension Test - Large Deformation Rheology

In these tests, maximum measured force stands for "maximum resistance (R_{max})" while the corresponding distance stands for "Extensibility", which are the rheological parameters illustrated in Figure 1. The extension tests showed that R_{max} and extensibility changed dramatically during the refrigeration storage. They were inversely correlated to each other. R_{max} was increasing in the early stages of storage and decreasing a little after ten days of storage (Figure 1A). From day 0 to day 10, R_{max} increased by more than two times and at the end of the storage - day 34, R_{max} was still 93.8 % higher than the one on day 0. In contrast, extensibility was decreasing during storage and then, it was increasing a little after day 6 (Figure 1B). From day 0 to day 10, extensibility decreased by 38.5%. On day 34, extensibility was only 32.9% of the initial value. It has been reported that the performance in these tests is largely determined by protein composition (Tronsmo et al., 2003).

Fundamental Rheology Measurement - Small Deformation Test

Many problems associated to the rheological characterization of dough have been mentioned in the literature (Zheng et al., 2000). Among them, slippage between the sample and the plates and sample dehydration appear to be of importance. To assess and minimize these potential artifacts under the conditions used to prepare and test the dough in this work, sand paper was attached to the plates to prevent slippage and a coating of oil was applied to the external surface of the sample to prevent dehydration. However, results did not vary significantly when the samples were tested without the sand paper and without the oil but with the sample slightly trimmed. The natural stickiness of the sample and some of

