



Efficient measurement of amylose content in cereal grains

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ABSTRACT

Rapid and economical measurement of amylose content in barley is important for genetic study and breeding improvement of this trait. Seventeen genotypes with a wide range of amylose contents were used to compare the amylose measurement accuracy of the cost-effective iodine–potassium iodide (I:KI) method to the commercially available enzyme-based Megazyme protocol. Comparable accuracy from I:KI was demonstrated in low amylose samples (below 10% dried base), as were limitations in regular or high amylose samples. To address the major cost of sample preparation in nutritional trait analysis, the I:KI method was also employed for amylose detection from β -glucan and free phosphate assay samples. Results indicated that samples used in β -glucan and phosphate assays could be further utilized for amylose measurement. Amylose detection accuracy using I:KI method from those assayed samples is comparable to that using the Megazyme method in low and regular amylose samples. Development of protocols for the double assays from one grain sample will significantly reduce the labor cost associated with sample preparation and will streamline the screening of early generation barley populations, where seed sample amounts are limited.

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1. Introduction

Starch is a major nutritional component in plant grains consisting primarily of amylose and amylopectin. Amylose is a linear polymer of α -(1 \rightarrow 4) linked D-glucose units with few side chains. In contrast, amylopectin has many α -(1 \rightarrow 6) linked glucose side chains attached to the main α -(1 \rightarrow 4) polymer. Regular grains contain about 20% amylose while waxy grains contain a much lower percentage. The chemical properties of the less-branched amylose molecule contribute to its nutritional value. Amylose is digested more slowly, providing beneficial effects on human health. Recent clinical studies indicate that amylose is important in reducing the glycemic and insulin impact of foods (Behall and Scholfield, 2005) and in increasing the body's fat burning ability which may help to maintain a healthy weight (Higgins et al., 2004). Scientific evidence of the health benefits may promote a demand for high amylose grains in future markets.

Improvement of the amylose content in barley requires a better understanding of the genetic mechanism controlling amylose metabolism, although variations in amylose content are affected by both genetic and environmental factors. The best known example

of a genetic effect on amylose content is the presence of 'waxy' mutations in crops including wheat (Nakamura et al., 1995; Yasui et al., 1997), barley (Bhatty and Rosnagel, 1997; Ishikawa et al., 1995), maize (Weatherwax, 1922), and rice (Murata et al., 1965). The common characteristic of these grain waxy mutants is low amylose content. Molecular evidence has confirmed that granule-bound starch synthase (GBSSI) is so far the sole gene responsible for the waxy mutations in different crop species (Patron et al., 2002; Vrinten and Nakamura, 2000; Wessler and Varagona, 1985). More studies with amylose mutations revealed that other genes also affect amylose content. Mutations in the starch synthase gene resulted in higher amylose content in barley (Morell et al., 2003), wheat (Yamamori et al., 2000), and maize (Gao et al., 1998). More recently, extremely high amylose wheat lines generated from RNA interference technology clearly demonstrated the function of the starch synthase gene in amylose metabolism (Regina et al., 2006). Another high amylose barley mutant of *amo 1* has been mapped to barley chromosome 1H (Schondelmaier et al., 1992; Swanston et al., 1995). Yield comparisons between high amylose barley and the corresponding wild-type lines have not been made. However, the association of high amylose with some undesirable traits such as decreased total starch and seed weight has been reported (Regina et al., 2006; Swanston et al., 1995). The genetic complexity of amylose metabolism provides opportunities for breeding high amylose content coupled with good agronomic characteristics.

Genetic studies and breeding for the amylose content require a rapid, inexpensive measurement method. Current measurement

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methods include the commercially available Megazyme assay method, which specifically precipitates amylopectin (Gibson et al., 1997), and colorimetric measurements such as the iodine–potassium iodide assay. The Megazyme amylose/amylopectin protocol is a well accepted technique. The drawback, in addition to the relative high cost per sample, is the difficulty of testing a large number of samples such as characterizing individuals in segregating populations or early generation breeding populations. Iodine–potassium iodide (I:KI) staining was first reported for amylose measurements in potato (Hovenkamp-Hermelink et al., 1988). It is a simple and rapid detection technique. Washington et al. (2000) compared the two methods in testing five waxy barley lines for amylose content. Both Megazyme and I:KI methods showed similar results, indicating that they may be equally reliable. But the sample number and the amylose content range of the tested lines were limited.

