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Variation in polar lipids located on the surface of wheat starch

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ABSTRACT

It is unknown whether starch isolated before dough development has the same surface lipid composition as starch isolated after dough development. The abundance of starch surface polar lipids is related to the physical hardness of the endosperm, but the variation in specific lipid classes and molecular species is unknown. The objective of this study was to determine the variation in polar lipids present on the surface of wheat starch granules. The experimental wheat lines used are, within each set, near-isogenic to each other but vary in endosperm hardness. Starch was isolated using two different processes: a dough and a batter method. Direct infusion electrospray ionization tandem mass spectrometry was used to identify and quantitatively determine the polar lipid species in wheat flour and on starch. Wide ranges in starch surface polar lipid concentrations were observed between the starch isolation methods. Starch isolation method provided a greater source of variation than did wheat kernel hardness. When dough is optimally mixed, lipids originally on the surface of wheat starch are dissociated, whereas in a batter system, starch surface lipids stay associated with the starch surface. The predominant starch surface polar lipids were digalactosyldigylcerol (DGDG), monogalactosyldigylcerol (MGDG) and phosphatidylcholine (PC) polar lipid classes.
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1. Introduction

Wheat (*Triticum aestivum* L.) starch constitutes 70–75% of the flour and is composed of three distinctive types of granules: A-type, B-type and C-type. The A-type granules are lenticular in shape with a diameter greater than 16 μm, the B-type granules are spherical in shape with a smaller diameter of 5–16 μm and the C-type granules are spherical and have the smallest diameter of less than 5 μm (Bechtel et al., 1990). During development of the wheat endosperm, A-type granules are synthesized first, beginning at 4 days after flowering. B-type granules begin developing at 10 days after flowering, and C-type granules begin developing at 21 days after flowering (Bechtel et al., 1990).

The internal structures of normal starch granules consist of the high-molecular-weight polymers, amylose and amylopectin. Amylose, the minor component, is a linear polymer of α-(1,4)-linked D-glucopyranosyl units, and amylopectin is a highly branched polymer of α-(1,4)-linked D-glucopyranosyl units with α-(1,6)-branch points. The non-reducing ends of amylopectin and amylose are orientated toward the granule's center, and the reducing ends are toward the surface of the starch granule. Wheat starch granules contain channels that allow some molecules to enter the granule matrix (Kim and Huber, 2008). A-type granules contain two types of channels: larger channels located on the equatorial groove and smaller channels located throughout the granule. B-type starch granules contain only one type of channel: large, void-like channels that are less defined than the channels on the A-type granules (Kim and Huber, 2008). Han et al. (2005) and Lee and BeMiller (2008) found that starch channels are lined with proteins and polar lipids. Han et al. (2005) identified the proteins as 38–40 kDa brittle-1 proteins. Lee and BeMiller (2008) identified the channel lipids in maize starch as lysophosphatidylcholine polar lipids with either palmitic acid (16:0) or linoleic acid (18:2) fatty acid moieties.

The compositional structure of the surface of wheat starch granules plays a crucial role in wheat endosperm hardness.

Abbreviations: Pin-A, puroindoline A; pin-B, puroindoline B; DGDG, digalactosyldigylceride; MGDG, monogalactosyldigylceride; PC, phosphatidylcholine; LPC, lysophosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PA, phosphatidic acid; PS, phosphatidylserine; PG, phosphatidylglycerol; LPE, lysophosphatidylethanolamine; LPG, lysophosphatidylglycerol; DGMG, digalactosylmonoglycerols; MGMG, monogalactosylmonoglycerol.

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Greenwell and Schofield (1986) identified a molecular marker for endosperm hardness as 15 kDa proteins that were found on the surface of water-washed starch granules from soft wheat. These proteins (called friabilin) were found in greater amounts on water-washed starch from soft wheat than on water-washed starch from hard wheat. The friabilin proteins were absent from water-washed starch from durum wheat (the hardest class of wheat). Jolly et al. (1993) and Morris et al. (1994) further discovered, through N-terminal sequencing, the existence of two proteins, puroindoline A (pin-A) and puroindoline B (pin-B), which together make up friabilin. Besides puroindoline proteins, 30 kDa glycoproteins and 60 kDa starch granule-bound starch synthase enzymes are located on the surface of starch granules (Baldwin, 2001).

As is the case for puroindoline proteins, Greenblatt et al. (1995) found that a pattern existed among polar lipids on the surface of starch granules. Both galactolipids and phospholipids were found, via thin layer chromatography, to be present in greater amounts on water-washed starch from soft wheat than on water-washed starch from hard wheat (Greenblatt et al., 1995). Konopka et al. (2005) found a negative correlation between starch surface lipids (polar and non-polar) and kernel hardness. Finnie et al. (submitted) found that 146 different lipid species were present on the surface of wheat starch granules in total mean amounts ranging from 89 to 482 nmol/g. The major polar lipid species identified were DGDG (36:4), MGDG (36:4), PC (36:4 and 34:2), and LPC (18:2 and 16:0).