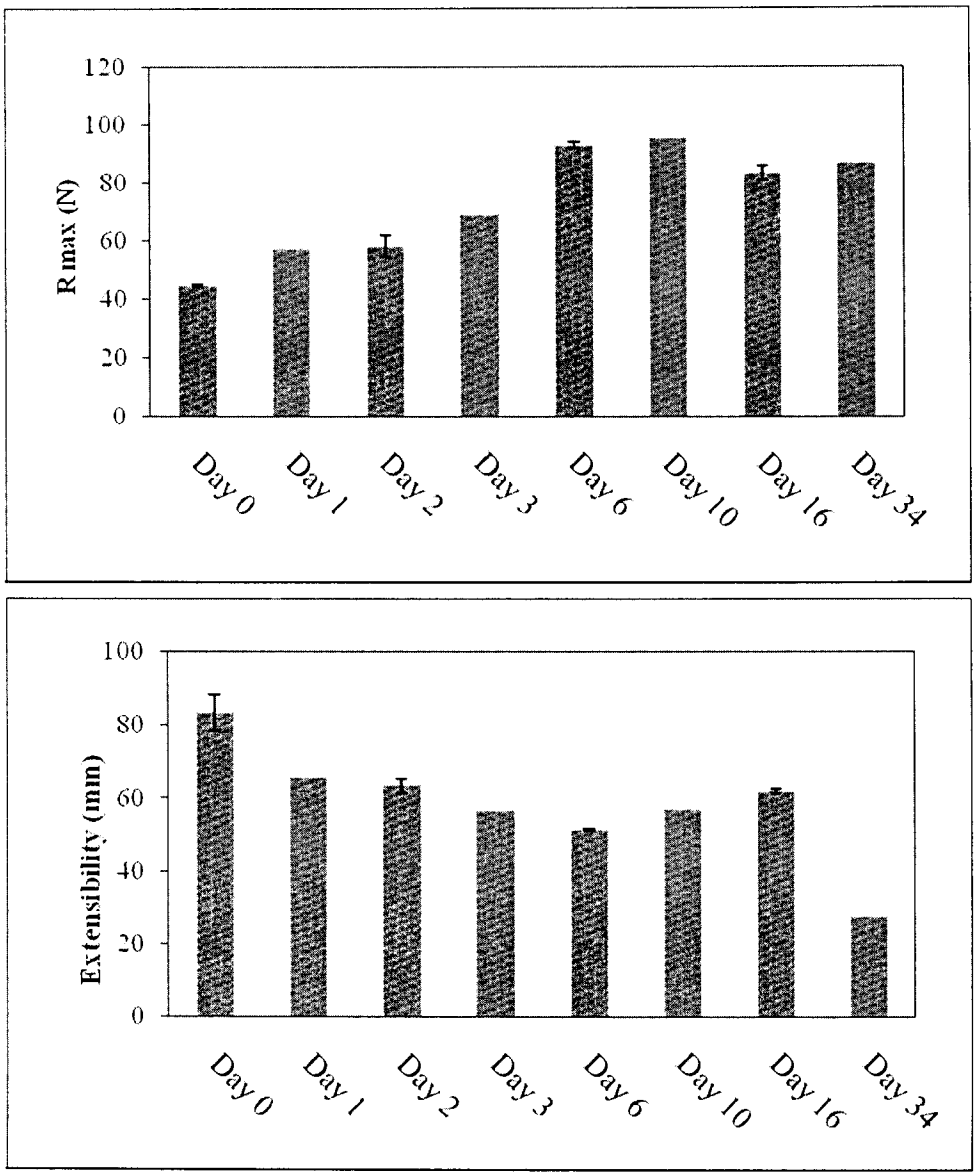

Figure 1. Analysis of refrigerated dough samples with TA-XT2*i* texture analyzer. (A) Maximum distance in millimetres dough strips stretched. (B) Maximum force in N required to tear dough strips. Error bars represent standard deviations.

the sample protruding the plates were conditions that prevented slippage and sample dehydration. The approach was preferred given the potential artifacts that may be introduced with the use of sand paper and oil. In the first case, the attachment of sand paper introduced some difficulties in setting and maintaining the selected gap, whereas the use of oil often may produce slippage due to migration of the oil to the interface between the plates and the samples.

Strain sweep

The validity of the calculated fundamental rheological parameters (G', G") requires that the samples are tested under conditions by which they can respond as linear viscoelastic materials (Menjivar, 1990). Wheat flour dough appears to behave linearly at low strain levels. Linear behavior under low strains implies that the small deformation does not damage to the dough's structure (Faubion & Hoseney, 1990). From the results of strain sweep tests, 0.5 % strain level was found to be within the linear viscoelastic range of the samples and used in all tests.

Frequency sweep

The elastic/storage modulus (G') and viscous/loss modulus (G") were determined as a function of the angular frequency (rad/s) at a strain of 0.5% for all dough samples (Figure 3). In agreement with the empirical tests, Figure 3 shows large changes on the rheological properties (both G' and G") with storage times. Given the viscoelasticity of dough, both moduli increased with the frequency, but G" increased at a lower rate than G'. In addition, values of G' were significantly higher than G" values, which shows the elastic nature of these samples. It is observed that these moduli decreased at a higher rate during the first

three days of storage (Figure 2). The decrease in G' due to the refrigeration storage suggests a decrease in dough elasticity.

Doughs exhibited the major decreases on the moduli on day 3 and day 16. By comparing the viscoelastic properties of day 0 and day 16, there is a decrease of 50% on the elastic modulus and a roughly 30% decrease in the loss modulus at the 150 rad/s (angular frequency).

By comparing the storage modulus for the doughs at different storage days, it can be observed that the dough exhibited the greatest elasticity with a storage modulus G' of approximately 34 kPa at an angular frequency 157 rad/s on day 0. These changes on rheological properties upon refrigerated storage could be also attributed to changes in protein composition (see below).

From the fundamental (small deformation) and empirical (large deformations) rheological measurements, dough samples exhibited high resistance to extension and high complex modulus (G*), calculated by using both the storage and the loss moduli as

$$G^* = \sqrt{G'^2 + G''^2}$$

Results are in agreement with those obtained by Abdelrahman and Spies (1986) who reported that protein composition has effect on storage modulus. Similarly, Kenny et al. (1999) also reported that protein composition was responsible for resistance, complex modulus and phase angle.

In summary, large deformation and small deformation rheological tests showed that upon refrigerated storage, the rheological properties of dough change significantly.