In addition to the chemical cost, sample preparation is another major cost consideration because of the labor intensive operations involved during seed cleaning, milling and weighing. Sample preparation is a common step for all the nutritional trait analyses in grains. Until a low-cost sample preparation method is available, the combination of nutritional trait assays using the same prepared samples is a good alternative way to reduce labor costs. In this paper, we report: (1) assays combining β -glucan and free phosphate sample preparations with amylose content determination; and (2) evaluation of I:KI amylose measurement across a range of barley genotypes.

2. Materials and methods

2.1. Plant materials

Seventeen barley genotypes and two starch controls were selected. Eight genotypes were the widely grown cultivars Azhul, Baronesse, CDC Alamo, Glacier, Harrington, Morex, Steptoe and Waxbar. Five genotypes were cultivars and breeding lines from the barley improvement program in Aberdeen, Idaho, and included Lenetah, Tetonia, 01AH2812, 03AH2214, and 03AH3043. The remaining test lines were mutations identified in our laboratory. The potato starch control was purchased from Sigma–Aldrich (St. Louis, MO) and the 70% amylose control was included in the assay kit from Megazyme International, Ireland. With the exception of Glacier, all the seeds were harvested from 2007 field increases grown in Aberdeen, Idaho.

2.2. Flour sample preparation

Thirty to 50 seeds were selected from each barley genotype and ground to pass a 0.5-mm screen using a laboratory cyclone mill (Udy Corporation, Fort Collins, CO). The mill was cleaned between samples.

2.3. β -glucan analysis

The enzymatic measurements of mixed-linkage β -glucan were performed using commercially available components (Megazyme Ireland International, Ltd., Bray, Ireland). In brief, the streamlined procedure of Megazyme (McCleary and Codd, 1991) calls for 80–120 mg of milled flour to be mixed with 200 μ l 50% (v/v) ethanol and 4 ml sodium phosphate buffer (20 mM, pH 6.5). This mixture is incubated with lichenase (200 μ l, 50 U/ml), then the enzymatic reaction is stopped with 5 ml sodium acetate buffer (200 mM, pH 4.0). After centrifugation, a 100 μ l aliquot is reacted to completion with 100 μ l β -glucosidase and the amount of glucose is

detected with 3 ml glucose oxidase/peroxidase GOPOD developing reagent.

2.4. Phosphate measurement

The single seed free phosphate assay was based on the protocol described by Bregitzer et al. (2008) using 96-well assay format. Both milled grain flour and crushed seed were tested.

2.5. Amylose measurement methods

The Megazyme amylose/amylopectin assay procedure, utilizing the commercial kit (Megazyme Ireland International, Ltd., Bray, Ireland) was followed according to the manufacturer's recommendation.

The iodine–potassium iodide method was adapted for high throughput testing from the protocol description in a previous report (Washington et al., 2000). Sample size and chemical amounts were reduced to conform with a 96-well assay. Absorbance values were measured at 620 and 535 nm using a Synergy HT auto-reader (Bio Tek Instrument, Inc., VT).

2.6. Amylose measurement using β -glucan assayed samples

The iodine–potassium iodide (I:KI) procedure was combined with β -glucan determinations by utilizing the remnant precipitated sample from the Megazyme mixed-linkage streamlined protocol. Following centrifugation, the supernatant was decanted and reserved as a backup to confirm β -glucan results, if necessary. The precipitate from the centrifuged sample was immediately resuspended in 1 ml of distilled water, which is the initial step in the I:KI amylose determination procedure. One ml of 2 M NaOH was added to extract the starch. After 30 min at room temperature, 2 ml of 1 M HCl neutralized the reaction and the suspension was diluted by adding 6 ml of distilled water. The extraction was further diluted 1:20 in cluster tubes arrayed in a 96-well format by aliquoting 50 μ l of sample into 950 μ l of distilled water, with gentle aspiration to mix. Potato starch and amylose controls were added to the assay set.

