THERMAL PROPERTIES OF STARCH FROM TRANSGENIC ISOLINES OF WHEAT
DIFFERING IN STARCH SURFACE COMPONENTS

by

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B.S, Federal University of Santa Catarina, Brazil, 2007

A THESIS

Submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Grain Science and Industry
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2010

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Abstract

Endosperm texture is an important characteristic in determining wheat processing and end-use. The presence of puroindoline proteins on the starch surface is the biochemical marker for wheat hardness. Near-isogenic samples overexpressing puroindolines have been used to assess the effect of wheat hardness on final product characteristics. The objective of this study was to determine differences among starch isolated from near-isogenic samples and to investigate the role starch surface components play in pasting. The use of near-isogenic samples overexpressing puroindolines combined with the use of two methods of starch isolation (batter and dough) was an effective means to create samples with varied amounts of surface components. Starch thermal properties were characterized and surface proteins and lipids were quantified. Starch isolated from hard wheat cultivars presented more similarities with starch isolated from its soft near-isogenic line when a dough method was used than when a batter method was used. Starch from soft experimental lines isolated using a batter method showed increased MVA peak viscosity, breakdown and swelling power. Increased levels of LysoPC in starch isolated from hard wheat cultivars or soft experimental lines by dough method could have complexed with amylose and restricted granule swelling. Thereby, decreasing peak viscosity, breakdown and swelling power.

Key words: near-isogenic, puroindoline, polar lipids, wheat starch, gelatinization, swelling power

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Acknowledgements

First of all, I would to express my endless gratitude to my major advisor Dr. Jon Faubion. This thesis would not be possible without his guidance, support and valuable counsels during this process. I am also deeply thankful to my committee members Dr. Hulya Dogan and Dr. Rebecca Miller who monitored my work and took time to read my drafts and provided valuable feedback. I owe my deepest gratitude to Dr. Rebecca Miller for the opportunity that she gave me to work at the Wheat Quality Laboratory. She could not realize how much I have learned from her.

I would like to express my gratitude to Dr. Richard Jeannotte and Mary Roth from the Lipidomics Center for their help and guidance throughout my lipid analysis. I also wish to thank Dr. John Tomich and Nozomi Caton from the Biochemistry Department for the all the support they provided for preparation of SDS PAGE. I am grateful for Dr. Shi for his numerous advices and patience in providing me with useful information on his expertise – starch. My sincere thanks go to Dr. Murray and Zhining Ou for their assistance in performing all statistical analysis.

I am indebted with many of my colleagues in Grain Science who supported me during my whole masters program. Special thanks for Dr. Sean Finnie who inspired me with his enthusiasm for his research and provided me good advices and valuable feedback on my work.

I thank the Department of Grain Science and Industry for providing me an excellent work environment for the past two years and all the staff for their joyful assistance.

Lastly, I offer my deepest gratitude to my family, friends, and my boyfriend Jaime Tobon for supporting me and helping me at all times.

Dedication

To my mom and dad.

CHAPTER 1 - Introduction

Wheat (*Triticum aetivum L.*) kernels are composed primarily of starch. For that reason, starch characteristics significantly influence end product quality. Many starch based foods are subjected to high temperatures and moisture during processing, causing starch granules to gelatinize. Pasting properties of starch are governed mainly by the granule's amylose/amylopectin ratio. In addition to amylose/amylopectin ratio, non-polysaccharide components that are present at the granule surface are important as some have been shown to interfere with granular swelling. Near-isogenic wheats overexpressing puroindolines have been used recently to assess the effect of wheat hardness on certain quality attributes of final products. However, no work has been done to evaluate how starches from near-isogenic wheats varying in surface components differ from each other functionally and how much of that difference can be attributed to surface components.

Therefore, the objective of this study was to identify differences among starches from near-isogenic wheats overexpressing puroindolines and to investigate the role that starch surface components play in pasting. Prime starch was isolated from two pairs of near-isogenic wheat lines differing in hardness using both batter and dough methods. The use of near-isogenic samples and two different starch isolation methods manipulates the amount of starch surface components without the use of aggressive chemical extraction. This approach is advantageous because the variables affecting starch pasting properties are, consequently, decreased considerably. Characterizing the relationships between starch granule surface components and pasting profiles should provide insight into how endosperm texture affects flour and dough characteristics. Additionally, characterizing near-isogenic samples should be quite helpful in

evaluating the overall effectiveness of using such samples as tools to assess the effect of wheat hardness on quality attributes of final products.

Objectives

The purpose of this research was to determine differences among starches from near-isogenic samples and to use the consequent difference that these samples exhibit in their starch surface components in order to investigate the role that starch surface components play in pasting.

The main objectives are:

1. To characterize the puroindoline proteins, polar lipids and thermal properties of starch isolated from two pairs of near-isogenic samples overexpressing puroindolines.

2. To investigate the role that starch surface components play in the pasting process by using batter and dough methods to both obtain the starch and manipulate its surface components.

CHAPTER 2 - Literature Review

2.1 Wheat endosperm hardness

Wheat (*Triticum aestivum L.*) is generally classified based on a number of factors; time of the year sown, kernel color, and hardness (Posner and Hibbs 1997). Wheat hardness, or texture, is an extremely important trait because it is used to define flour end-use (Pomeranz and Williams 1990). Soft wheat is generally used in the manufacture of non-fermented bakery goods, such as noodles, cakes and cookies, while hard wheat is mainly used for breadmaking (Delcour and Hoseney 2010). The strength of the interaction between protein matrix and starch granule is the primary contributor to endosperm hardness (Barlow et al. 1973). Using micropenetration techniques, Barlow et al. (1973) found that starch and protein from soft and hard wheat endosperm have equivalent hardness. Therefore, starch or protein hardness alone cannot be responsible for wheat endosperm hardness. The explanation advanced was that in soft wheat endosperm, the protein matrix adheres less tenaciously to the starch granules than it does in hard wheat endosperm.

Now it is known that wheat hardness is a heritable trait controlled by a gene localized at the *Hardness* (Ha) locus on the short arm of the 5D chromosome (Symes 1965, Doekes and Belderok 1976). However, the exact mechanism which produces hard or soft texture in wheat endosperm is yet to be established. The *Hardness* gene is responsible for the presence of a 15kDa protein (friabilin) which is associated with the starch surface of the wild (soft) state of wheat. Durum wheat (*T. turgidum L. var. durum*), used for pasta products, lacks the 5D chromosome and is the hardest wheat variety (Greenwell and Schofield 1986). The absence of or mutations at this locus will cause wheat endosperm to have a hard texture (Morris 2002).

Friabilin is itself composed of two polypeptides called puroindolines a (*pin*a) and b (*pin*b) (Jolly et al. 1993, Morris et al. 1994). Greenwell and Schofield (1986) suggested that puroindolines act as "nonstick" agents, decreasing the strength of the interaction between starch granules and the protein matrix.

As is true for puroindolines, Greenblatt et al. (1995) found a relationship between the amount of polar lipids associated with the starch surface and wheat endosperm softness. Konopka et al. (2005) and Feiz et al. (2009) implied a link between the content of starch surface lipids and wheat hardness. Although soft and hard wheat have essentially equal levels of polar lipids present in the endosperm, starch isolated from soft wheat using a batter method, has greater levels of polar lipids on its surface than does equivalently isolated hard wheat starch (Finnie et al. 2009). These studies indicate the existence of a relationship between polar lipids and puroindolines on the surface of soft wheat starch.

Extending that argument, the nature of the starch surface, (i.e the presence or absence of proteins and polar lipids) directly affects milling behavior and consequently, flour characteristics (Martin et al. 2001, Hogg et al. 2005). Hard and soft wheats behave differently during milling and, for that reason, they require different processing flows. The weak structure of soft wheat endosperm breaks more easily into very fine particles, and also results in less damaged starch granules when compared to hard wheat (Posner and Hibbs 1997).

2.2 Structural characteristics of wheat starch

Wheat (*Triticum aestivum* L.) flour contains on average 63-72% starch (Lineback and Rasper 1988). Mature wheat starch granules are classified into three distinct groups termed A, B, and C-type, according to their size, shape, and time of biosynthesis (Bechtel et al. 1990). Type-A are the first granules to be synthesized (within 4 days after flowering). They are lenticular in

shape, and have the largest diameter (~16μm). In contrast, B-type granules are smaller (5-16 μm) and spherical in shape. Their synthesis does not start until 10 days after flowering. The third wheat starch class, type-C, is the smallest (< 5 μm) of the three classes and the last to be synthesized (21 days after flowering). Whether type-C is a distinct class or simply late synthesis of B-type is still not completely established as many researchers still consider wheat starch distribution as being bimodal (Bechtel et al. 1990, Peng et al. 1999). Whereas type-A granules constitute the highest proportion of flour starch, B and C-type are greater in number and surface area (Soulaka and Morrison 1985, Bechtel et al. 1990, Peng et al. 1999).

Like other cereal and tuber starch granules, wheat starch consists of two polymers: amylose and amylopectin. Amylose is an essentially linear polymer of α-D-glucose joined by α-1-4 bonds. Amylopectin is also composed of α-D-glucose connected by α-1-4 linkages but, amylopectin is a highly branched molecule connected at branch sites by α-1-6 linkages. The amylopectin chains are classified as A, B or C-chains according to their type of substitution (Figure 2.1-A). A-chains are unsubstituted, while B-chains hold one or more chains and are attached to the amylopectin by α-1-6 linkages. Each amylopectin molecule has only one C-chain which carries the reducing end (Sajilata et al. 2006, Copeland 2009). Generally, the proportion of amylopectin to amylose is ~ 3:1, although amylose enriched or depleted (waxy) types exist. One important aspect of amylose polymers is their ability to form a right handed helix with a lipophilic interior capable of interacting with non-polar molecules and iodine. Amylose iodine-binding capacity is the basis of some amylose quantification tests (Lineback and Rasper 1988).

Native starch occurs as partially crystalline structures because of the unique branching structure of amylopectin. Starch granules possess alternating layers of crystalline and amorphous regions forming the so called "growth rings" (Morrison 1995). The crystalline regions are

formed mainly by amylopectin, whereas the amorphous regions contain amylose and the areas around branching points of amylopectin (Figure 2.1-B) (Sajilata et al. 2006, Copeland 2009). Starch crystallinity; which is a property of amylopectin, affects gelatinization temperature and degree of swelling of starch granules in excess water (Tester and Morrison 1990). Sasaki (2005) reported that starch containing high amounts of amylopectin (waxy) had greater gelatinization temperature and enthalpy than did normal starches. Waxy starches swell faster but cannot maintain viscosity under shear. Because of this, they show a greater breakdown and reduced final viscosity when compared to non-waxy starch (Sasaki 2005).

The existence of pores and channels in wheat and other cereal and tuber starches has been described (Fannon et al. 1992, Fannon et al. 1993, and Fannon et al. 2003). A-type granules from soft wheat starch contain large channels located in the equatorial groove region and fine channels at other places on the granule. B-type granules possess larger and less defined channels than do A-type. These channels affect the permeability of the granule and influence starch behavior during processing (Kim and Huber 2008). Han et al. (2005) and Lee and BeMiller (2008) detected the presence protein and phospholipids (mainly lysophosphatidylcholine) in granule channels of maize starch.

2.3 Components associated with starch surface

2.3.1 Lipids

Lipids are a minor component of cereal grain, representing only about 1.2% of wheat flour (Table 2.1) (Lillford and Morrison 1997). Even though present in small amounts, flour lipids have been shown to affect bread quality by stabilizing the foam structure of dough (MacRitchie and Grass 1973). Sroan and MacRitchie (2009) found that endogenous flour lipids

did not affect dough biaxial extensional rheology, supporting the idea that the effect in baking is due to stabilization of gas cells by their action as surface active agents.

Lipids in the endosperm are divided into total flour lipids and nonstarch lipids. Total flour lipids are extracted using hot polar solvents, while nonstarch lipids are extracted at room temperature without granule swelling. Nonstarch lipids include all the lipids present in the endosperm that are not incorporated into or onto the starch granules. The main non-starch polar lipids are glycolipids (GL) and phospholipids (PL) (Morrison 1988). A further classification scheme for flour lipids divides them into free or bound, also based on method of extraction. Free lipids are extracted first using nonpolar solvents (e.g. hexane). Bound lipids are extracted in sequence with a more polar solvent (isopropanol-water) (Greenblatt et al. 1995).