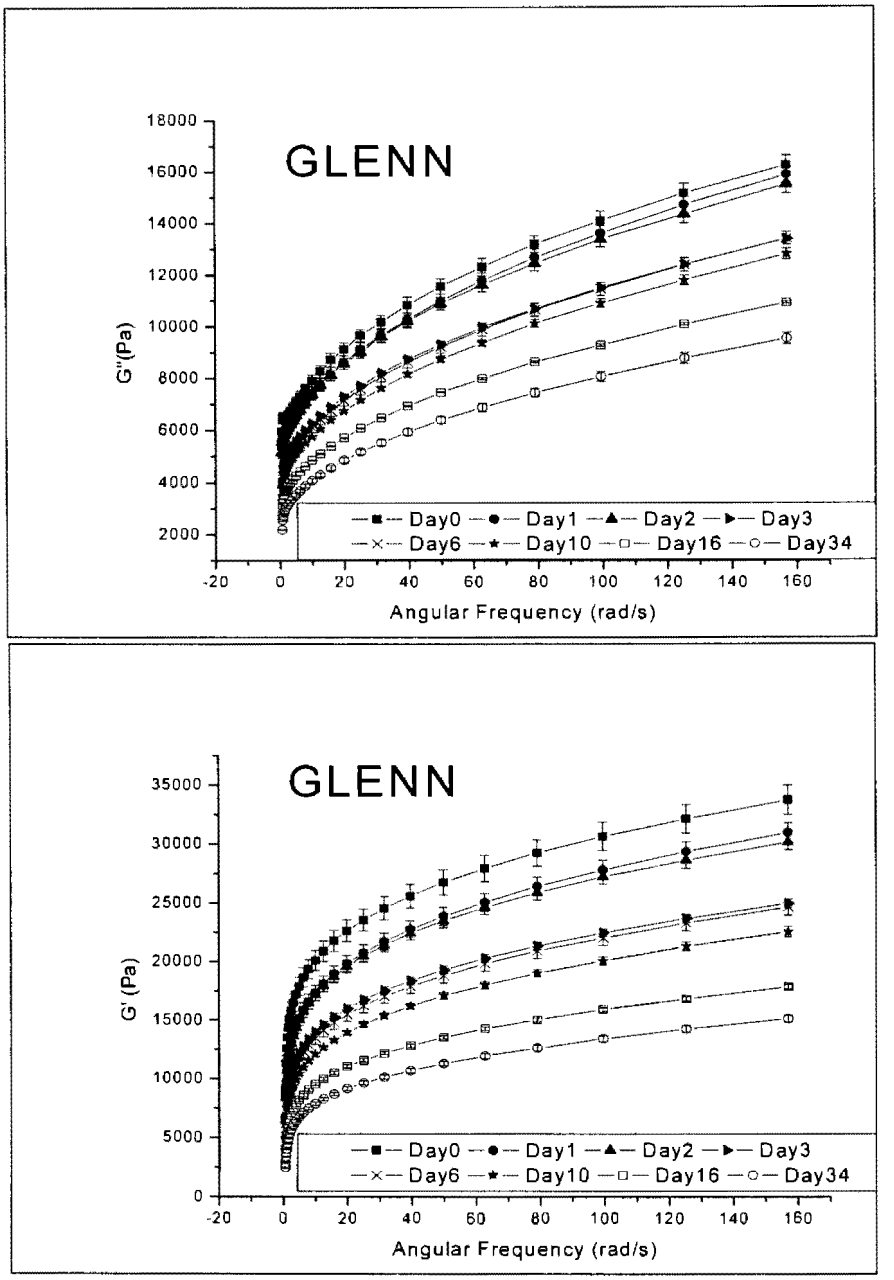

Figure 2. Changes in elastic modulus (G') and viscous modulus (G") during refrigerated storage for dough samples. Error bars represent standard deviations.

Empirical rheological measurements generally provide good correlations with bread-making performance, but they are empirical in nature, so these tests cannot be interpreted in terms of molecular interactions between the material components (Menjivar, 1990). Conversely, in small deformation oscillatory testing, interactions caused by protein-protein, starch-starch, starch-protein interactions could be assessed under specific testing conditions, however, they cannot be resolved in terms of final product quality and thus the results are not often correlated with baking quality (Janssen et al., 1996; Khatkar & Schofield, 2002).