For amylose detection, 100 μ l of a 1:8 dilution of 0.3%I:1.5%KI stock solution was placed in each reaction well in a 96-well assay plate, 25 μ l of the diluted sample was added with gentle aspiration, and the plate was held at room temperature 5–10 min to allow for color development. A blank consisting of I:KI working solution and the test reagents was included. The assay plate was read at 620 and 535 nm in a BioTek Synergy HT plate reader using the KC4 program. The absorbance ratio of 620:535 estimates amylose content by categorizing samples into very low, waxy, normal, intermediate or high amylose levels. The amylose percentage was calculated from the formula $1.4935 * \exp^{(2.7029 * (OD_{620}/OD_{535}))}$ as determined by Washington et al. (2000) from comparisons with the Megazyme amylose/amylopectin detection assay.

2.7. Amylose measurement using free phosphate assayed samples

The single kernel assay for free phosphate has been developed for use in a 96-well format (Raboy et al., 2000). Single barley seed was weighed, placed into either cluster tubes or sample wells in heavy-duty assay racks, and then crushed with fitted adapters and a hydraulic press. Ten μ l of 0.4 M HCl per mg of tissue is added and the samples are refrigerated overnight to allow for extraction. From this extract, 10 μ l is transferred to assay plates for free phosphate determination using Chen's reagent. The remainder of the extract is discarded.

The residue extract, still in the original assay racks, was used for amylose content estimation. The pH of the extract was adjusted by the addition of 300 μ l of 2 M NaOH with aspiration to mix, then the suspension was held at room temperature for 30 min to allow for starch extraction. The reaction was neutralized with 200 μ l of 1 M HCl and the mixture was diluted with 100 μ l of distilled water. The extraction was further diluted 1:10 in cluster tubes, maintaining the 96-well format.

Amylose detection followed the standard I:KI protocol. Absorbances were read at 620 and 535 nm and the ratios were used to estimate amylose content and to calculate the percentage of amylose.

2.8. Statistical analysis

Results from the 17 barley genotypes and starch controls were used to perform statistical analysis to compare the amylose detecting accuracy between methods. All the tests were repeated at least twice in the enzymatic protocol and I:KI procedures. Correlation analyses were conducted with JMP 6.0 software (SAS Institute Inc., Cary, NC).

3. Results

3.1. Comparison between Megazyme and I:KI methods for the accuracy of amylose measurement

To compare amylose measurements from Megazyme and I:KI protocols (Washington et al., 2000), 17 barley genotypes with a wide range of amylose levels plus two starch controls were analyzed. Results indicated that the I:KI and Megazyme methods are comparable for the samples whose amylose is low (Table 1). The results from three low amylose samples, including Waxbar, 01AH2812, 03AH2214, M38 showed the same measurements in both I:KI and Megazyme methods. For other three low amylose samples of Azhul, CDC Alamo, and 03AH3043, the amylose content detected from I:KI is higher than that from Megazyme but the absolute values are within 5% variation. The remaining regular

amylose lines (about 20%), including Baronesse, Harrington, Lenetah, Morex, Steptoe, Tetonia, mutant CM13 and mutant M351, the I:KI method gave significantly lower values to the Megazyme assay. Absolute amylose values in those regular amylose lines differed 9.0–12.2% from two methods (Table 1). However, for the high amylose samples of Glacier and Harrington mutant M436, the amylose measurements are considerably lower with more than 15% using the I:KI method (Table 1). The amylose control sample, with a widely accepted amylose content of 70–74%, is the check supplied with the Megazyme kit. Our repeated results showed that amylose quantitative measurements gained from the I:KI protocol in regular and high amylose lines (above 20% with Megazyme measurement) tended to be low. The current report from systematic comparisons between the two methods using a relatively large number of samples provides strong evidence that the I:KI method may be used in early generation selection or segregation population analysis where plants are simply categorized as normal or low amylose. However, caution is advised concerning the precise measurement in regular or high amylose materials. Megazyme procedure may be needed to confirm the regular or high amylose measurements when they are concerned.