Lipid analysis techniques include traditional, such as thin layer chromatography, newer methods of lipid profiling including gas chromatography, HPLC and, more recently, electrospray ionization tandem mass spectrometry (ESI-MS/MS). The last technique provides highly detailed characterization of lipids present in biological samples (Welti and Wang 2004, Devaiah et al. 2006). Lipid profiling is defined as "a targeted metabolomics platform that provides a comprehensive analysis of lipid species with high sensitivity" (Devaiah et al. 2006). Finnie et al. (2009) used lipid profiling to fully characterize and quantify lipid classes present in wheat whole meal, flour and starch. A total of 146 lipid species were found to be present in these wheat fractions. The main polar lipids for all wheat fractions were digalactosyldiglyceride (DGDG), monogalactosyldiglyceride (MGDG), phosphadidylcholine (PC) and lysophosphadidylcholine (Lyso-PC). Greater amounts of mono-acyl polar lipids were present in starch internal lipid fractions, suggesting that lipids present in wheat granule channels were also extracted in that study (Finnie et al. 2009). Starch isolation method can be a significant source of variation for

starch surface lipid concentrations (Finnie et al. 2010). According to Finnie et al. (2010) the most abundant surface lipids present on starch isolated using a batter method were: PC, DGDG and MGDG. Lyso-PC was the most abundant lipid class present on the surface of starch isolated using a dough method.

2.3.2 Proteins

A thorough review of starch granule-associated proteins (SGAPs) has been presented by Baldwin (2001). Starch granule-associated proteins have been separated into two groups according to their molecular weights (M_w). The first group is comprised of low M_w proteins (5, 8, 15, 19 and 30 kDa), also classified as "surface" proteins. The second group includes internal granular proteins with M_w ranging from 60 to 149 kDa. However, the designation of "surface" and "internal" is simplistic, because proteins with M_w of 60 kDa (more commonly known as *waxy* protein) and the 30 kDa glycoproteins were reported to be present internally and on the surface of the starch granule (Baldwin 2001).

To date, the only protein that has been shown to be present exclusively on the starch surface with no documentation of its presence inside the starch granule is the biochemical marker of wheat hardness, the 15 kDa protein friabilin (Baldwin 2001). Due to its unique tryptophan-rich domain, the two polypeptides compromising the friabilin group were named puroindoline a and b (*pin*a, *pin*b) (Blochet et al. 1993). Puroindolines are basic proteins and contain five intrachain disulfide bridges. The presence of the tryptophan-rich domain and the ability to extract them with Triton-X114 suggests that these proteins are able to tightly bind polar lipids and interact with membranes (Blochet et al.1993, Kooijman et al. 1996). Oda and Schoefield (1997) studied the location of *pin* proteins in intact starch granules using immunolocation techniques. They found that *pin*a is mainly located in the protein matrix, while *pin*b is generally located on

the surface of starch granules. However, when the flour was wetted, *pin*a may be adsorbed on the starch surface, equalizing the amounts of both *pin* proteins present on the starch surface. Finnie et al. (2010) reported that the presence of puroindolines on the starch granule surface can be depleted or maintained depending on starch isolation method. These studies indicate that concentration of *pin*a and *pin*b proteins on the surface of starch granules may then be considered "partially artifactual", as it was described by Baldwin (2001).

The amphiphilic nature of *pin* proteins, especially *pin*a, which has a greater *in vitro* affinity for phospholipids and glycolipids (Le Guerneve, 1998), greatly increases foam stability in the presence of polar lipids (Dubreil et al. 1997). The addition of small quantities of puroindoline has a considerable effect on dough rheology and baking quality (Dubreil et al. 1998). Lately, increased attention has been paid to possible effects of puroindoline proteins in baking. Ruille et al. (2005) observed better gas cell stability and finer crumb grain in French bread dough with the addition of only 0.1% puroindolines. Near-isogenic samples overexpressing puroindolines have been used as a tool to assess the effect of wheat hardness on quality attributes of final products (Hogg et al. 2005, Martin et al. 2007, Martin et al. 2008). Hogg et al. (2005) found that high levels of puroindoline negatively affect bread loaf volume and crumb grain in near-isogenic samples overexpressing these proteins. However, the different levels of damaged starch produced in hard and soft wheat were not taken into consideration in that study. Martin et al. (2008) reported no improvement in white salted noodles characteristics for wheat lines overexpressing *pin* proteins.

2.4 Effect of heat and moisture on starch properties

Gelatinization, pasting and retrogradation are terms used to describe changes starch undergoes when heated and sheared in the presence of water (Atwell et al. 1988). When thermal energy is applied to a starch suspension, hydrogen bonds in amylopectin double helices are broken while new hydrogen bonds are formed with water molecules. At that point, starch granules begin to swell and amylose is leached out of the granule (Morrison 1995). Disruption of the molecular order of the polymers within starch granules is commonly called "gelatinization". Gelatinization is an irreversible process and is observed by loss of birefringence under polarized light (microscopy), loss of crystallinity (X-Ray diffraction), granule swelling and the start of starch solubilization. Pasting is defined as the process which follows gelatinization under continued shear and increasing temperature with total disruption of the granule only achieved at high temperatures. The viscosity versus temperature curve obtained using a viscometer, such as the Rapid Visco Analyzer (RVA) or Brabender Micro Visco Amylograph (MVA) is called a "pasting" curve. During cooling and storage starch molecules start to reassociate to form a more ordered structure by a process referred to as "retrogradation" (Atwell et al. 1988).

The pasting properties of starch are strongly influenced by amylose/amylopectin ratios (Zeng et al. 1997). Synthesis of amylose is controlled by the enzyme granule-bound starch synthase (GBSS) encoded by the waxy genes: *Wx-Al, Wx-Bl* and *Wx-Dl*. Waxy wheat lines containing only amylopectin have null alleles at the three loci encoding the GBSS (Nakamura et al. 1995). In addition to amylose/amylopectin ratio, non-polysaccharide components that are present at the granule surface also have been shown to interfere with granule swelling (Han and Hamaker 2002, Debet and Gidley 2006). Starch ghosts (nonsolubilized portions of gelatinized granules) with concentrated amounts of protein present on their surface were able to maintain their granular structure after gelatinization. Conversely, starches from waxy maize and amylose-

free potato (which lack GBSS) were shown to be fragile after gelatinization. It was suggested that the presence of granule-associated proteins is partly responsible for maintaining ghost integrity (Han and Hamaker 2002). In a later study, Debet and Gidley (2007) found that protein and lipid present at the wheat starch surface determines both the size and robustness of starch granule ghosts. The granular structure of starch ghosts is relevant because it influences the viscosity and breakdown of pastes. Gels with increased levels of intact swollen granules, produce a "short" texture, different from the "long" viscous texture of gels from solubilized polysaccharides (Debet and Gidley 2007).

Many techniques are used to evaluate thermal and physical properties of starch suspensions. Cooking behavior of starch is generally assessed by viscometric analyses such as, RVA or MVA. These instruments record changes in viscosity of a starch suspension under constant stirring and controlled time and temperature. Several factors are involved with changes in viscosity observed by MVA curves. According to Miller et al (1973) the sharp increase in viscosity observed during the heating cycle of the MVA is attributed only partially to intensity of granule swelling. Instead, they postulated that the rapid increase in viscosity which occurred after granule swelling was stabilized by a "continuous and complex filamentous network" present at the starch suspension formed by leaching of starch molecules at high temperatures (90^oC).

Differential Scanning Calorimetry (DSC) is also a powerful tool used to illustrate gelatinization behaviors and to determine glass transition temperature of starch granules. DSC is capable of measuring changes occurring inside individual granules. The endothermic curve of gelatinization is the sum of the energy absorbed by each granule during that process. Broad gelatinization curves represent a heterogeneous sample with granules gelatinizing at different

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temperatures (Biliaderis 2009). Examples of RVA and DSC curves with the main parameters generally analyzed are shown in figures 2.2 and 2.3 respectively.

Many studies have attempted to assess the effects of starch surface components on gelatinization and pasting properties (Melvin 1979, Eliasson et al. 1981, Nierle et al. 1990, Debet and Gidley 2006). Results varied greatly according to sample preparation and methods of surface extraction used in the studies.

2.5 Technological relevance of starch surface components

A number of authors have investigated the influence of starch in breadmaking and concluded that normal wheat starch is unique and there is yet no better replacement for it (Harris and Sibbit 1941, Harris and Sibbit 1942, Sandstedt 1961, Hoseney et al. 1971, Kusunose et al. 1999). Frequently, starch based foods undergo thermal processes which involve high temperatures and moisture causing starch granules to gelatinize. Because starch is the main constituent of wheat flour, starch pasting characteristics have an important role in dough expansion and loaf volume (Kusunose et al. 1999). One important characteristic of normal wheat starch is that it is able to retain its integrity during baking and interact with the gluten phase of the dough to form a continuous gas phase, which prevents further shrinking of the dough (Kusunose et al. 1999). Hearth bread produced with waxy wheat had significantly reduced weight and a more open structure then did bread made with normal wheat starch (Sahlstrom et al. 2006). Differences observed in gelatinization and pasting curves as well as the inability of waxy starch to retain some portion of its granular integrity after gelatinization could be the explanation for the lack of potential of waxy wheat in creating an acceptable bread crumb (Kusunose et al. 1999, Sahlstrom et al. 2006).

Wheat starch isolated from flour frequently contains non-starch components such as minerals, lipids, and proteins (Baldwin, 2001). These non-polysaccharide components, mainly proteins and lipids, are relevant to starch technology because they have been shown to affect important characteristics of starch-based products as well as starch itself. Nierle et al. (1990) evaluated rheological properties of surface-extracted wheat starch and reported clear differences in pasting temperatures and viscosity between extracted and non-extracted wheat starch. Debet and Gidley (2006) found that wheat starch extracted with 2% SDS (sodium dodecyl sulphate) at room temperature swelled faster and to a greater extent than did native starch. However, the effect of surface proteins and lipids restricting granule swelling is minor for high amylose starches, suggesting that swelling behavior is primarily controlled by carbohydrate composition (Debet and Gidley 2006).

Other authors characterized starch properties after lipid and protein were extracted using SDS and found evident correlation among starch surface extracted granules, thermal behavior, and granule swelling (Melvin 1979, Eliasson et al. 1981, Debet and Gidley 2006). On the other hand, Seguchi (1995) demonstrated that an aqueous solution of 1% SDS containing 1% 2-mercaptoethanol was able to destabilize the starch granule structure after the second extraction. He observed that starch structure is weakened by these solvents, not only by extraction of surface components, but also by dissolution of starch polymers at the granule surface.

The high proportion of starch in flour provides a large surface area for interaction with gluten proteins. For that reason, there is increased attention on the properties of the starch surface and the effects of starch surface modifications on dough rheology. Eliasson et al. (1981) analyzed starch derived from flours with good and poor breadmaking performance and concluded that differences in thermal and rheological properties of the assessed starches were

strongly related to the presence of lipids on the starch surface. Additionally, Larsson and Eliasson (1997) reported that modifications at the starch surface using heat, adsoption of lecithin, and a wheat protein fraction affected dough rheological performance, whereas the same components exerted no effect on dough rheology when they were added directly to the flour.

The application of chlorine gas to pastry flour is a common technique used to improve final quality of batter-based products such as cakes and pancakes (Segushi and Matsuki 1977, Finnie et al. 2006). Such improvements are attributed to changes on the starch surface from hydrophilic to hydrophobic. That change is, itself, believed to be affected by changes in the starch surface proteins (Segushi 1985, Segushi 1987). Furthermore, Baldwin et al. (1997) showed that chlorination removes phosphocholine groups present at the starch surface. This also helps to increase starch surface hydrophobicity.

Caution is required when comparing different studies on the effects of surface components on starch properties because different methods of starch isolation and surface molecule extraction greatly affect the outcomes. For that reason, disagreements are frequently found among studies concerned with changes in thermal behavior of starch pastes caused by extraction of proteins and lipids present on the surface of native starch. The technique chosen is relevant to the purpose of the study because it affects starch surface components (Finnie et al. 2010). The most common method used to isolate starch from wheat flour is the traditional dough development method. However, a batter method is recommended for studies involving starch surface components because when dough is mixed to optimum gluten development, lipids and puroindoline proteins tend to interact preferentially with the gluten phase of the dough. In a batter method, where water is abundant and there is minimal gluten formation, these components are much more likely to remain associated with the starch granule surface (Finnie et al. 2010).