Protein Changes during the Storage

HP-SEC has been extensively used to analyze molecular weight distribution of wheat proteins (Bietz, 1984; Ohm et al., 2008). The analysis of wheat proteins using HP-SEC exhibited five main protein fractions corresponding to several molecular weights. These are: very high (3.6-4.0 min retention time), high (4.0-4.3 min) and low (4.3-6.15 min) molecular weight polymeric protein, gliadin (6.15-6.85 min), and albumin and globulin (6.85-7.7 min) fractions (Figure 3A). Gluten proteins are a heterogeneous class with a mixture of polymeric glutenin having molecular weight from 80,000 Da to several million Da and monomeric gliadins of molecular weights ranging from 30,000 Da to 80,000 Da. Albumins and globulins are non-gluten proteins whose molecular weights were reported to be <25,000 Da (Veraverbeke & Delcour, 2002).

The results obtained in the present work appear to reflect protein-protein interaction, and they can be relevant to affect the rheology of dough, as changes were also determined in the protein upon storage. In the extractable protein of doughs, the peaks of polymeric protein fractions and gliadins became lower with the storage (Figure 3B)

indicating that the protein was hydrolyzed during refrigeration storage. As noted in the Figure 3B, the peak of soluble protein fractions (albumin and globulin) increased and was calculated as 41.1%. These results were in agreement with changes in the rheological properties of dough. Effects of protein amount on rheological properties of dough have already been reported in the literature (Abdelrahman & Spies, 1986; Dong, 1992; Petrofsky & Hoseney, 1995).

The correlation coefficients (r) for extensibility, R_{max}, G' and G" against Absorbance Area (AA) of the total and extractable protein at each elution time were determined. Extensibility was positively correlated to AA while R_{max} was negatively correlated. Extensibility showed higher significant correlation with the AA of proteins eluted at different times (Figure 4). The high (3.87-4.10 min) and low (4.61-5.15 min) molecular weight polymeric protein, and gliadin (5.92-6.56 min) were highly and positively correlated to extensibility ($r > 0.83$). Soluble protein (7.20-7.06 min) was highly and negatively correlated to extensibility ($r < - 0.83$). The maximum correlation coefficient (r_{max}) in extensibility corresponded to an elution time near 4.07 and 6.51 min (Figure 4). For R_{max}, r was highly significant only to the polymeric protein fraction (high molecular weight protein fraction). Also, G' and G" were highly correlated to polymeric protein, gliadin and soluble protein. The r_{max} corresponded to an elution time near 4.02 min. G' was negatively correlated to soluble protein fraction in the unextractable protein HPLC chromatogram (Figure 5). While the soluble protein fraction became larger because of hydrolysis during storage, G' became lower. Previous work has shown that there is a critical molecular size above which polymeric proteins contribute to dough strength