Addition of water and energy to wheat flour produces a unique viscoelastic dough capable of retaining gases during fermentation and proofing. When dough is mixed to optimum (i.e., with sufficient hydration and work applied), the gliadin and glutenin proteins interact and form a continuous protein macropolymer (gluten) surrounding the starch granules (Pomeranz, 1988; Singh and MacRitchie, 2001). Few research articles have documented the interaction between gluten proteins (gliadin and glutenin) and the starch granule surface as it relates to flour functionality (Hoseney et al., 1971; Larsson and Eliasson, 1997; Sandsted, 1961). Larsson and Eliasson (1997) used *in vitro* modification of the starch granule surface (heat treatment, absorption of wheat protein and absorption of phosphatidylcholine) and found that these treatments affected the rheological properties of the dough, thereby indicating the importance of the starch granule surface components in dough functionality. Because the starch granule surface components appear to interact with the other components in dough, their abundance on the starch granule surface, their composition or both could vary depending on whether the starch was isolated before or after dough development (i.e., before or after interactions).

The results presented in Finnie et al. (submitted) were derived from starch isolated with a batter method, thus representing the starch granule surface polar lipids absent of dough development. If the starch granule-gluten interactions are mediated or affected by the starch granule's surface composition, the polar lipids on the surface of the starch granules might not be present in the same abundance or ratios after dough development. Additionally, the results of Greenblatt et al. (1995) indicate that the abundance of the starch granule surface polar lipids is related to the physical hardness of the endosperm (implying a relationship between puroindoline proteins and polar lipids). Results of Greenblatt et al. (1995) indicated a genetic source of variation in the relative abundance of starch granule polar lipids but did not indicate how the specific classes and molecular species of the polar lipids were affected by puroindoline protein expression. Therefore, the objective of this study was to quantitatively determine the variation in polar lipids present on the surface of wheat starch granules. Variation in the starch granule surface polar lipids was evaluated as resulting from genetic variation (from differences in wheat endosperm hardness) or induced by the starch isolation processes. Genetic variation studies were conducted using three pairs of near-isogenic wheat lines that varied in their puroindoline expression and kernel endosperm hardness. Induced variation studies were conducted using two distinctly different starch isolation processes: a dough-ball method (Wolf, 1964), representing starch after experiencing dough development, and a batter method, representing starch that had not experienced dough development.

2. Experimental

2.1. Wheat samples

A series of unique experimental wheat lines (Table 1) were collected that vary in their starch granule surface components. The experimental wheat lines used are, within each set, near-isogenic to each other but vary in their grain endosperm hardness. One set was derived from the hard cultivar Hi-Line (PI 549275), the second set was derived from the hard cultivar Bobwhite, and the final set was derived from the soft cultivar Alpowa (PI 566596) (Hogg et al., 2005; Morris and King, 2008).

The wheat samples derived from Hi-Line and Bobwhite were grown near Bozeman, MT at Montana State University Post Agronomy Farm. The Alpowa-derived samples were grown near Pullman, WA by the USDA-ARS Western Wheat Quality Laboratory. The wheat was grown in replicated plots in 2007. Once the wheat lines were harvested and cleaned, single kernel hardness was determined with the Single Kernel Characterization System 4100 (Perten Instruments North America, Inc., Springfield, IL). To provide enough wheat for the experiments, the two field replicates were bulked into one sample (hardness values were compared to ensure no combinations of multiple wheat lines).

2.2. Milling

The wheat lines were milled into straight-grade flour with a Bühler experimental mill per AACC International Approved Method 26–31 (AACC International, 2008). Modifications included a short tempering time (20 min) for the Hi-Line and Bobwhite derived samples instead of the specified 16–24 h time. The 20-min tempering was short enough to ensure that the water did not penetrate into the endosperm but provided enough time for the bran to become plastic and not break into small particles, which could contaminate the flour. The Alpowa-derived samples were tempered (24 h) per AACC International 26–31. All wheat samples were tempered to 14% moisture content, and the wheat was milled at a reduced feed rate of 100 g/min.

Table 1
Cultivar and corresponding experimental line, puroindoline haplotype, molecular change and SKCS hardness value of the wheat samples.

Cultivar/experimental line	Puroindoline haplotype	Molecular change from wild-type[†]	Hardness (SKCS)[§]
Alpowa	*Pina-D1a/Pinb-D1a*	–	31
Alpowa/hard-nil	*Pina-D1a/Pinb-D1e*[*]	Pinb nullTrp-39 to stop	68
Hi-Line	*Pina-D1a/Pinb-D1b*	Pinb Gly46 to Ser	82
HGAB18	*Pina-D1a*[**]/*Pinb-D1a*[**]	–	9
Bobwhite	*Pina-D1b/Pinb-D1a*	Pina null	84
BW2	*Pina-D1a*[**]/*Pinb-D1a*	–	24

[†] Wild-type defined as *Pina-D1a/Pinb-D1a* puroindoline haplotype.
[§] SKCS single kernel characterization system hardness index value.
[*] Indicates puroindoline gene modified through backcross breeding method.
[**] Indicates puroindoline gene modified through transgenic method.