The extensive literature addressing starch functionality and the effect of starch surface chemistry on starch properties and dough behavior provides evidence for the technological importance of understanding how starch surface components affect overall quality of starch and starch-based foods.
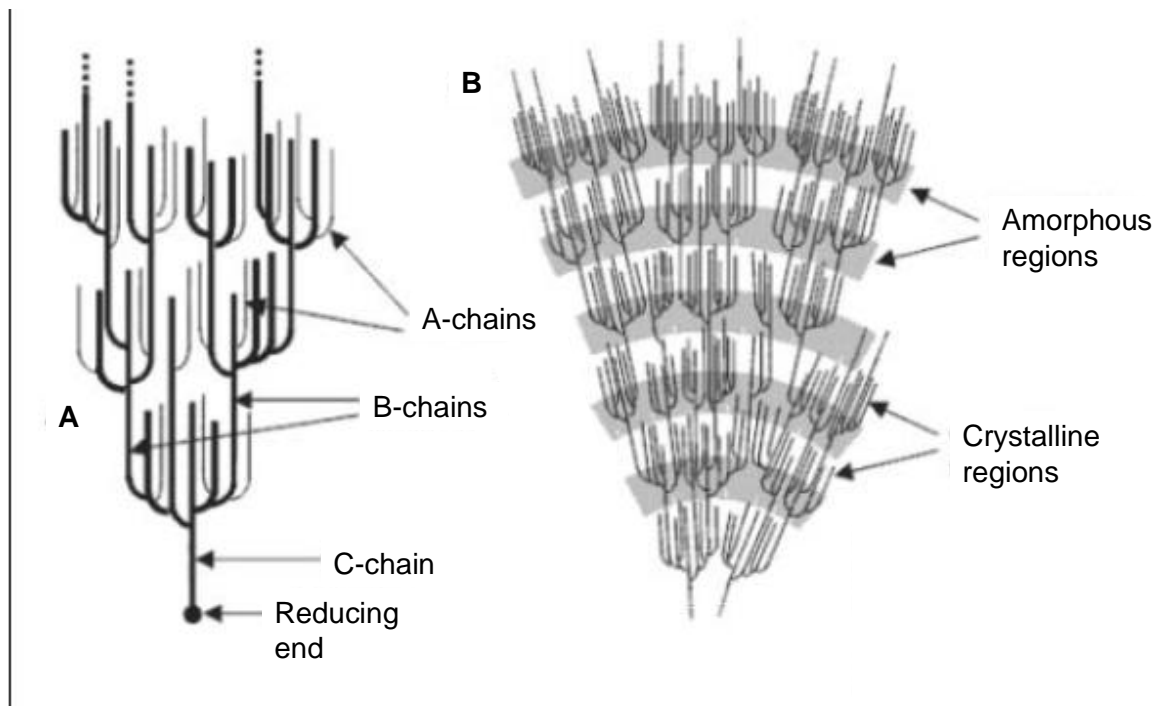
Figure 2.1(A) Model of amylopectin showing A, B and C-chains. (B) Organization of amorphous and crystalline regions of a starch granule (Adapted from Sajilata et al. 2006).
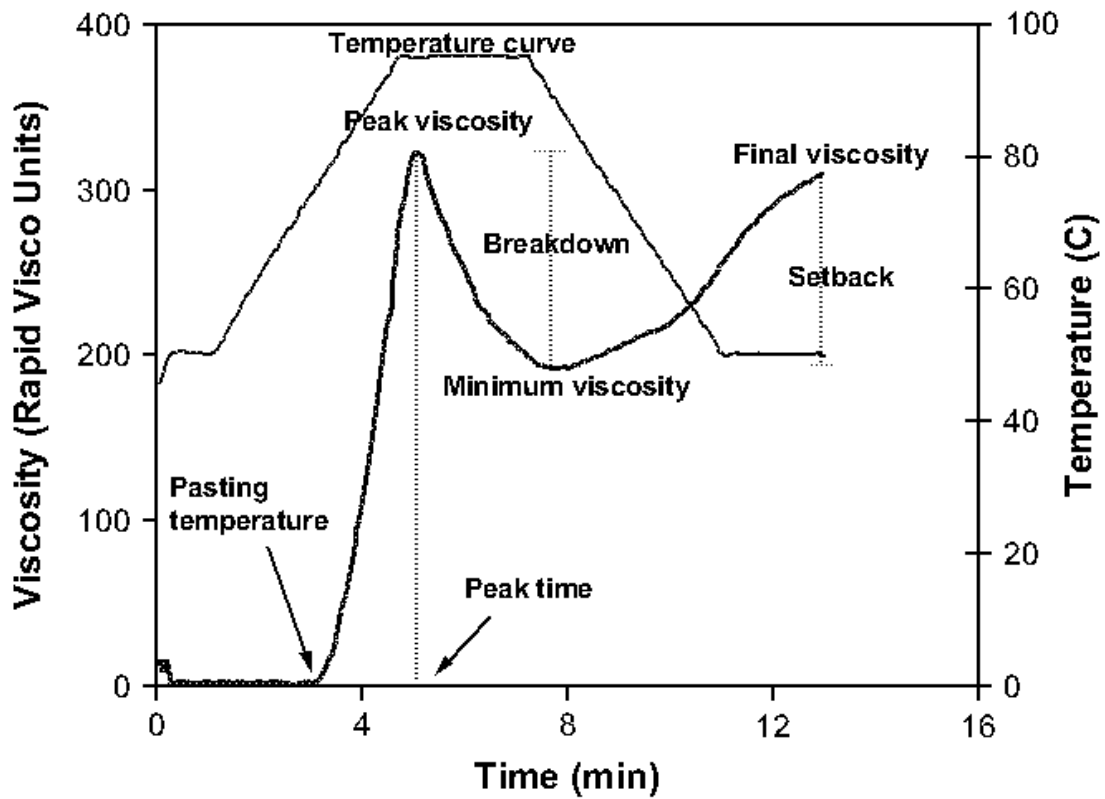
Figure 2.2 Example RVA pasting curve of rice starch showing the main parameters generally analyzed (Copeland et al. 2009).
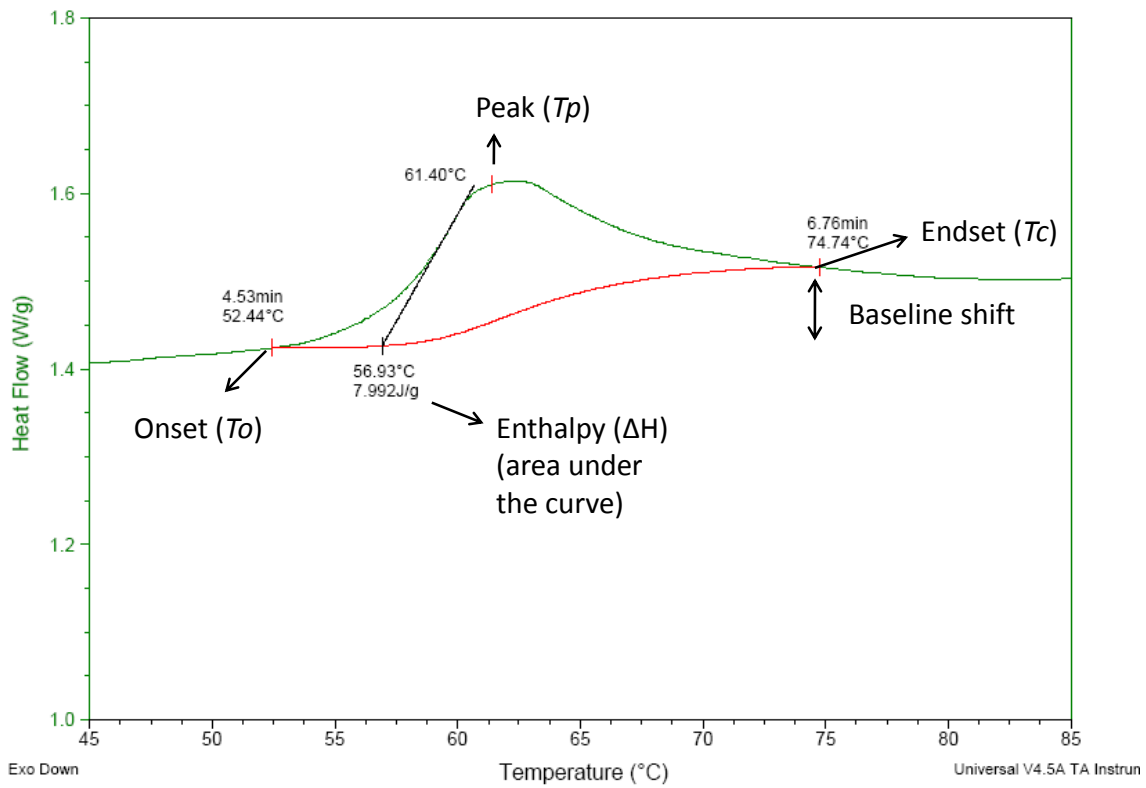
Figure 2.3 Example of a Differential Scanning Calorimetry (DSC) curve of wheat starch in excess water showing the main parameters generally analyzed.

Table 2.1 Composition of wheat starch granules (wild type) (Lillford and Morrison 1997).

Component		*Level (%, dry basis)*
Amylose		23-27
Amylopectin		73-77
Lipids		
	Surface	0.02-0.6
	Internal	0.1 – 0.6
Proteins		
	Surface	0.006-0.5
	Internal	0.07

CHAPTER 3 - Material and Methods

3.1 Wheat samples

Unique pairs of near-isogenic wheats varying in surface components (Table 3.1) were generously supplied by Dr. Mike Giroux (Montana State University). Hi-Line (PI 549275) (Lanning et al. 1992) is a hard red spring wheat cultivar which has an amino acid change (Glycine 46 to Serine) in *pin*b (Giroux and Morris 1997). In order to produce its soft textured near-isogenic pair (identified as HGAB18), genes *Pina-Dl* and *Pinb-Dl,* located in the hardness locus on chromosome 5D, were transgenically modified to the wild type (Hogg et al. 2005). Milling and baking characteristics of these samples were reported by Martin et al. 2007. Bobwhite is a hard wheat winter cultivar which possesses a *pin*a null gene. Bobwhite 2 (BW2), its pair, possesses a soft endosperm. This was accomplished by genetically changing the *pin*a gene to its wild type (Martin et al. 2006). Hi-Line, HGAB18, Bobwhite, and BW2 were grown in the 2009 crop year at Bozeman, MT close to Montana State University. Two field replications were cultivated for each sample, and bulked into a single sample. Wheat was cleaned using a Dockage Tester (Carter-Day Company, Minneapolis, MN) and each line's hardness was assessed using the SKCS 4100 (Single Kernel Characterization System) (Perten Instruments North America, Inc. Springfield, IL).

3.2 Milling

Wheat lines were milled at the USDA-ARS Western Wheat Quality Laboratory, Pullman, WA. Hard wheat samples were tempered to 16% moisture whereas soft wheat samples were tempered overnight to 14.5%. Samples were tempered overnight and then milled using a Buhler

MLU-202 pneumatic laboratory mill (Buhler Inc. Uzwil Switzerland) to obtain straight grade flour following Approved Method 26-31 of the American Association of Cereal Chemists (AACC International 2009). A slightly slower feed rate was used for soft varieties (100g/min) than for hard varieties (130g/min).

3.3 Starch isolation

Starch was isolated from flour in three replicates by two different washing techniques; a dough-ball method and a batter method. The traditional dough-ball method was adapted from Wolf (1964) and the AACC International (2009) gluten hand-wash method 38-10.01. Flour (40g) and distilled water (~23mL) were mixed for 3-4 min in a 100g *pin* mixer (National Manufacturing Co., Division of TMCO, Lincoln, NE) until a coherent mixed dough was formed. The dough was placed in a plastic container and soaked in excess distilled water at room temperature for 10 min, and then hand washed. After dough washing, the resultant starch suspension was filtered thru a 75μm sieve (Dual MFG. Co, Chicago, IL). This procedure was repeated until the water from the gluten phase became clear. After filtration, the starch suspension was centrifuged at 4800x g for 10 min and the supernatant discarded. To ensure that no gluten protein was present, starch was filtered and centrifuged a second time under the same conditions. Tailings were removed from the top of the starch pellet with a spatula, leaving only prime starch in the bottom of the tube. Prime starch was then dried for 48 hours at room temperature and ground using an analytical mill (Tekmar, model A-10, Mason, Ohio).

The batter method for starch isolation was that used by Finnie et al. (2009) which was, in turn adapted from Knight and Olson (1984). Distilled water and flour at 4.7:1 (v/w) were slurried for 5 min to form a homogeneous batter. The slurry was then transferred to polypropylene tubes, centrifuged at 100x g for 5 min and rested for 10 min. After resting, batter was filtered through

the following series of sieves: 425μ, 400μ, 180μ, 106μ, and 75μ (Dual MFG. Co, Chicago, IL). The resulting starch milk (thrus) was centrifuged, dried and ground the same way as the starch isolated using the dough method.

The dough method was not performed on hard endosperm samples (Hi-Line and Bobwhite) because previous studies demonstrated that these lines contain greatly reduced puroindoline protein levels on the starch surface (Martin et al. 2006; Giroux and Morris 1997; Finnie et al. 2009). Finnie et al. (2010) reported that differences in starch surface polar lipids between batter and dough was greater for soft lines (BW2 and HGAB18) than for the hard lines (Bobwhite and Hi-Line). For that reason, little variation was expected in starch characteristics of Hi-Line and Bobwhite starch isolated using either batter or dough method.