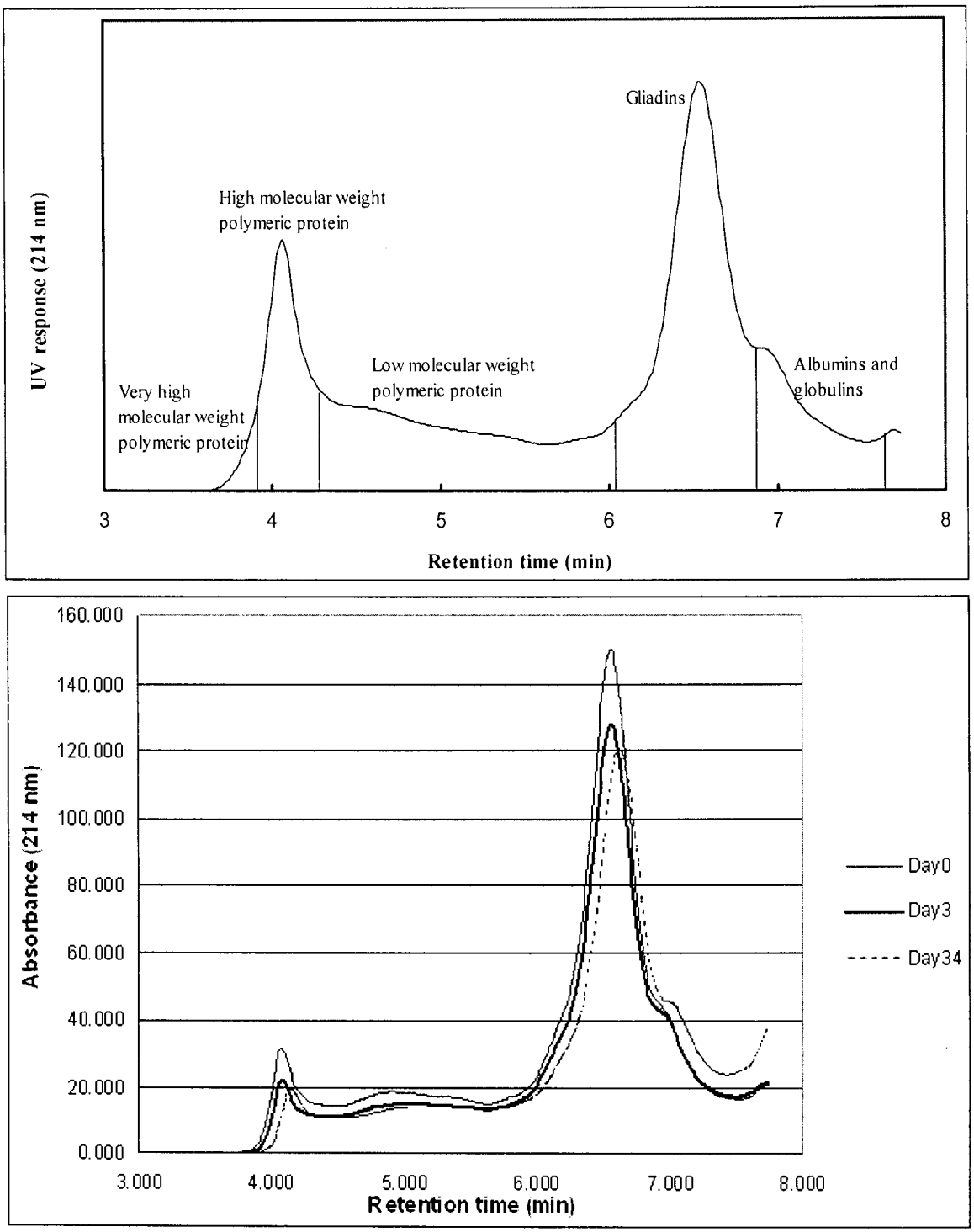

Figure 3. (A) The main fractions of wheat proteins using High Performance Size-Exclusion Chromatography (HP-SEC), (B) Chromatograms of the changes in the extractable protein of doughs during refrigeration storage.