2.3. Protein determination

Total protein was determined for all sample fractions using the combustion method per AACC International Approved Method 46-30 (AACC International, 2008), N X 5.7 for all fractions.

2.4. Starch isolation

Prime-starch was isolated using two different processes: a dough-ball method (Wolf, 1964) and a batter method (Finnie et al., submitted). In the dough-ball method, dough was mixed to optimum (3–4 min) with a 100-g pin mixer (National Manufacturing Co., Division of TMCO, Lincoln, NE). After dough washing the starch slurry was sieved through a sieve with 75 μm openings (Dual MFG. Co., Chicago, IL).

2.5. Lipid extraction

Because Finnie et al. (submitted) found that free lipids (hexane extractable) were extracted in relatively minor proportions compared with the bound lipid extracts (mol% basis), only bound lipids were analyzed in this study. For a detailed description of the lipid extraction, see Finnie et al. (submitted).

2.6. Lipid quantification

The polar lipids targeted for analysis were PC, phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidic acid (PA), phosphatidylserine (PS), phosphatidylglycerol (PG), LPC, lysophosphatidylethanolamine (LPE), lysophosphatidylglycerol (LPG), DGDG, MGDG, digalactosylmonoglycerol (DGMG), and monogalactosylmonoglycerol (MGMG).

An automated electrospray ionization tandem mass spectrometry approach was used, and data acquisition and analysis, and acyl group identification were carried out as described previously (Devaiah et al., 2006; Finnie et al., submitted).

2.7. Puroindoline isolation and determination

SDS-PAGE precast gels with a Tris–HCl gradient of 10–20% were used (Bio-Rad, Hercules, CA). Puroindoline proteins were extracted by using the modified method of Giroux et al. (2003), which uses a Triton X-114 non-ionic detergent phase partitioning step. Modification includes the use of SYPRO Tangerine protein stain (Invitrogen, Carlsbab, CA) instead of Coomassie blue to visualize the protein bands. Gel images were obtained with a UVP (Upland, CA) Multidoc-It Imaging System equipped with Doc-it LS Analysis software.

2.8. Statistical analysis

For wheat flour polar lipid determination, five replicated lipid extractions were conducted on each wheat flour sample. Differences in flour means were assessed using Scheffé's multiple comparison procedure, $P = 0.05$. Wheat starch polar lipid determination was conducted using a completely randomized block experimental design. "Day of starch isolation" was the blocking factor (three blocks with two duplicated lipid extractions each, $n = 6$). To determine significant sources of variation in lipid content found on the surface of wheat starch, a two-way ANOVA was conducted using starch isolation method and wheat endosperm hardness classification as the sources of variation. Data for starch surface lipids were non-normally distributed. To fulfill the assumption of normality, data were *log* transformed. Multiple mean comparisons of starch isolation and genotype were conducted using Scheffé's test, $P = 0.05$. All statistical analyses were conducted using the General Linear Model in the Statistical Analysis System (SAS) software (SAS version 9.1, SAS Institute, Cary, NC). Type III sums of squares are reported.

3. Results and discussion

3.1. Wheat flour bound polar lipids

Wheat flour bound polar lipid composition and content were found to be similar to the results in Finnie et al. (submitted). The four major lipid classes for all the flour samples were DGDG, MGDG, PC, and LPC. DGDG was the most prevalent lipid class, representing 42–59 mol% of the total bound polar lipids. In general, wheat lines within the cultivar/experimental line pair were more similar than different. Mean comparisons for each class within the cultivar/experimental line pairs showed only slight differences. The overall polar lipid totals were not significantly different between the cultivar/experimental line pairs for all the samples (Table 2). The Bobwhite/BW2 pair had the most significantly different lipid class means, with BW2 containing greater amounts of DGDG and PA lipids and Bobwhite containing greater amounts of MGDG, PC, LPC, PE, MGMG, PI and LPE (Table 2). Within the Hi-Line and HGAB18 pair, Hi-Line contained greater amounts of PC and PI, and HGAB18 contained greater amounts of DGDG, DGMG, MGMG, PA and PS (Table 2). The Alpowa/Alpowa hard-nil pair had the least difference between the lipid classes, with Alpowa containing greater amounts of DGDG, LPG and PS lipids and Alpowa/hard-nil containing greater amounts of PI lipids (Table 2). Only DGDG and PI lipid classes were found to be significantly different between all the cultivar/experimental line pairs. It is worth noting that flour from the soft-textured wheat lines (Alpowa, BW2 and HGAB18) contained significantly greater concentrations of DGDG lipids, and flour from the hard-textured wheat lines contained significantly greater concentrations of PI lipids (Table 2).