3.4 Lipid extraction and quantification

Lipids were extracted from prime starch according to Greenblatt et al (1995). Free lipids were extracted by adding 5mL of hexane to 1g of starch in a glass centrifuge tube (Kimble HS 15mL, 18 x 102, screw cap with PTFE liner). The suspension was stirred on a vortex mixer every 15 min for one hour then centrifuged at 3000x g for 5 min. The supernatant was discarded and 3.75mL isopropanol:water (90:10) solution was added to the starch sample to extract bound lipids. The tubes were stirred on a vortex mixer every 15 min for 1 hour then centrifuged at 3000 x g for 5 min. The supernatant was decanted into another test tube and evaporated under a stream of nitrogen until completely dry. Chloroform (1 mL) was added to the dried lipids using a glass syringe (1 mL) and the tubes were covered with aluminum foil to protect from light and frozen at -20°C. Eleven polar lipid classes present on the starch surface were quantified using automated electrospray ionization (ESI)-tandem mass spectrometry (MS/MS) according to the method described by Devaiah et al. (2006) and Finnie et al. (2009). The lipid classes analyzed were:

phosphatidylcholine (PC), phosphatidylethanolamine (PE), lysophosphatidylcholine (LysoPC), lysophosphatidylethanolamine (LysoPE), digalactosyldiglycerides (DGDG), monogalactosyldiglycerides (MGDG), phosphatidylglycerol (PG), lysophosphatidylglycerol (LysoPG), lysophosphatidilcholine (LysoPC), phosphatidylethanolamine (PE), lysophosphatidylethanolamine (LysoPE), phosphatidylinosotol (PI), phosphatidylserine (PS) and phosphatidic acid (PA). Lipid extraction was performed in triplicate for each replicate of starch isolation, totaling 54 samples. The average of the triplicates for each sample was used for statistical analysis.

3.5 Protein extraction and quantification

Proteins associated with the starch granule surface were isolated according to the method of Giroux et al (2003), in which Triton X-114 non–ionic detergent diluted in Tris-buffered saline is used as the separation step. Extracted proteins were than fractionated using SDS-PAGE (precast gel) on a 10-20% Tris-HCl gradient (BIO-RAD, Hercules, CA). Known concentrations of bovine serum albumine (BSA, fraction V, 2.0mg/mL in a 0.9% aqueous NaCl solution containing sodium azide) were used as standards for band density. Gels were stained overnight in Coomassie Brilliant Blue R250 (BIO-RAD, Hercules, CA, catalog# 161-0436), and de-stained in 30% methanol and 10% acetic acid until clear. Images were taken with an Epson^TM Twain 5 scanner and band density was analyzed with Image Quant^TM TL Software (GE Healthcare Bio-science Corp., Sweden). A standard curve of pixels versus BSA concentration was prepared and puroindoline concentration was calculated by using the linear equation for the standard curve.

3.6 Thermal analysis

3.6.1 Microviscoamylograph

The viscosity curve of a 10% (w/v) starch suspension was analyzed with a Micro Visco Amylograph (MVA) (C. W. Brabender Inc., Germany). Samples were heated at 2°C/min from 30°C to 95°C, held at 95°C for 5 min, cooled to 50°C at the same rate and held for 5 more min. Six parameters were assessed: a) pasting temperature (beginning of gelatinization); b) peak viscosity (maximum viscosity at 95°C); c) breakdown (difference between peak viscosity and viscosity at the end of holding at 95°C); d) setback; e) peak time; f) final viscosity. Setback rate was calculated as the slope of the curve during the cooling period (95°C to 50°C).

3.6.2 Differential Scanning Calorimetry

Thermal analysis was performed using a Q100 Differential Scanning Calorimeter (DSC) with a refrigerated cooling system (TA Instruments, New Castle, DE). The DSC was previously calibrated with indium. Starch (5.0-7.0 mg) was weighed into a stainless steel pan. Distilled water was added to make a 30% starch suspension, and the pan was hermetically sealed. Before heating, samples were allowed to rest for 15 min at room temperature then scanned with an empty pan used as a reference. Each replicate of starch isolation was scanned in duplicate. Settings and operation were: heat from 10°C to 140°C at 10°C /min. Onset temperature (T_o), peak temperature (T_p), and enthalpy of starch gelatinization (ΔH_g – expressed as J/g of sample) were recorded. The manufacture's software program was used to analyze and plot the data. A sigmoid curve was used to calculate the enthalpy instead of a linear curve in order to correct for the shift in the baseline after gelatinization.

3.6.3 Swelling power

The swelling power of the starch samples was obtained following the method described by Mangalika et al. (2003). A small (200 mg) sample of starch was weighed into a previously tared screw cap test tube (Kimble HS 15 mL, 18 x 102) and 5 mL of distilled water was added. The starch suspension was then vortexed for 10 seconds and placed in a 70°C water bath for 20 min. While in the water bath, tubes were inverted every 2 min. The tubes were then transferred to a 20°C water bath for 5 min to cool, and then centrifuged at 1,700x g for 4 min. The supernatant was carefully removed by aspiration and discarded. The tubes containing swollen starch were weighed and the swelling power was calculated as the weight of swollen starch per 1 g of dry starch. Swelling power was performed in duplicate for each replicate of starch isolation.

3.7 General analysis

The moisture content of starch samples was determined according to AACC International (2009) method 44-15A. Starch total protein contents were assessed by nitrogen combustion (N% x 5.70) Approved Method 46-30 (AACC International 2009) (Leco Corp. St. Joseph, MI). Damaged starch was quantified using Megazyme starch kit (Megazyme Inc., Wicklow, Ireland) according to AACC International Approved Method 76-31 (AACC International 2009). All values were reported on a dry weight basis. All general analysis was performed in duplicate for each replicate of starch isolation.

3.8 Statistics

There are three sets of genotype and isolation process combinations in this study, resulting in six combinations (Hi-Line batter, HGAB18 batter, HGAB18 dough, Bobwhite batter, BW2 batter, BW2 dough). Starch isolation was conducted randomly within each set. A completely randomized block design was used. One full day was necessary to process each

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combination, totaling six days (a week) of starch isolation for each set. To test for starch isolation and characterization measurements, "week of starch isolation" was used as the blocking factor. Starch isolation method (batter or dough) and genotype (Hi-Line, Bobwhite, HGAB18, BW2) were the treatment factors. The outcomes were evaluated using pair-wise comparison Analysis of Variance (ANOVA) where treatment and replicate were the source of variation. Significance was determined at a level of 0.05. Fisher's least significant difference (LSD) was used to determine significant difference within treatments. MVA and SDS-PAGE for *pin* protein was performed only once for each replicate. All statistical analysis was conduced using Statistical Analysis System software (SAS, version 9.1, SAS Institute Inc., Cary NC).

Table 3.1 Sample identification and corresponding source, puroindoline haplotype, molecular change and SKCS hardness index value of the wheat samples.

Sample Identification	Class	Puroindoline Haplotype	Molecular change from 'wild-type'†	Hardness Index
Hi-Line	Hard	*Pina-D1a/Pinb-D1b*	*Pin*b Gly46 to Ser	82
HGAB18	Soft	*Pina-D1a**/Pinb-D1a***	none	9
Bobwhite	Hard	*Pina-D1b/Pinb-D1a*	*Pin*a null	80
BW2	Soft	*Pina-D1a**/Pinb-D1a*	none	25

† 'Wild-type' defined as *Pina-D1a/Pinb-D1a* puroindoline haplotype

** Indicates puroindoline gene transgenic modified

CHAPTER 4 - Results and Discussion

4.1 Observations during starch isolation by batter and dough methods

During isolation of the starch from soft lines (HGAB18 and BW2) using a batter method, the protein network became dispersed and spread out more across the various sieves than did the hard lines (Hi-Line and Bobwhite) (Fig. 4.1). Doughs prepared with the soft line flours, especially BW2, were weaker and stickier than were those of the hard cultivars. Those observations suggested that the overexpression of *pin* proteins might affect protein interactions (formation of the gluten network), and water absorption which could, in turn, affect end use properties. Higher levels of damaged starch present on flour from the hard cultivars compared to their soft experimental line could also have had an effect on such behavior. It is expected that near-isogenic pairs of wheat over-expressing *pin* would have essentially the same quality and quantity of gluten forming protein. However, total flour total protein obtained by LECO has shown slight differences between hard cultivar and their soft experimental lines. Hi-Line and HGAB18 contained 13.12% and 12.53% of total protein, respectively. Bobwhite and BW2 contained 10.59% and 9.6% total protein, respectively. Martin et al. (2007) reported that it was not clear from their study if changes in end use properties could be attributed to increased mixograph water absorption (due to greater levels of damaged starch produced during milling for hard lines) or if the presence of *pin* proteins actually had an effect. More research is necessary in order to verify if the differences observed on dough handling of hard and soft near-isogenic samples is attributable mainly to starch surface components or if other factors, such as levels of damaged starch and flour protein, or protein make-up, exert a greater effect.

Prime starch yield was calculated for all four genotypes for both batter and dough methods. Overall starch yield was considered low (25.8% to 49.4%), but it is in agreement with

yields obtained by Finnie et al. 2010 (results not published). The removal of tailings from the top of the starch pellet resulted in some loss of prime starch during that process. The dough method resulted in a better starch yield than did the batter for the soft lines HGAB18 and BW2. Starch isolation by a dough method was filtered only by one fine sieve (75 μm). On the other hand, isolation by a batter method used a stack of 5 sieves, which gave more surface area for the starch slurry to be trapped resulting in higher losses. In addition, it was easier to wash the starch from the dough, where a single dough ball is formed and the gluten remained together in one big piece, than when a batter method was used and the gluten was spread in many individual strings. When comparing yield using the batter method within near-isogenic pairs, Hi-Line produced greater yield than did HGAB18, but Bobwhite did not differ significantly from BW2 (Table 4.1). Bobwhite flour is low in total protein and has poor dough handling. The gluten network from Bobwhite was not as strong as Hi-Line and when a batter method was used the gluten was not aggregated in one piece, but spread throughout the surface of the first sieve (Figure 4.1). That behavior could be one of the reasons why Bobwhite and BW2 did not differ significantly in starch yield when a batter method was used.

4.2 Starch surface lipids profile

Polar lipid profiles from two pairs of near-isogenic samples differing in starch surface components were evaluated in this study. Lipid profiling using an automated ESI-MS/MS allowed the detailed characterization of multiple (in this case eleven) lipid classes. The profiles from the two pairs of near-isogenic wheats were found to be similar to those reported by Finnie et al. (2010). The major polar lipids classes were: DGDG, MGDG, PC and LysoPC (Fig. 4.2). This is not surprising because the same wheat cultivars grown in a different crop year were used. This provides evidence of the stability of these profiles across crop years. Among the major polar

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lipid classes found on the samples, each class contained one or two dominant molecular species. For DGDG and MGDG the major molecular species were 34:2 (total acyl carbons: total double bonds) and 36:2. For LysoPC, 16:0 and 18:2 were predominant. High amounts of 34:2 were found for PC.

Hard wheat cultivars Hi-Line and Bobwhite exhibited similar lipid profiles. The predominant lipid class for both hard cultivars was LysoPC, followed by DGDG, MDGD and PC (Fig 4.3). The reason why hard lines, and soft lines isolated using a dough method, contained greater amounts of LysoPC on the starch surface compared to soft lines isolated by batter method is unknown. Starch from the soft cultivars that were isolated using a batter method contained predominantly diacyl lipids DGDG and MDGD. Concentrations of PI, PA, and PS were zero or close to zero for all starch samples (data not shown).

As previously demonstrated by Finnie et al. (2010), starch isolation method can effectively manipulate the concentration of polar lipid on the starch surface without the use of chemical treatments. In this study, the two hard cultivars (Hi-Line and Bobwhite) contained almost equal amounts of total polar lipids on the starch surface (Fig 4.4). The soft wheat line HGAB18 exhibited higher standard variation for total polar lipid contents within replicates of starch isolation than did BW2.

Polar lipid concentration of flour was not evaluated on this study but it was quantified by Finnie et al. (2010) who reported that HGAB18 flour contained greater amounts of total polar lipids than did BW2 and that both soft wheat lines contained greater amounts of DGDG then did hard textured lines. For that reason, it is reasonable to conclude that differences in starch polar lipid concentration between HGAB18 and BW2 were due to different concentrations in the

original flour of each cultivar. Because all samples are from the same year crop and were grown in the same location, environmental effects can be excluded.

For both near-isogenic line pairs, starch isolated using a batter method retained greater amounts of polar lipids on its surface than did starch isolated by dough method. Hard cultivars demonstrated less variation within replicates than did their respective soft experimental lines when using a batter method. These differences could be caused by environmental variations for different days of starch isolation, or because soft lines contained greater levels of lipids giving more opportunity for variance.