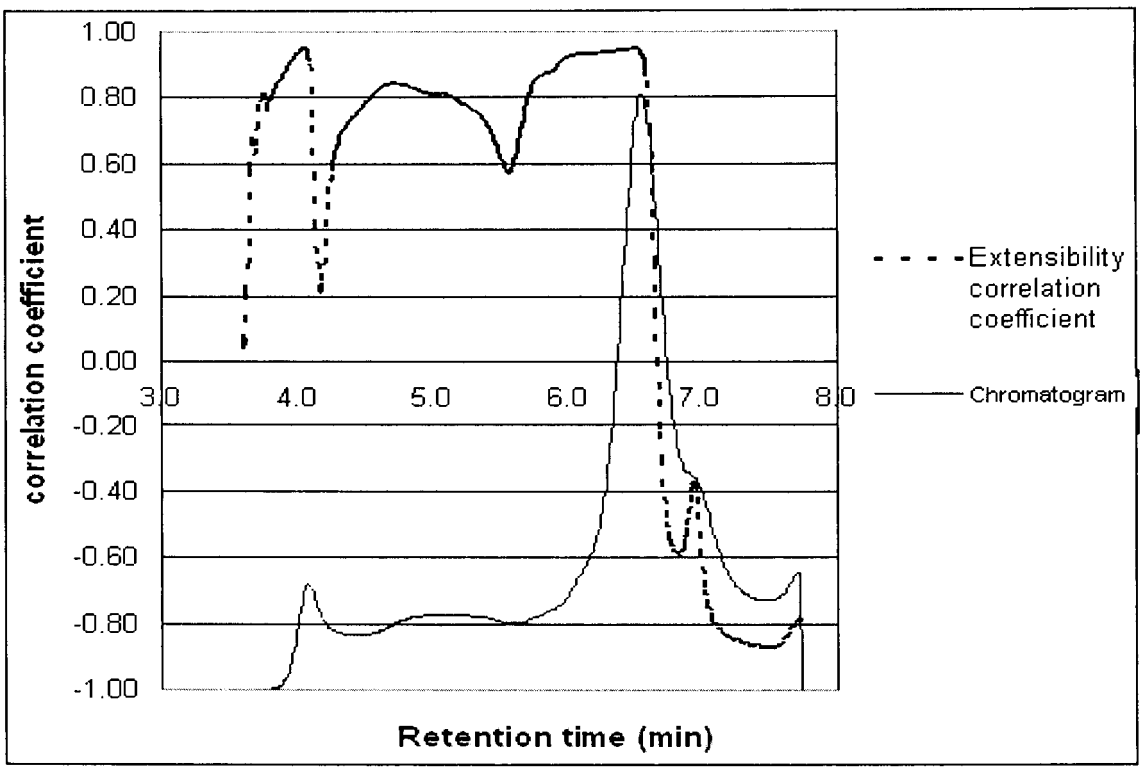

Figure 4. Correlation coefficient of extensibility (distance) against Absorbance Area (AA) of the extractable protein.

(Bangur et al., 1997) and those gliadins and glutenins are mainly responsible for the functional properties such as dough extensibility and elasticity. Our study also proved the high molecular weight protein fractions are important in dough rheological properties, but at the same time, the soluble protein (small molecular weight) also affects the rheological properties of dough.

The elastic modulus and the viscous moduli decreased during refrigeration storage. In terms of the elastic modulus, a measure of dough elasticity, results show that upon storage the dough becomes less elastic. These results showed that the rate in the decrease of elasticity was different at the different stages of storage. As rheological changes are a

macroscopic reflection of chemical and structural changes in the sample, it could be hypothesized that during refrigerated dough storage, proteins may have an influence. Although previous studies have considered that the syruping phenomenon is associated to the presence of endoxylanases (Gys et al., 2004), this study shows that significant rheological changes are observed and cannot be attributed solely to the presence and