Mean differences among the cultivar/experimental line pairs were significantly different. The Hi-Line derived samples contained the greatest concentration of total polar lipids (3638 nmol/g) followed by Alpowa-derived samples (3288 nmol/g), and Bobwhite derived samples (3055 nmol/g). Overall, these results indicate there was more variability in total polar lipid content among the cultivar/experimental line pairs than within the pairs.

3.2. Variation in starch polar lipids

In this study, two starch isolation methods were evaluated for differences in their effects on starch granule surface lipids. The two isolation methods represent starch isolated before dough development (batter method) and after dough development (dough method). In the randomized complete design where block represented the day of starch isolation and treatment was the starch isolation method, the blocking effect had no significant influence, but the treatment was a significant source of variation in polar lipid concentration (data not shown).

Wide ranges in starch surface polar lipid concentrations were observed between the two starch isolation methods. Included in this study were three near-isogenic wheat line pairs that varied in their puroindoline expression levels and thus were expected to vary in starch granule surface polar lipid concentrations. The combined polar lipid class means showed a broad range in polar lipid concentrations among the samples studied (Table 3). Minimum mean values for the four major starch surface polar lipids were 0.01, 0.04, 0.01 and 0.94 nmol/g, DGDG, MGDG, PC and LPC, respectively, and their maximum values were 551, 222, 195 and 39 nmol/g. This broad range in lipid concentrations indicates that

Table 2
Mean values (nmol/g of sample) of different bound polar lipid classes from wheat flour cultivars and corresponding experimental wheat lines.

Lipid class	Cultivar	Experimental line	Cultivar	Experimental line	Cultivar	Experimental line
	Alpowa	Alpowa/hard-nil	Bobwhite	BW2	Hi-Line	HGAB18
DGDG	1984 a ± 41	1764 b ± 34	1334 b ± 41	1509 a ± 37	1549 b ± 17	1762 a ± 27
MGDG	684 a ± 14	691 a ± 15	530 a ± 14	482 b ± 10	700 a ± 18	726 a ± 11
PC	475 a ± 7	489 a ± 19	743 a ± 16	626 b ± 17	887 a ± 45	719 b ± 6
LPC	82 a ± 2	80 a ± 4	165 a ± 4	147 b ± 3	176 a ± 2	165 a ± 4
PE	55 a ± 1	58 a ± 2	133 a ± 2	107 b ± 2	93 a ± 24	84 a ± 2
PG	32 a ± 1	31 a ± 2	33 a ± 1	34 a ± 1	54 a ± 1	58 a ± 1
DGMG	20 a ± 1	19 a ± 1	18 a ± 1	17 a ± 1	16 b ± 1	18 a ± 0
MGMG	14 a ± 0	15 a ± 1	15 a ± 1	13 b ± 1	14 b ± 0	16 a ± 0
LPG	11 a ± 0	10 b ± 1	13 a ± 0	14 a ± 0	19 a ± 0	20 a ± 0
PA	9 a ± 0	9 a ± 1	23 b ± 1	26 a ± 0	31 b ± 1	44 a ± 1
PI	8 b ± 0	16 a ± 1	58 a ± 2	29 b ± 1	49 a ± 1	24 b ± 1
PS	6 a ± 0	4 b ± 0	11 a ± 0	11 a ± 0	13 b ± 0	17 a ± 1
LPE	5 a ± 0	5 a ± 0	10 a ± 0	9 b ± 0	10 a ± 3	12 a ± 1
Total Polar	3385 a ± 61	3192 a ± 60	3086 a ± 77	3023 a ± 67	3664 a ± 54	3611 a ± 47

Value represent mean nmol of polar lipids per gram of sample ±SE, $n = 5$. Letter difference represents significant difference between cultivar and corresponding experimental line, $P < 0.05$.

the starch granule surfaces are not equal in their ability to interact with lipids, and that other competing forces of lipid interaction are present.

ANOVA results identifying the sources of variation among the samples are presented in Table 3. Data were non-normally distributed as determined by residual plots. Consequently, data were *log* transformed to satisfy the normality assumption. Interactions between the main effects (starch isolation method and wheat endosperm hardness) were not significant for any lipid classes except LPC and LPE (Table 3). The interactions between the main effects in the LPC and LPE lipids were caused by mean crossovers, in which the starch isolated using the dough method contained greater amounts of lipids in the soft-textured samples and the starch isolated using the batter method contained greater amounts of lipids in the hard-textured samples (Table 3 and Fig. 1). Both main effects were found to be significant sources of variation in DGDG, MGDG, PC, PE, PG, DGMG, MGMG, PI, PS and PA lipid classes (Table 3). According to ANOVA *F*-values for the polar lipid classes (Table 3), starch isolation method constituted a greater source of variation than did wheat kernel hardness.