4.3 Variation in starch surface protein

Total protein and *pin* protein concentrations were evaluated for all samples and are shown in Tables 4.4 and 4.5. Puroindoline protein was quantified by SDS PAGE by determination of the band density in comparison to known concentrations of BSA standards (Fig. 4.5). The reagent Coomassie Brilliant Blue binds to the protein present on the gel. Therefore, the density of the blue color at the 15k band is proportional to the concentration of puroindoline. As expected, soft wheat starch isolated using a batter method possessed greater amounts of protein on the starch surface than did hard lines or soft wheat starch isolated by a dough method. From ANOVA pairwise comparison results (Table 4.6), the differences in starch total protein between batter and dough method for HGAB18 was significantly different from the difference between batter and dough method for BW2 (*P-value* 0.0068). On the other hand, differences in *pin* content were not significantly different between the two near-isogenic pairs, suggesting that HGAB18 might have increased amounts of proteins other than *pin* present on the starch surface when compared to BW2. As expected, Hi-Line and Bobwhite starch were devoid of *pin* protein as no bands were detected by SDS PAGE at 15k (data not shown). Isolation of starch by dough

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method significantly reduced *pin* concentration on starch surface for all starch samples (Tables 4.4 and 4.5). These results are in agreement with Finnie et al. (2010) who demonstrated that, when dough is formed, *pin* proteins tend to interact with the gluten phase of the dough instead of remaining on the surface of the starch. During the preparation of the dough there is more mechanical force applied that can facilitate the interaction between *pin* and the gluten phase of the dough. In a dough system the gluten matrix and the starch are closer together than in a batter system, which also facilitates the contact between *pin* proteins and lipids present on the starch surface and the hydrophobic protein matrix. In a study to localize *pin*a and lipids in bread dough, Dubreil et al. (2002) found *pin*a to be localized mainly at the protein matrix where it was associated with lipids. On the other hand, when defatted flour was used, *pin*a was mainly localized around gas cells, suggesting that the interaction between *pin*a and the protein matrix is probably lipid mediated. That fact also helps to explain the reduced amount of polar lipid for samples with lower amount of *pin* protein on the starch surface

4.7 Effect of surface components in thermal properties of starch

Three replicates of starch isolation were performed in this study where "week of starch isolation" was used as the blocking factor. Blocking by week of starch isolation helped to reduce experimental error variations caused by a non-uniform environment (Kuel 2000). For each parameter analyzed, a mean value was calculated for each treatment and compared to other treatments means using pair-wise comparison. No significant difference in starch thermal properties was found for "replicate" as source of variation for the near-isogenic pair Hi-Line/HGAB18 (Table 4.7). These results suggest that the blocking factor "week of starch isolation" was effective in reducing data variation caused by experimental error. On the other hand, the near-isogenic pair Bobwhite/BW2 showed significant differences for replicate as

source of variation for three parameters analyzed (setback, final viscosity and DSC first peak-To) (Table 4.8). That means that analysis of treatment effect (genotype*isolation method), was compromised for these three parameters.

The two pairs of near-isogenic samples analyzed in this study could not be directly compared because they would present variables other than starch surface components, such as, amylose:amylopectin ratios. To overcome this issue, the two pairs were compared statistically by contrasting the differences in the parameters analyzed in one pair (Hi-Line/HGAB18) with the differences observed within the other pair (Bobwhite/BW2). Even though the two pairs of near-isogenic lines contained different levels of total protein and lipids on the starch surface, our results showed that differences observed in thermal properties between HGAB18 starch using batter and dough isolated samples were not significantly different from the differences observed between BW2 starch isolated by batter and dough methods (Table 4.6).

Starch isolated from HGAB18 and Hi-Line by the batter method exhibited somewhat different pasting profiles (Fig. 4.6). Starch isolated from HGAB18 (batter) had slightly faster viscosity development during the heating phase than did Hi-Line and HGAB18 (dough). When surface components were depleted from HGAB18 by dough method isolation, the setback was shifted to a lower viscosity. Because Hi-Line and HGAB18 are near-isogenic to each other, only differing in starch surface components, it is expected that removal of surface components from HGAB18 by the dough method would result in pasting behavior closer to that of Hi-Line than HGAB18 (batter). This hypothesis was, in fact, supported for maximum peak viscosity and swelling power which was reduced for Hi-Line, greatest for HGAB18 (batter) and intermediate for HGAB18 (dough) (Table 4.7). Variations in the levels of surface components within replicates of starch isolation could be one of the reasons for the high standard deviations

observed for MVA parameters. Starch from HGAB18 (batter) swelled more and had greater peak viscosity than did Hi-Line. On the other hand, HGAB18 (dough) was not significantly different from either HGAB18 (batter) and Hi-Line. The sample HGAB18 (batter) had the greatest peak viscosity and, consequently, significantly greater breakdown. The breakdown is the measurement of loss of viscosity during the holding period with constant shearing. The loss in viscosity is caused by the alignment of solubilized starch polymers and also by the rupture of highly swollen granules. Starches that swell more, such as, *waxy* starch, are more fragile to shearing and tend to show a greater breakdown (Zeng et al. 1997). Han and Hamaker (2002) and Debet and Gidley (2007) reported that lipids and proteins present on the surface of starch granules helped maintain the integrity of swollen granules. In this study, we observed that when constant shear is applied to concentrated starch pastes (10%), the breakdown of the starch paste was more affected by the extent of granule swelling than by the total levels of lipids and proteins present on the starch surface.

Martin et al. (2008) reported that HGAB18 flour had greater swelling than did Hi-Line, but starch swelling volume was the same for both genotypes. They attributed the inconsistency between flour and starch swelling to the fact that proteins and lipids might have been removed during starch isolation (using a dough method). The present study supports their hypothesis by showing that when a batter method was used and starch surface components were retained the swelling power was actually increased.

The fact that soft wheat starch, which contains greater amounts of lipids and proteins on the granule surface, demonstrated slightly increased peak viscosity and swelling power compared to the hard cultivar is to some extent contradictory from previous studies considering effects of starch surface components in pasting. Nierle et al. (1990) reported that starch extracted with 1%

SDS and 80% ethanol exhibited a lower pasting temperature and higher peak viscosity than did native control, suggesting that proteins and lipids act as a protective "film" on the starch surface, inhibiting granule swelling. Other authors also studied chemically extracted granules and reported similar conclusions (Eliason et al. 1981, Debet and Gidley 2006). That said, there is very little literature extent analyzing effects of surface components on starch that has not been subjected to chemical extraction. Brites et al. (2007) studied wheat starch from hard and soft varieties with different *pin* alleles and found that starch from the wild type genotype (soft) exhibited greater peak viscosity and breakdown than that from hard cultivars. However, their samples were not near-isogenic, thus the results may have been affected by other variables such as, amylose/amylopectin ratio and damaged starch level. In the present study, the samples are near-isogenic and the variables affecting the outcomes are significantly minimized.

One possible explanation for the increased peak viscosity and swelling power for the soft experimental lines using batter method lies in the lipid profile for those samples. As discussed earlier (section 4.2), soft lines isolated by the batter method contained a significantly reduced amount of LysoPC on the starch surface. When added to starch suspensions, monoacyl lipids with long chain length, as well as diacyl lipids, have inferior amylose complexing ability compared to short chain monoacyl lipids (Siswoyo and Morita 2001; Siswoyo and Morita 2003). Complexes between amylose and monoacyl lipids are present on native wheat starch or they are formed during gelatinization (Morrison 1995). When heated in the presence of excess water the amylose-lipid complex can be observed in the DSC as a second endothermic transition around 100ºC (Eliasson 1985). Amylose that is complexed with lipids or iodine is known to restrict granule swelling and increase the enthalpy of DSC second peak (Patel et al. 2006; Tester and Morrison 1990). Complexes between amylose and lipids formed during gelatinization could have

prevented amylose from leaching out of the granule resulting in reduced peak viscosity, swelling power and breakdown as observed for samples with increased amounts of LysoPC. This hypothesis is supported by MVA and swelling power results, but not supported by DSC data. Greater enthalpy values for amylose-lipid melting peak represents greater amounts of complex formed between amylose and lipids. Since starch from hard cultivars and soft experimental lines isolated using a dough method had increased amounts of LysoPC, it is expected that these samples would have greater enthalpy values for DSC second peak. Even though the mean values followed that expectation, when analyzed statistically treatments showed not to be significantly different from each other for the pair Hi-Line/HGAB18 (Table 4.7).

The second pair of samples evaluated in this study (Bobwhite/BW2) is also near-isogenic to each other but varying in starch surface components. For that set of samples, starch isolated from Bobwhite (batter) showed pasting profiles very similar to BW2 (batter). However, Bobwhite exhibited significantly less breakdown than did BW2 (batter). Even though BW2 (batter) possessed greater amounts of total polar lipids, Bobwhite starch exhibited significantly higher amount of monoacyl lipid LysoPC. These results suggests that starch breakdown is affected not only by the extent of peak viscosity and the total amount of polar lipids on the surface, but also by the presence of specific lipid species, such as LysoPC. In agreement with what was found for Hi-Line/HGAB18, when starch surface components were depleted from BW2 by a dough method, the pasting curve shifted to a reduced viscosity (Fig. 4.6). Close examination of BW2 (batter) and BW2 (dough) parameters reveal that the second pair of near-isogenic sample responded to the treatment applied in a very similar fashion that of HGAB18 (batter) and HGAB18 (dough) (discussed previously). These parameters include; significantly reduced peak viscosity, breakdown, setback and swelling power for BW2 (dough) compared to

BW2 (batter). Because Bobwhite starch behaves in such an unexpected manner, it is possible and perhaps likely that there are more differences between this hard cultivar and its respective soft near-isogenic line than only starch surface components. That is supported by the data shown in Table 4.6 where some parameters are shown to be significantly different when contrasting the difference between a hard cultivar and its soft experimental lines using identical methods for starch isolation. These facts lead to the conclusion that both pairs evaluated in this study presented similar responses to the treatment applied to the soft lines and both samples have potential to be used as a tool to assess the effects of starch surface components in starch thermal properties. On the other hand, the different pasting behavior recorded for Hi-Line and Bobwhite when compared to their soft near-isogenic line demonstrated that starch from hard and soft near-isogenic lines are different. Those differences can be attributed to more than starch surface components. Therefore, studies of final product characteristics involving those materials need to take into consideration that (beyond surface protein and lipids), flour damaged starch is probably not the only other variable affecting results when comparing hard and soft near-isogenic lines.

It is well known that damaged starch absorbs greater amounts of water than does native undamaged granules and that increased levels of damaged starch increases flour water absorption (Greer and Stewart 1959). Gelatinization enthalpy and pasting viscosity of wheat starch suspensions have been demonstrated to decrease when damaged starch levels increase (Leon et al 2006). All samples analyzed in this study had very similar levels of damaged starch, ranging from 2.17 to 1.67% for hard cultivars and from 0.81 to 1.27% for soft lines (Tables 4.7 and 4.8). Even though damaged starch was shown to be significantly different within treatments, all damaged starch levels were very low compared to other published studies. Therefore, it is not

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likely that differences in damaged starch could explain the differences observed in starch thermal properties in the present study.

Because the isolation method resulted in variable amounts of protein and lipids for each replicate, Pearson correlation coefficients were calculated between starch thermal properties and starch surface components using each replicate as an individual sample (Table 4.9). Total protein and *pin* protein were significantly correlated with breakdown, swelling power and second peak enthalpy for the near-isogenic pair Hi-Line/HGAB18. LysoPC was significantly negatively correlated with breakdown but, surprisingly, not significantly correlated with peak viscosity, swelling power and second peak enthalpy. Peak viscosity was significantly correlated only with *pin* contents ($r = 0.887$). For the second pair of samples (Bobwhite/BW2), total protein was positively correlated with peak viscosity ($r = 0.752$) and breakdown ($r = 0.753$). Concentration of *pin* protein was significantly correlated with breakdown ($r = 0.885$), swelling power ($r = 0.717$) and negatively correlated with second peak enthalpy ($r = - 0.780$). Increased amounts of LysoPC decreased granule swelling and, for that reason, LysoPC was negatively correlated with peak viscosity, breakdown and swelling power.

Table 4.1 Prime starch yield (percentage based on as is flour weight) for Hi-Line and Bobwhite using batter method, and for BW2 and HGAB18 using both batter and dough methods.

Sample	Isolation Method	Percentage Yield
Hi-Line	batter	37.38a ± 1.91
HGAB18	batter	25.83b ± 1.79
HGAB18	dough	49.37c ± 8.47
Sample	*Isolation Method*	*Percentage Yield*
Bobwhite	batter	36.60a ± 1.82
BW2	batter	36.32a ± 5.41
BW2	dough	49.23b ± 5.82

Values represent mean starch yield percent ± standard deviation, $n = 3$ (three replicates of starch isolation).
Letter difference represents significant difference within cultivar (batter) and respective experimental line (batter and dough) with a column at $P = 0.05$.

Table 4.2 Mean values (nmol/g of sample) of the bound polar lipid classes and subclasses extracted from the starch surface of Hi-Line (batter method) and its respective near-isogenic pair HGAB18 using batter and dough methods.