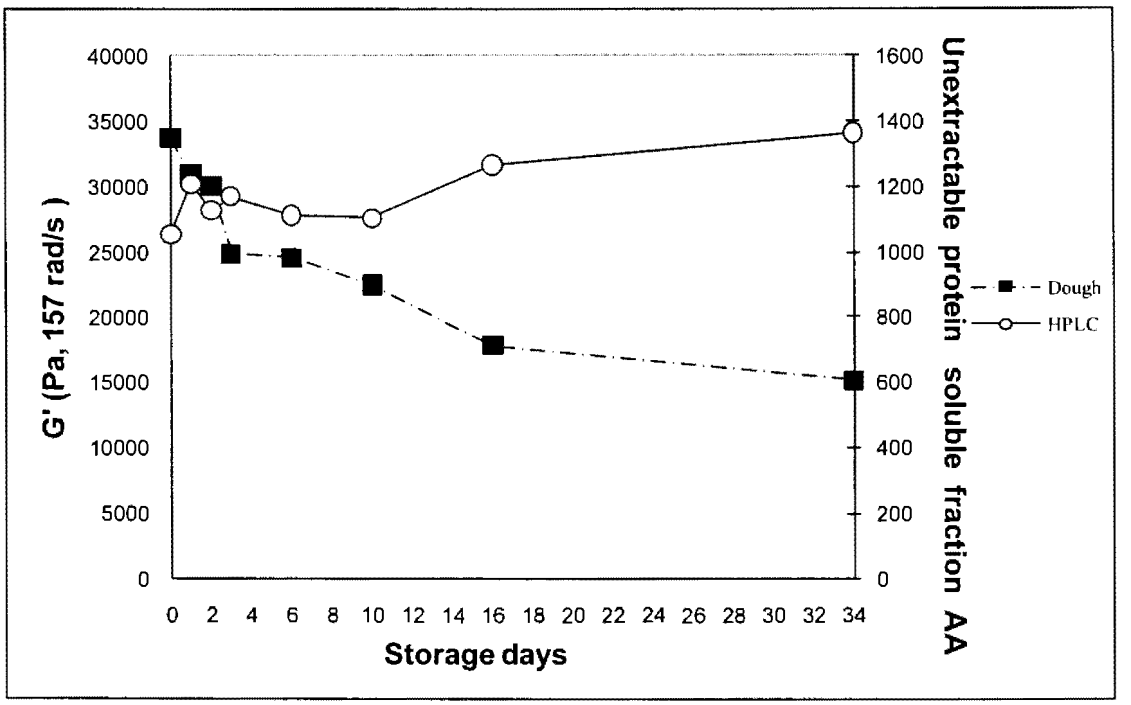

Figure 5. The correlation between G' of dough and soluble protein fractions in unextractable proteins using HP-SEC.

degradation of AX by these enzymes. Important changes in the protein composition during storage may contribute to changes in the rheology of these doughs.

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GENERAL CONCLUSION

This thesis focused on: 1) the structural and chemical analysis of polysaccharides and protein components in dough samples using two Hard Red Spring Wheat (HRSW) varieties with different amylose content, 2) characterization of arabinoxylans (AX) and starch functionality and identification of their changes during extended refrigeration storage, and 3) determination of dough rheological properties associated with dough syruping, using both empirical and fundamental rheological methods. The overall purposes of this study were to explain the structural and chemical basis for deterioration of refrigerated dough quality.

The first paper investigated the structural changes of AX in relation to their physicochemical properties and the degree of dough syruping (DDS). It can be concluded that the dough sample initially exhibited a considerable increase on DDS and then DDS increased slowly compared to the first few days of storage. Water unextractable AX (WUAX) was hydrolyzed and water extractable AX (WEAX) was degraded due to the presence of endogenous xylanase activity during refrigerated storage of dough, resulting in the formation of dough syruping. By comparing WEAX extracted from the flour and from the dough on storage day 34, the high molecular weight fraction decreased up to 17%, while the low molecular weight fraction peak III increased up to 11%.

The second paper investigated the structural changes of starch in relation to its physicochemical properties. Starch properties changed in many aspects during refrigerated storage in both normal wheat (Glenn) and partial waxy (Parshall) wheat samples. The relative percentage of amylopectin in starch structural profile decreased up to 10.51%. More dramatic changes were detected in starch properties of rapid viscosity analyzer

(RVA) profiles compared to the X-ray diffraction and differential scanning calorimetry (DSC) patterns. Compared to Glenn, Glenn had less change on pasting properties and the molecular weight distribution of starch component during storage.

The third paper investigated the changes of dough rheological properties in relation to the protein chemistry. The rheological strength of refrigerated dough declined throughout storage, which was reflected by a decrease of elastic modulus (G'). Significant rheological changes were correlated to the important changes in the protein composition during storage. G' and viscous modulus (G") were highly correlated to polymeric protein, gliadin and soluble protein fractions.

Results from the three papers in this study lead to a conclusion that the reduction of water holding capacity of the dough resulted in syrup formation, which is associated with the physiochemical changes in the components of dough, such as AX, starch and protein. Syrup formation indicates water redistribution in the dough system and separation of water molecules from the hydrophilic components with which they are normally associated. The enzymes endogenously existing in the flour, i.e. endoxylanase, amylase and probably proteases, are activated when water is added and play critical roles in determining the functional characteristics of the flour and dough. As AX are degraded by endoxylanase and lose their strong water holding capacity, more free water is released from the dough system. Alpha-amylase and proteases can become more active and cause the degradation of starch and protein component. Also, the decrease in hydrophobic forces, which stabilize gluten, may contribute to the slow deterioration of gluten functionality. In refrigerated dough, the degradation of AX should be avoided at all times because of its utmost

important role in water holding capacity. Flours with minimum endoxylanase activity are suitable for refrigerated dough industry.

The findings of this study will benefit the refrigerated dough industry in terms of providing them a better understanding of the dough syruping phenomenon and the changes in dough macromolecules at the micro-structural level. Further research is needed to investigate the effect of dough syruping on the end-product quality after extended refrigeration storage, relate the refrigerated dough properties to the baked product quality, and develop methods to control dough syruping efficiently and economically without affecting the texture and flavor.