All the model R^2 values for the di-acyl lipid classes (DGDG, MGDG, PC, PE, PG, PA, PI and PS) were relatively high, ranging from 0.69 to 0.89 (data not shown). The high R^2 value indicated a good model fit in which 69–89% of the variation in the data can be explained by this two-way ANOVA. The mono-acyl lipid classes (LPC, DGMG, MGMG, LPG) exhibited lower R^2 values, ranging from 0.14 to 0.35 (data not shown), indicating that the two-way ANOVA only explained 14–35% of the total variation in those lipid classes and that other sources of variation may exist for the mono-acyl lipids. Possible sources of variation other than isolation method and endosperm hardness could be differences in the extractability of the lyso-phospolipids from the channels of the wheat granule between the samples. Differences in lipid acyl-hydrolase activities or specificities of the various wheat flour samples could also exist.

Figs. 1 and S1–S5 present the differences in polar lipids between starch isolation method and wheat endosperm hardness on a nmol/g basis. Fig. 1 provides information regarding the polar lipid class differences, and Figs. S1–S5 provide information regarding molecular species differences within classes. For the molecular species figures, the *y*-axes within a lipid class were kept at a constant scale, providing the ability to visually observe the lipid contents in a "mol% of total" basis as well as the labeled nmol/g. It is evident that starch isolation method had a significant influence on the abundance of the polar lipids; starch isolated using the batter method contained greater amounts of polar lipids than did starch isolated using the dough method. The only non-significant difference between isolation methods was observed in the mono-acyl lipid classes (Figs. 1 and S1–S5). All the di-acyl lipids were in greater concentration on the batter-isolated starches (Figs. 1 and S1–S5). The predominant lipid classes (DGDG, MGDG, PC and LPC) were different between the batter- and dough-isolated starch samples. For the dough-isolated starch samples, LPC was the most predominant lipid class, but in the batter-isolated samples, DGDG, MGDG and PC lipid classes were found in greater quantities (Fig. 1).

The molecular species present in this study were similar in composition to those identified previously (Finnie et al., submitted). The most prevalent molecular species were those that contained 16:0 and 18:2 fatty acids (36:4, 34:2 and 16:0). In general, the effect of starch isolation method was non-discriminant to the molecular species within a class; differences between the dough and batter starch lipids were relatively constant among all the molecular species (Figs. S1–S5).

The difference in starch surface lipids between the two isolation procedures provides insight into the role starch surface lipids play in the development of gluten. During gluten formation, the majority of flour polar lipids become incorporated into the gluten phase of dough (Chung and Tsen, 1975). It appears that as gluten is being developed, polar lipids originally located on (or near) the surface of

Table 3
Sample statistics and *F*-values from two-way ANOVA of starch lipids according to starch isolation method and wheat endosperm hardness sources of variation

Statistic/source	DGDG	MGDG	PC	LPC	PE	PG	DGMG	MGMG	LPG	PA	PI	PS	LPE
Minimum	0.01	0.04	0.01	0.94	0.00	0.04	0.01	0.08	0.13	0.00	0.02	0.00	0.00
Mean	92	35	29	17	2	1	1	1	2	2	0.5	0.5	1
Maximum	551	222	195	39	11	7	3	2	4	12	1	2	4
Isolation method	258	399	396	ns	209	439	11	4	ns	140	220	122	9
Hardness	61	77	80	5	33	94	24	19	ns	ns	4	29	7
Interaction	Ns	ns	ns	5	ns	ns	ns	ns	14	ns	ns	ns	ns

Sample statistics are expressed as nmol of lipid per gram of sample. *F*-values are significant at $P = 0.05$; ns, not significant. Data used for two-way ANOVA were transformed to satisfy normality assumption using log. *F*-values derived from Type III sums of squares.