Lipid class	Cultivar	Experimental line		Source of Variation	
	Hi-Line	HGAB18	HGAB18	Treatment	Replicate
	Batter	Batter	Dough	P-value	
DGDG(34:2)	1b ± 0	42a ± 23	8b ± 4	0.0393	ns
DGDG(36:4)	3b ± 1	133a ± 92	20ab ± 12	ns	ns
Total DGDG	**5b ± 2**	**218a ± 139**	**35b ± 20**	**ns**	**ns**
MGDG(34:2)	0b ± 0	6a ± 3	1b ± 0	0.0297	ns
MGDG(36:4)	2b ± 1	79a ± 54	14b ± 7	ns	ns
Total MGDG	**2b ± 1**	**97a ± 64**	**17ab ± 9**	**ns**	**ns**
Total PG	**0b ± 0**	**2a ± 1**	**0b ± 0**	**0.0005**	**ns**
Total LysoPG	**2a ± 1**	**0b ± 0**	**2a ± 1**	**0.019**	ns
LysoPC(16:0)	9a ± 4	4b ± 2	10a ± 5	0.0046	ns
LysoPC(18:2)	5a ± 2	2b ± 1	7a ± 3	0.0150	ns
Total LysoPC	**15a ± 7**	**7b ± 3**	**19a ± 8**	**0.0037**	**ns**
Total LysoPE	**1a ± 0**	**1a ± 0**	**1a ± 0**	**ns**	**ns**
PC(34:2)	1b ± 0	27a ± 13	3b ± 1	0.0231	ns
PC(34:1)	0c ± 0	6a ± 2	1b ± 0	0.0001	ns
PC(36:4)	0b ± 0	14a ± 8	1b ± 0	0.0411	ns
PC(36:3)	0b ± 0	6a ± 3	1b ± 0	0.0063	ns
Total PC	**2b ± 1**	**64a ± 26**	**7b ± 3**	**0.0115**	**ns**
Total PE	**0a ± 0**	**6a ± 5**	**1a ± 0**	**ns**	**ns**
Total lipids	**31b ± 2**	**391a ± 245**	**94b ± 29**	**ns**	**ns**

Values represent mean nmol of polar lipids per gram of sample (db) ± SE, $n = 3$ (three replicates of starch isolation).
Letter difference represents significant difference within cultivar and respective experimental line within the same row at $P = 0.05$ using LSD test.
F-values derived from Type III sums of squares.

Table 4.3 Mean values (nmol/g of sample) of the bound polar lipid classes and subclasses extracted from the starch surface of Bobwhite (batter method) and its respective near-isogenic pair, BW2, using batter and dough methods.

Lipid class	*Cultivar*	*Experimental line*		*Source of Variation*	
	Bobwhite	BW2	BW2	Treatment	Replicate
	Batter	Batter	Dough	*P-value*	
DGDG(34:2)	1b ± 1	21a ± 10	4b ± 0	0.0015	ns
DGDG(36:4)	3b ± 2	56a ± 27	8b ± 2	0.0034	ns
Total DGDG	**6b ± 3**	**100a ± 46**	**15b ± 3**	**0.0024**	**ns**
MGDG(34:2)	0b ± 0	2a ± 1	0b ± 0	0.0003	ns
MGDG(36:4)	2b ± 1	27a ± 15	4b ± 1	0.0057	ns
Total MGDG	**2b ± 1**	**35a ± 19**	**6b ± 1**	**0.0044**	**ns**
Total PG	**0b ± 0**	**1a ± 1**	**0b ± 0**	**0.0250**	**ns**
Total LysoPG	**1a ± 0**	**0b ± 0**	**1a ± 1**	**0.0292**	**ns**
LysoPC(16:0)	9a ± 4	4a ± 2	9a ± 8	ns	ns
LysoPC(18:2)	4a ± 2	2a ± 1	4a ± 4	ns	ns
Total LysoPC	**14a ± 7**	**6b ± 3**	**24c ± 2**	**0.0002**	**ns**
Total LysoPE	**0b ± 0**	**0b ± 0**	**1a ± 0**	**ns**	**ns**
PC(34:2)	1b ± 0	10a ± 5	1b ± 1	0.0003	ns
PC(34:1)	0b ± 0	3a ± 1	0b ± 0	0.0007	ns
PC(36:4)	0b ± 0	5a ± 3	1b ± 0	0.0106	ns
PC(36:3)	0b ± 0	2a ± 1	0b ± 0	0.0001	ns
Total PC	**2b ± 1**	**22a ± 10**	**3b ± 1**	**0.0005**	**ns**
Total PE	**0b ± 0**	**1a ± 1**	**0b ± 0**	**0.0253**	**ns**
Total lipids	**31b ± 1**	**194a ± 44**	**50b ± 2**	**0.0032**	**ns**

Values represent mean nmol of polar lipids per gram of sample (db) ± SE, $n = 3$ (three replicates of starch isolation).
Letter difference represents significant difference within cultivar and respective experimental line within the same row at $P = 0.05$ using LSD.
F-values derived from Type III sums of squares.

Table 4.4 Total protein and *pin* protein mean values from starch isolated from Hi-Line (batter method) and its respective near-isogenic pair, HGAB18, (batter and dough method) using treatment (hardness*starch isolation method) and replicate as sources of variation

	Cultivar	*Experimental line*		*Source of variation*	
	Hi-Line	HGAB18	HGAB18	Treatment	Replicate
	Batter	Batter	Dough	*P – value*	
Total protein	0.50b	0.98a	0.59b	0.0064	ns
(%db)	± 0.10	± 0.06	± 0.13		
Pin protein	0*c	1.27a	0.61b	0.0085	ns
(µg/mg starch)	± 0	± 0.43	± 0.18		

Values represent means for three replicates of starch isolation ±SE (db).
Total protein was assessed by LECO. *Pin* protein was assessed by SDS PAGE.
* *Pin* protein in Hi-Line was zero or not detectable by SDS PAGE
Different letters within cultivar and respective experimental line represent significant difference for treatment within the same row at $P = 0.05$ using LSD test.
ns: not significant
F-values derived from Type III sums of squares.

Table 4.5 Total protein and *pin* protein mean values from starch isolated from Bobwhite (batter method) and its respective near-isogenic pair, BW2, (batter and dough method) using treatment (hardness*starch isolation method) and replicate as source of variation

	Cultivar	*Experimental line*		*Source of variation*	
	Bobwhite	BW2	BW2	Treatment	Replicate
	Batter	Batter	Dough	*P – value*	
Total protein	0.55b	0.66a	0.51b	0.0014	0.0006
(%db)	± 0.09	± 0.10	± 0.11		
Pin protein	0*c	1.16a	0.36b	< 0.001	ns
(µg/mg starch)	± 0	± 0.33	± 0.05		

Values represent mean protein percent for three replicates of starch isolation ±SE (db).
Total protein was assessed by LECO. *Pin* protein was assessed by SDS PAGE.
 * *Pin* protein in Bobwhite was zero or not detectable by SDS PAGE
Different letters within cultivar and respective experimental line represent significant difference for treatment within the same row at $P = 0.05$ using LSD test.
ns: not significant
F-values derived from Type III sums of squares.

Table 4.6 Pair-wise comparison ANOVA comparing differences in thermal properties and surface components between the two pairs of near-isogenic samples.

Technique	Parameter	Contrast	
		HGAB18(ba) - HGAB18(do) = BW2(ba) - BW2(do)	Hi-Line(ba) – HGAB18(ba) = Bobwhite(ba) – BW2(ba)
		P – value	
MVA[1]	Pasting (°C)	ns	ns
	Peak viscosity	ns	0.0153
	Breakdown	ns	ns
	Peak time (min)	ns	ns
	Setback	ns	ns
	Final viscosity	ns	0.0203
	Setback rate	ns	0.0183
DSC	To (°C)	ns	ns
First peak	Tp (°C)	ns	ns
	Enthalpy (J/g)	ns	ns
Swelling Power[2]		ns	ns
Damaged starch (%)		ns	0.0048
Total protein (% db)		0.0068	0.0005
Puroindoline		ns	ns
Total Lipid (nmol/mg)		ns	ns
DGDG		ns	ns
LysoPC		ns	ns

ns: not significant

1 – Brabender Micro Visco Amylograph parameters are given in Brabender Units (BU). Setback rate is the slope of the curve during the cooling period.
2 - Weight of swollen starch per 1g of dry starch.

Table 4.7 Mean values for starch properties and *P*-values from pair-wise comparison ANOVA for Hi-Line and HGAB18 starch using treatment (hardness*starch isolation method) and replicate as sources of variation.

Technique	Parameter	*Treatment*			*Source of variance*	
		<u>Hi-Line</u> Batter	<u>HGAB18</u> Batter	<u>HGAB18</u> Dough	Treatment	Replicate
					P - value	
MVA[1]	Pasting (°C)	71.1a ±3	68.8a ±1	70.8a ±4	ns	ns
	Peak viscosity	400a ±16	469b ±28	449ab ±14	0.0326	ns
	Breakdown	0b ±0	15.7a ±4	0b ±0	0.0011	ns
	Peak time (min)	20.7a ±2	19.5a ±0	20.2a ±3	ns	ns
	Setback	254b ±9	339a ±38	258b ±32	0.0349	ns
	Final viscosity	652b ±25	783a ±70	706ab ±33	0.0304	ns
	Setback rate	329a ±13	371a ±25	370a ±12	ns	ns
DSC First peak	To (°C)	51.06a ±0.23	52.00a ±0.64	52.01a ±1.37	ns	ns
	Tp (°C)	60.43a ±0.02	60.88a ±0.54	61.13a ±0.81	ns	ns
	Enthalpy (J/g)	9.34a ± 0.77	8.83a ±0.54	7.73b ± 0.77	0.0317	ns
DSC Second peak	To (°C)	96.48a ±1.92	96.35a ±1.06	97.30a ±1.27	ns	ns
	Tp (°C)	107.32a ±0.95	105.81a ±2.14	107.29a ±1.05	ns	ns
	Enthalpy (J/g)	1.13a ±0.23	0.81a ±0.11	0.95a ±0.09	ns	ns
Swelling Power[2]		7.51b ±0.14	7.90a ±0.06	7.70ab ±0.13	0.0205	ns
Damaged starch (%)		2.17a ±0.06	1.01b ±0.05	1.16c ±0.03	< 0.001	ns

Values are means of three replicates of starch isolation.

Letter difference represents significant difference between cultivar and corresponding experimental line on the same row, P < 0.05

1 – Brabender Micro Visco Amylograph parameters are given in Brabender Units (BU). Setback rate is the slope of the curve during the cooling period.

2 - Weight of swollen starch per 1g of dry starch.

Table 4.8 Mean values for starch properties and *P*-values from pair-wise comparison ANOVA for Bobwhite and BW2 starch using treatment (hardness*starch isolation method) and replicate as sources of variation.

Technique	Parameter	Treatment			Source of variance	
		Bobwhite Batter	BW2 Batter	BW2 Dough	Treatment	Replicate
					P - value	
MVA[1]	Pasting (°C)	65.9a ±1	66.0a ±1	66.6a ±0	ns	ns
	Peak viscosity	476a ±13	494a ±6	442b ±18	0.0141	ns
	Breakdown	1b ±2	12.7a ±6	0b ±0	0.0160	ns
	Peak time (min)	17.6a ±1	17.9a ±1	18.4a ±0	ns	ns
	Setback	322ab ±66	349a ±47	281b ±29	ns	0.0320
	Final viscosity	794b ±69	822a ±50	721ab ±45	0.0399	0.0326
	Setback rate	400a ±6	396ab ±6	378b ±13	ns	ns
DSC First peak	To (°C)	51.43a ±1.23	51.22a ±1.35	52.28a ±0.44	ns	0.0326
	Tp (°C)	59.14b ±0.33	59.42ab ±0.56	60.64b ±0.61	ns	ns
	Enthalpy (J/g)	7.08a ±0.79	7.17a ±0.86	7.17a ±1.67	ns	ns
DSC Second peak	To (°C)	96.36a ±1.88	96.15a ±1.68	96.72a ±0.85	ns	ns
	Tp (°C)	107.79a ±1.25	107.77a ±2.09	107.67a ±0.62	ns	ns
	Enthalpy (J/g)	1.03b ±0.10	0.73a ±0.02	0.85ab ±0.06	0.0241	ns
Swelling Power[2]		8.19b ±0.25	8.59a ±0.16	8.25b ±0.14	0.0325	ns
Damaged starch (%)		1.67a ±0.14	0.81b ±0.03	1.27c ±0.09	< 0.001	ns

Values are means of three replicates of starch isolation.
Letter difference represents significant difference between cultivar and corresponding
experimental line on the same row, P < 0.05 by LSD.
ns: not significant.

1 – Brabender Micro Visco Amylograph parameters are given in Brabender Units (BU). Setback
rate is the slope of the curve during the cooling period.
2 - Weight of swollen starch per 1g of dry starch.

Table 4.9 Correlation and level of significance between concentrations of proteins and lipids present on the starch surface and the most relevant of the parameters analyzed.

Near-isogenic pair	Parameter	Total Protein	Pin	Total lipid	DGDG	LysoPC
Hi-Line /HGAB18	Peak viscosity	0.645 ns	0.887**	0.457 ns	0.466 ns	-0.349 ns
	Breakdown	0.896**	0.841**	0.651 ns	0.660 ns	-0.886**
	Swelling power	0.867**	0.854**	0.582 ns	0.589 ns	-0.597 ns
	Enthalpy 2nd peak	-0.482 ns	-0.744*	-0.366 ns	-0.377ns	0.5392ns
Bobwhite/ BW2	Peak viscosity	0.752*	0.471 ns	0.564 ns	0.589 ns	-0.772**
	Breakdown	0.753*	0.875**	0.753*	0.780*	-0.803**
	Swelling power	0.088 ns	0.717 *	0.792*	0.802 **	-0.700*
	Enthalpy 2nd peak	-0.463ns	-0.824**	-0.780*	-0.762*	0.411ns

**, * Significant at P = 0.01 and P = 0.05, respectively.

ns: not significant.

	HGAB18 ↓	Hi-Line ↓
1st sieve (425µm) ⟶		
2nd sieve (400µm) ⟶		

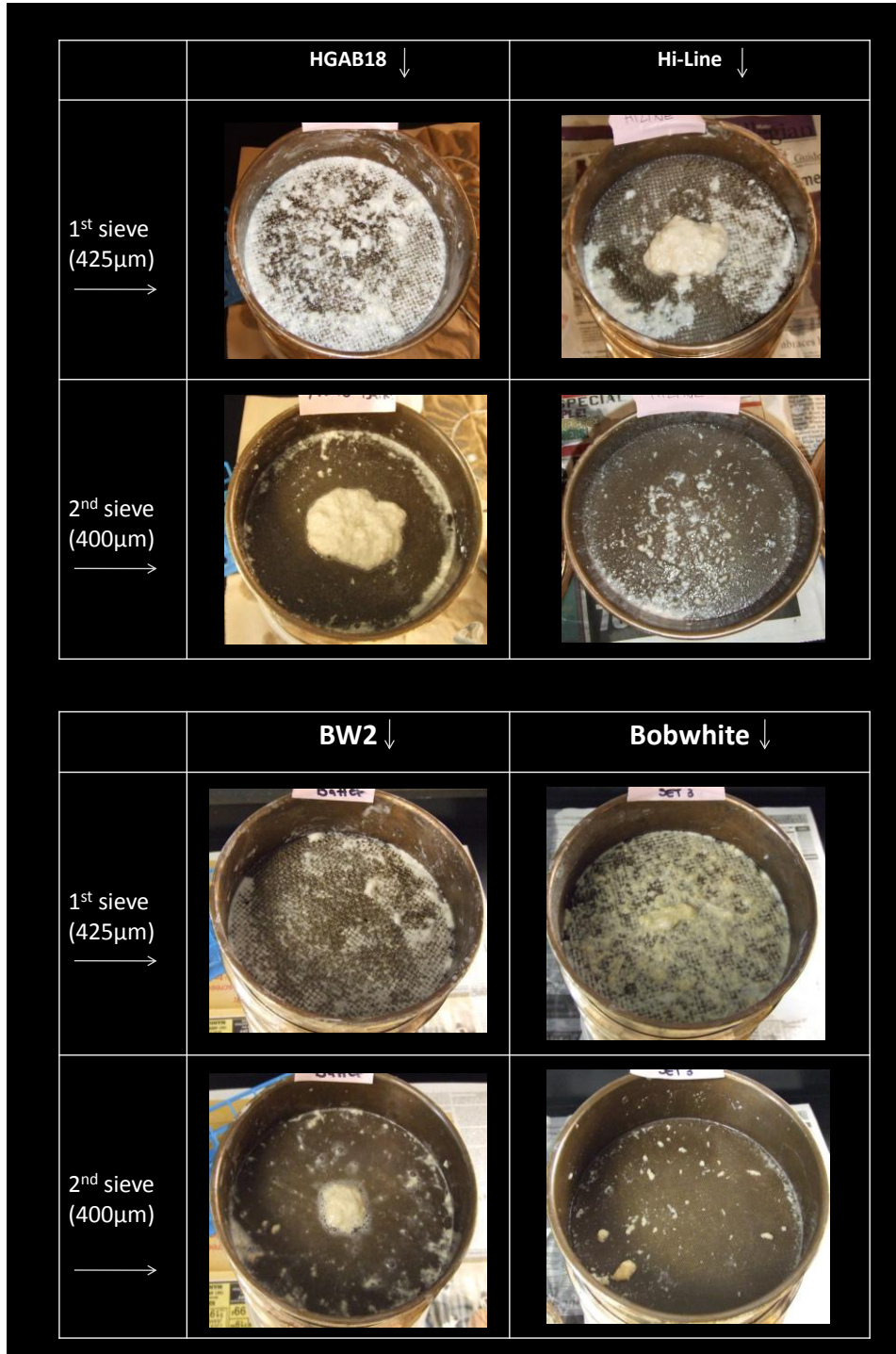|	BW2 ↓	Bobwhite ↓
1st sieve (425µm) ⟶		
2nd sieve (400µm) ⟶		

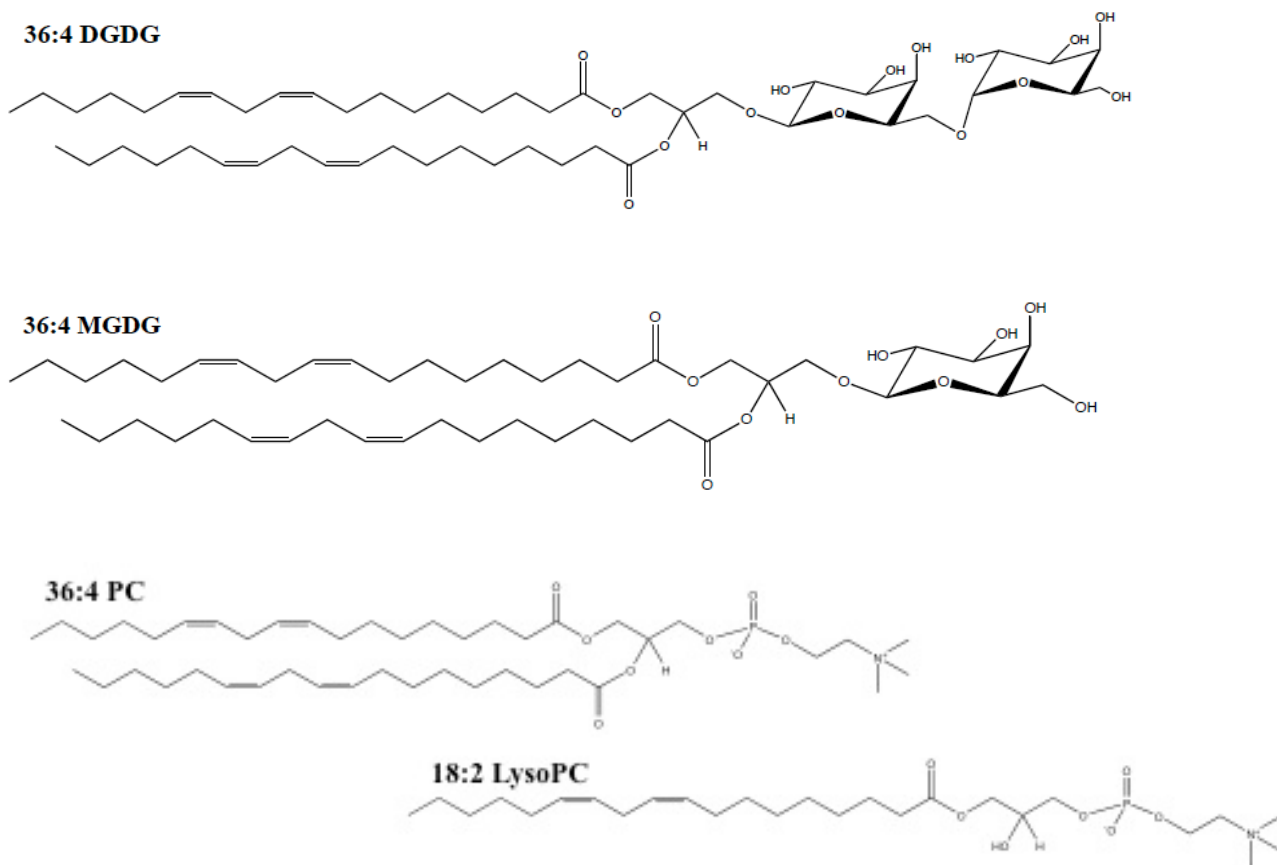**Figure 4.1 Top sieves (425 and 400µ) during starch isolation using the batter method.**