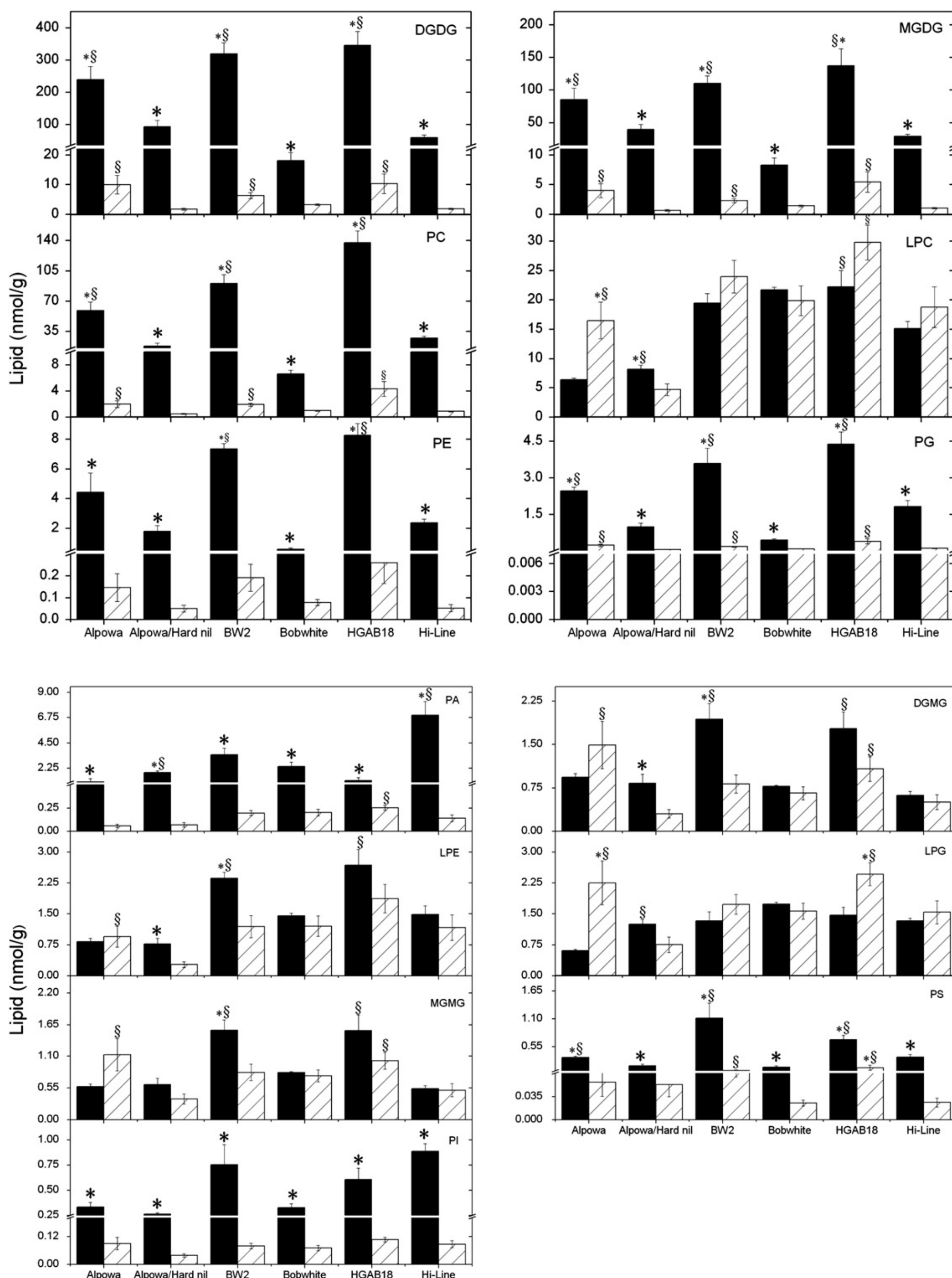

Fig. 1. Polar lipid class means (nmol/g) from starch isolated using batter (solid bars) and dough (striped bars) methods. Values are means and ±SE ($n = 6$). * indicates significantly greater lipid content between the starch isolation method for the specific wheat sample, § indicates significantly greater lipid content between cultivar and corresponding experimental line, both at $P < 0.05$.

the starch granules become incorporated into the gluten phase of the dough. Starch granule surfaces are thus dynamic and depending on their surface surrounding environment, their composition can be significantly altered.

Wheat endosperm hardness also had a significant effect on the composition and concentration of the polar lipids located on the surface of starch (Table 3 and Figs. 1 and S1–S5). Endosperm hardness was a significant source of variation in the two-way ANOVA for all the lipid classes except LPG and PA. Overall, starch from soft-textured wheat contained greater amounts of polar lipids than did starch from hard-textured wheat (Figs. 1 and S1–S5). An exception to this pattern was the PA and PI lipids from the Hi-Line derived samples. The starch isolated from Hi-Line flour (hard-textured wheat) contained greater amounts of PA and PI lipids than did the starch isolated from HGAB18 flour (soft-textured wheat). One possible explanation for this finding is that the activity of

phospholipase D might be greater in the Hi-Line wheat sample than the other wheat samples. Products of phospholipase D reactions are PA lipids. The molecular species difference between the starches isolated from soft- and hard-textured wheat also appears to be non-discriminating, with relatively similar differences between the near-isogenic pairs (Figs. S1–S5).

For some batter food systems (some cakes and pancakes), soft wheat flour is treated with chlorine gas to improve its color and baking quality (Finnie et al., 2006; Montzheimer, 1931). The starch granule properties are considerably altered because of chlorination (Johnson et al., 1980; Sollars, 1958). Seguchi (1987) attributed the starch granule property change to an increase in hydrophobicity of the starch granule surface. Because the results of the current study show that starch from the soft-textured wheat samples contained significantly greater amounts of polar lipids, the increase in starch granule surface hydrophobicity in chlorinated flour might be caused by a change in the polar lipids. The change in hydrophobicity could be the result of a change in polar lipid structure (e.g., physical removal of the polar components) or by an indirect change, in which the non-polar components (acyl groups) of the polar lipid become more accessible for association. Even though the results of the current study provide possible insight into the effects of chlorination on the starch granule surface lipids, more research is needed to fully determine how chlorination of wheat flour alters the properties of the starch granule surface especially because, other research has shown that the improving effect of chlorine gas on starch granules is due to the oxidative depolymerization of starch molecules in the granule (Johnson et al., 1980).