Figure 4.2 Structural representation of main polar lipid species extracted from the surface of wheat starch. Labels represent total acyl carbons : total double bonds and specific class of lipid (from Finnie et al. 2009)
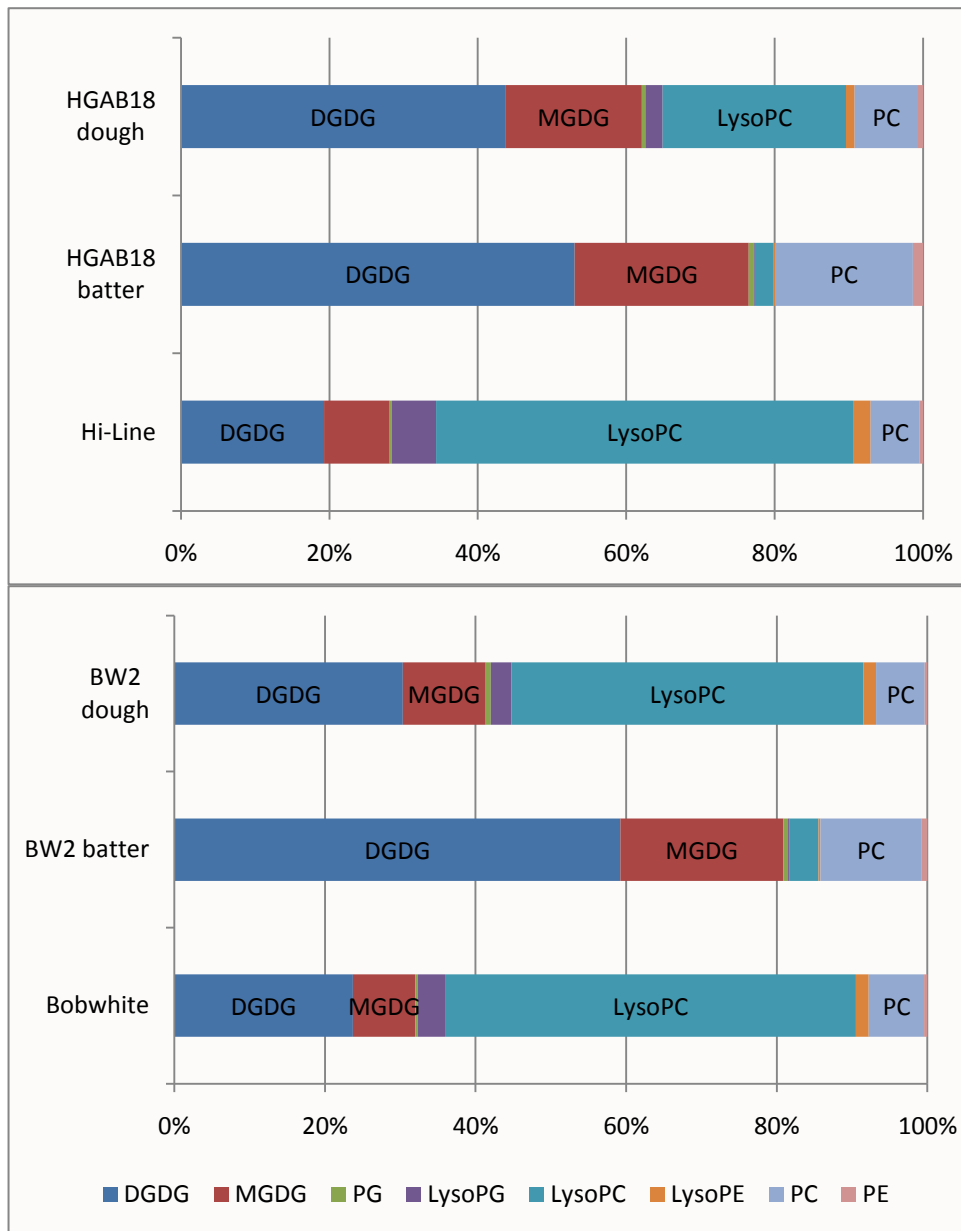
Figure 4.3 Polar lipid class means (mol%) from starch isolated using batter and dough methods (HGAB18 and BW2) and batter method (Hi-line and Bobwhite).
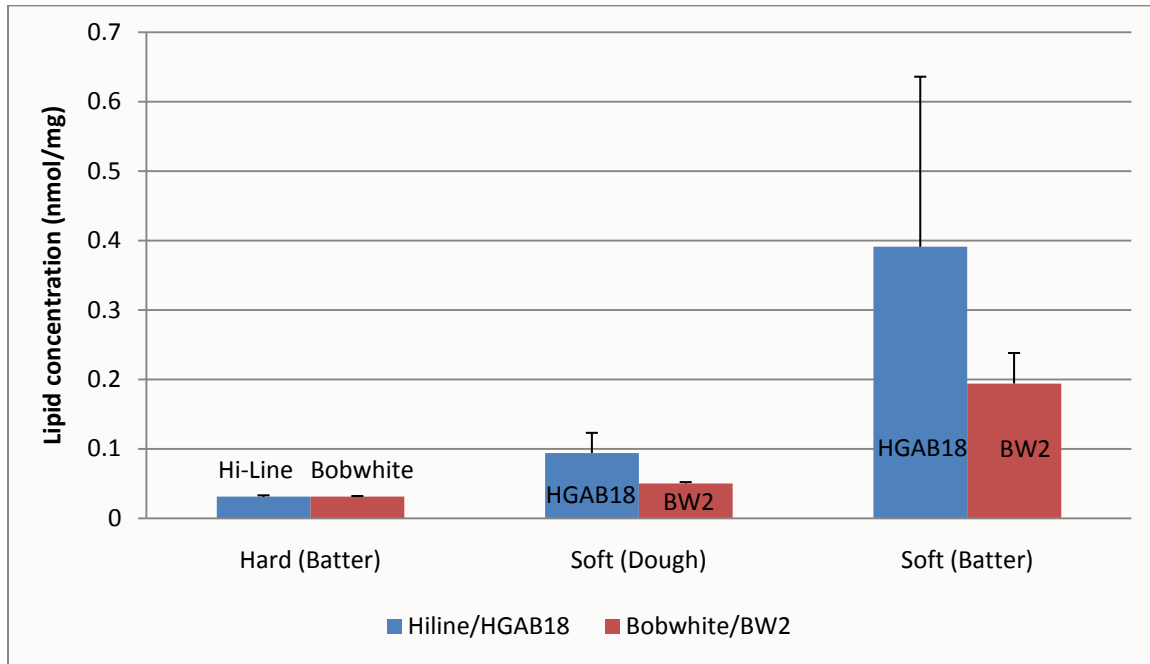
Figure 4.4 Total bound polar lipid present on the surface of starch isolated using batter and dough methods from the two pairs of near-isogenic wheat lines.
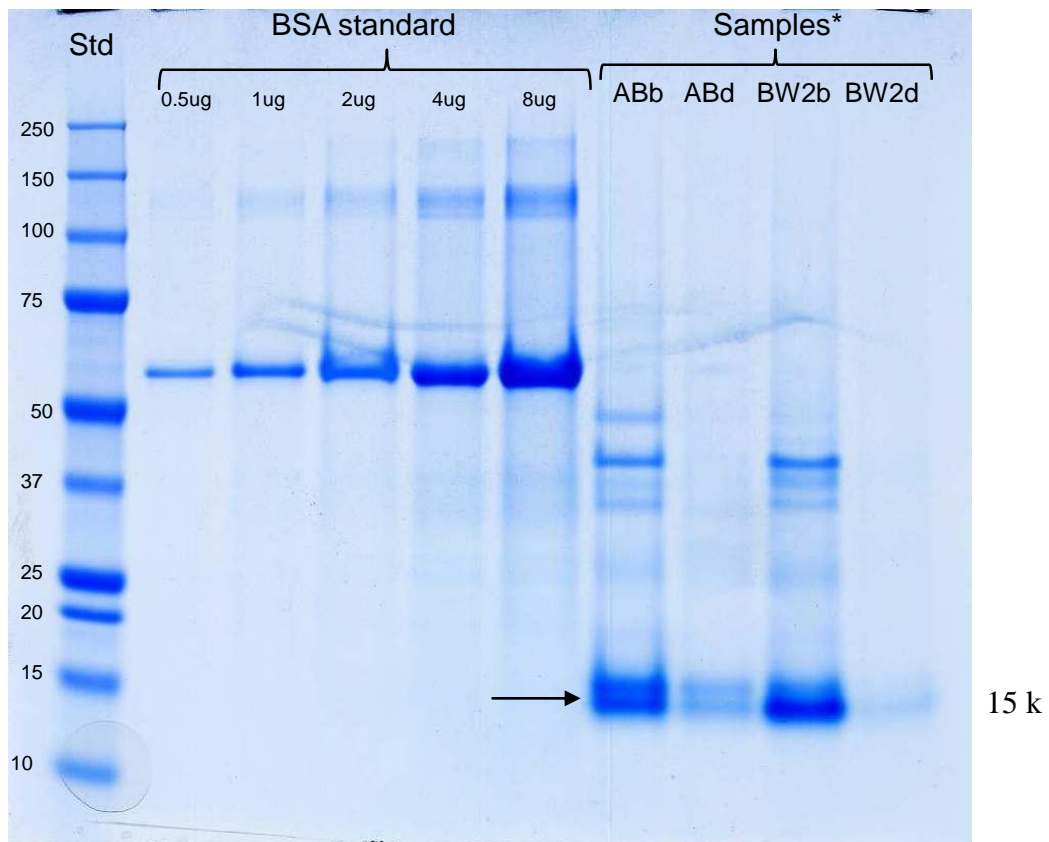
Figure 4.5 SDS PAGE image of *pin* protein extracted from starch surface of HGAB18 by batter (ABb) and dough (ABd) methods, and BW2 by batter (BW2b) and dough (BW2d) methods. Standard (Std) molecular markers with molecular weight ranging from 10 to 250 kDa. Bovine serum albumin (BSA) was used as standard for concentration.

* Sample abbreviations: HGAB18 batter – ABb; HGAB18 dough – ABd; BW2 batter – BW2b; BW2 dough – BW2d.

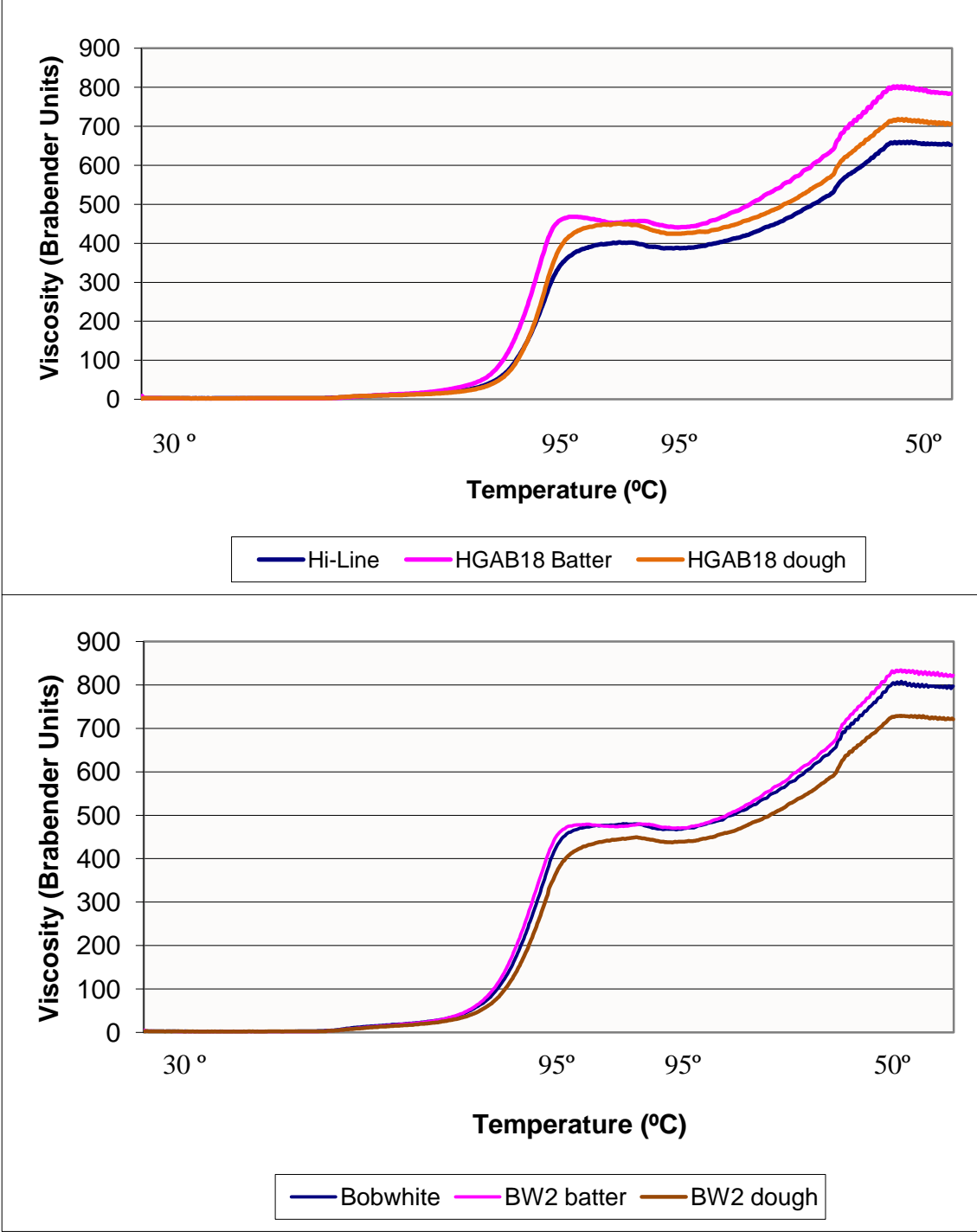Arrow indicates the 15k band representing puroindoline concentration

Figure 4.6 Brabender MVA viscosity curves of a 10% starch suspension for the six samples analyzed. Each curve is an average from three replicate of starch isolations.

CHAPTER 5 - Conclusions

Objective 1. To characterize the puroindoline proteins, polar lipids and thermal properties of starch isolated from two pairs of near-isogenic samples overexpressing puroindolines.

- Starch isolated using a batter method retained greater amounts of protein and bound polar lipids on its surface than starch derived from dough method.

- Starch isolated from hard cultivars presented more similarities with starch isolated from its soft near-isogenic line when a dough method was used than when a batter method was used.

- Starch isolated from the hard cultivars using a batter method and starch isolated from their soft experimental lines using a dough method exhibited significantly higher amounts of LysoPC.

- Results suggested that starch from hard and soft near-isogenic lines is different and that difference is not solely attributed to starch surface components.

Objective 2. To investigate the role that starch surface components play in the pasting process by using batter and dough methods to both obtain the starch and manipulate its surface components.

- Starch isolated from soft experimental by a batter method exhibited increased MVA peak viscosity, breakdown, setback and swelling power.

- Increased amounts of LysoPC was negatively correlated with peak viscosity, breakdown and swelling power for the near-isogenic pair Bobwhite/BW2, and negatively correlated with breakdown for Hi-Line/HGAB18.

- The presence of specific lipid species, such as LysoPC, on the starch surface may exert a greater effect on starch pasting profile than does the total amount of lipids and protein present on starch surface.

CHAPTER 6 - Future Studies

Both near-isogenic lines evaluated in this study showed potential to be used in future studies assessing the effect of wheat hardness on final products characteristics. Such samples could be used to determine functionality of starch surface components on dough formation, dough rheology and breadmaking quality. It was observed during this study that transgenic modification of hard cultivar into soft wheat lines by *pin* mutation produced flour with different dough handling properties and gluten formation. It would be useful to investigate dough rheology of these samples by using other equipment such as, the Chopin Mixolab. Breadmaking quality of near-isogenic lines overexpressing *pin* proteins was investigated in the past. However, the different levels of damaged starch produced during milling were not corrected on previous studies. Therefore, it would be interesting to evaluate breadmaking properties of near-isogenic samples after damaged starch levels were equalized on soft wheat cultivars. Preliminary studies done during this thesis showed that damaged starch could be successfully produced in the soft lines by multiple passages of the flour through the smooth rolls of a Ross mill. It would be also useful to evaluate properties of near-isogenic flours with intermediate hardness.

Some of the mechanisms that explain wheat hardness and interaction of protein and lipids on starch surface are still hypothetical. Therefore, more research is necessary to understand the exact mechanisms in which proteins and lipids interact with the polymers on surface of the starch granule and how the presence of these components can affect dough formation and starch properties. It would also be interesting to address the reasons why starch isolated using batter method from soft lines had depleted amounts of Lyso-PC present on the starch surface. A precise interpretation of the increased peak viscosity and breakdown observed in samples with greater

amounts of starch surface components is not yet possible and further investigation will be necessary. Finally, the exact differences between starch isolated from hard and soft near-isogenic samples is yet to be determined. Future studies should include more than only two pairs of near-isogenic samples varying in starch surface components.

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Appendix A - DSC endothermic curves for starch isolated from Hi-Line, HGAB18 (batter) and HGAB18 (dough)