3.3. *Resting time effect on starch surface polar lipids between the two isolation methods*

During starch isolation, the time in which the dough or batter was allowed to rest after initial hydration was different between the two methods. The samples prepared using the dough method were allowed to rest for 60 min after mixing, whereas the batter method samples were hydrated and rested for 10 min prior to starch isolation. To determine if the difference in resting time affected the starch granule surface lipids, a study was conducted using Alpowa flour with two starch isolation methods and three resting times (0, 30 and 60 min).

Various resting times produced different results depending on the isolation method (Fig. 2). The four major lipid classes in the batter-isolated starch exhibited increases in their class totals over time. When results were evaluated on a nmol/g basis, DGDG, MGDG and PC class totals increased significantly between 0 and 30 min, after which no further increase was observed. The LPC class totals increased over the 0, 30 and 60 min rests but were not statistically different from each other. When the same data were analyzed on the mol% of total basis, no difference was observed between resting times (Fig. 2). The lack of differences in the mol% of total data show that even though the actual amount of lipids increased from 0 to 30 min in the major polar lipid classes, each class increased in relatively equal proportions.

The four major lipid classes from the dough-isolated starch exhibited a different response to time than did those from the batter-isolated starch. On a nmol/g basis, LPC, the most predominant polar lipid in the dough-isolated starch, increased between 0,

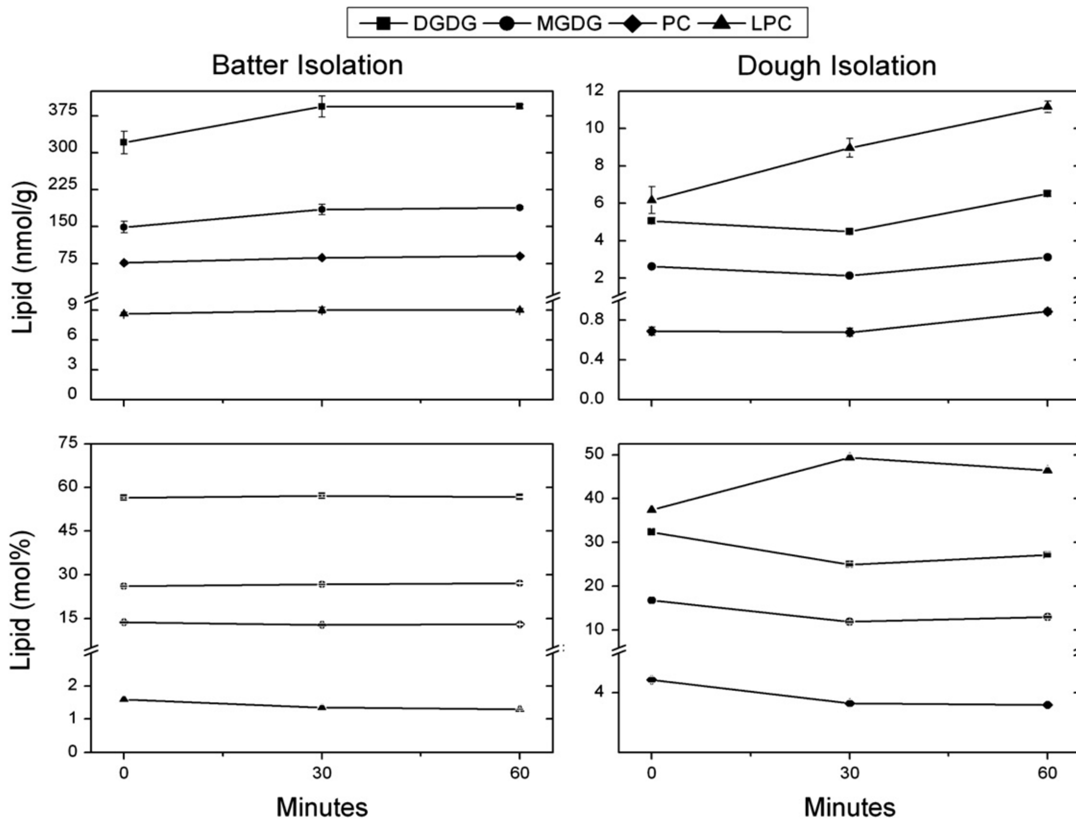

Fig. 2. Major polar lipid classes (digalactosyldigylceride, monogalactosyldigylceride, phosphatidylcholine, and lysophosphatidylcholine) in starch isolated from Alpowa flour using the batter and dough methods with three different resting periods (0, 30 and 60 min) prior to fractionation. Values are means and ±SE of two starch isolation replicates with five lipid extractions each ($n = 10$). Top graph represents data in nmol/g, and bottom graph represents data in mol% of total.

Table 4
Protein mean values and *P*-values from one-way ANOVA for wheat endosperm hardness source of variation in the various wheat fractions.

Cultivar/experimental line	Hardness class	Protein (%)			
		Meal	Flour	Dough-isolated starch	Batter-isolated starch
Bobwhite	Hard	14.32 ± 0.00	13.51 ± 0.01	0.25 ± 0.04	0.36 ± 0.02
BW2	Soft	14.40 ± 0.02	13.00 ± 0.03	0.26 ± 0.03	1.42 ± 0.28
Alpowa	Soft	10.12 ± 0.05	8.84 ± 0.04	0.25 ± 0.02	0.75 ± 0.20
Alpowa/hard-nil	Hard	10.74 ± 0.05	8.92 ± 0.00	0.22 ± 0.02	0.50 ± 0.09
Hi-Line	Hard	15.07 ± 0.04	13.98 ± 0.08	0.27 ± 0.06	0.57 ± 0.06
HGAB18	Soft	15.17 ± 0.01	12.70 ± 0.03	0.29 ± 0.03	1.52 ± 0.28
Source of variation		*F*-value			
Hardness		ns	ns	ns	16.92

Value represent mean protein % of samples and ±SE. Meal and flour samples are on a '14 % moisture' basis, whereas the starch samples are on an 'as is' basis. *F*-values are significant at $P = 0.001$; ns, not significant.

30 and 60 min (6.2–11.2 nmol/g) (Fig. 2). DGDG, MGDG and PC had an initial significant decrease between 0 and 30 min followed by a significant increase between 30 and 60 min (Fig. 2) (5.0–6.5, 2.6–3.1 and 0.7–0.9 nmol/g, respectively). The mol% of total value shows an initial increase between 0 and 30 min for the LPC lipids and an initial decrease in DGDG, MGDG and PC. After this point, the mol% of total LPC decreased and the mol% of total for DGDG, MGDG and PC increased. These results provide evidence that the starch surface lipids (DGDG, MGDG and PC) are removed during dough development and that the internal starch lipids (LPC) are not affected.

Even though the results for the resting time study showed differences caused by resting time, the differences were minor compared with the variation between the starch isolation methods. For future work evaluating starch surface lipids, use of the batter method with a 30-min resting period is recommended.

3.4. *Effect of starch isolation method on starch surface proteins*

A study was conducted to determine if the starch granule surface proteins (puroindoline A and B) exhibited the same response between the two starch isolation methods. The same starch samples used in the lipid extractions for Bobwhite and BW2 were used to extract puroindoline proteins and measure their relative abundance via SDS-PAGE gels (Fig. S6). The results showed that starch isolated using the batter method contained more puroindoline proteins than did starch isolated using the dough method. The results between the soft- and hard-textured samples were expected; the starch from the soft-textured wheat (BW2) contained greater puroindoline proteins than did the Bobwhite starch.

The results from the SDS-PAGE were further evaluated by analyzing the various sample fractions for total protein (Table 4). The whole-meal and flour fractions contained total protein ranges of 10.1–15.2 and 8.8–14.0%, respectively (Table 4). Hardness class was not a significant source of variation for the whole-meal and flour fractions. Within the starch isolated fractions, the dough-isolated starch ranges were from 0.22 to 0.29% and the batter-isolated starch ranges were from 0.36 to 1.52% (Table 4). Hardness class did not contribute to the variation in total protein in the dough-isolated starch, however, hardness class was a significant source of variation in the batter-isolated starch (Table 4). The batter-isolated starch from the soft-textured wheat flour contained greater amounts of total protein than did the starch isolated form hard-textured wheats.

4. Conclusions

This research provides evidence that as dough is mixed to optimum, lipids on the surface of wheat starch granules become incorporated into the gluten phase of the dough, whereas in a batter, the starch surface lipids stay associated with the granule surface. The puroindoline proteins located on the surface of starch granules also are removed during the mixing of dough. Consequently in lower water systems where gluten develops (i.e., doughs), the partitioning forces for both lipids and puroindolines are greater in gluten compared to the starch granule surface. This research also indicates that the predominant starch surface polar lipids are di-acyl lipids of DGDG (36:4), MGDG (36:4) and PC (34:2 and 36:4) polar lipid classes, and the predominant internal starch polar lipids are LPC (16:0 and 18:2) lipids. We recommend that future studies evaluating starch surface components use a starch isolation method that involves no (or slight) gluten formation. We recommend further use of the batter method involving a 30-min resting period as described in the methods section.

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Appendix A. Supplemental material

Supplementary information for this manuscript can be downloaded at doi:10.1016/j.jcs.2009.09.007.

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