3.2. Amylose measurement from β -glucan assay samples using the I:KI method

Limited amounts of seed for biochemical assays are available in early breeding generation and mutant population lines. Assay cost should be reduced if the same sample can be used for multiple trait assays. We explored the possibility of using the same flour sample for both β -glucan and amylose measurements. Barley flour samples were first assayed for β -glucan content and the residues were tested for amylose using the I:KI method. The amylose levels from the dual assay correlated closer with the Megazyme results than did those of the same lines assayed for amylose alone using the same detection technique. The detailed comparison showed that amylose measurements were overall higher in most samples using β -glucan assayed samples than that using the flour samples (Table 2). One possible explanation is that β -glucan degradation may have contributed to better extraction of the amylose from the solution, or

Table 1

Amylose percentage measurement comparison between the iodine–potassium iodide and the Megazyme amylose/amylopectin methods for 17 barley and two starch controls. I: KI represents the amylose measurements directly from flour samples using the iodine–potassium iodide protocol. Megazyme indicates the percentage amylose detected with the Megazyme amylose/amylopectin assay kit. Values presented are the means of four test condition replications. Total starch contents were measured from flour samples using the Megazyme Kit and presented as the percentage in dry basis.

Genotype	I:KI	Megazyme	Total starch
Azhul	6.6 \pm 0.1	2.8 \pm 0.0	46.57 \pm 3.1
Baronesse	18.8 \pm 0.7	27.8 \pm 2.4	51.31 \pm 0.8
CDC Alamo	7.4 \pm 0.3	3.4 \pm 0.7	44.99 \pm 0.2
Glacier	19.4 \pm 0.5	38.5 \pm 2.3	47.36 \pm 0.8
Harrington	14.3 \pm 0.9	23.4 \pm 0.9	44.79 \pm 0.6
Lenetah	16.9 \pm 0.8	24.7 \pm 2.5	56.61 \pm 0.8
Morex	13.9 \pm 0.2	24.1 \pm 2.7	57.72 \pm 2.7
Steptoe	11.2 \pm 0.7	23.4 \pm 2.3	40.78 \pm 2.3
Tetonia	15.8 \pm 0.0	25.0 \pm 3.2	56.74 \pm 0.2
Waxbar	8.5 \pm 0.6	9.6 \pm 0.5	57.18 \pm 2.6
01AH2812	8.2 \pm 0.2	8.1 \pm 0.1	53.69 \pm 0.9
03AH2214	7.9 \pm 0.2	9.4 \pm 1.4	53.83 \pm 0.3
03AH3043	7.2 \pm 0.5	3.6 \pm 0.2	54.79 \pm 2.0
CM13	11.5 \pm 1.3	20.9 \pm 2.9	43.53 \pm 0.3
M38	8.0 \pm 0.3	11.1 \pm 0.4	35.59 \pm 2.2
M351	13.9 \pm 1.5	24.3 \pm 0.8	44.84 \pm 1.5
M436	14.5 \pm 0.6	35.9 \pm 2.2	49.17 \pm 2.8
Potato starch ck	23.4 \pm 0.2	27.4 \pm 4.1	94.91 \pm 0.2
Amylose ck	61.2 \pm 3.2	73.8 \pm 3.2	94.25 \pm 0.2

Table 2

Amylose percentage comparisons for various sample sources using the iodine–potassium iodide colorimetric assay of 17 barley lines and two starch controls. The I:KI designation indicates that amylose measurements were made directly from flour samples specifically prepared for amylose testing. I:KI BGp describes amylose levels from β -glucan assay precipitated residue. I:KI Lpa represents the amylose measurements using the residual extract from the free phosphate protocol. Values presented are the means of four test condition replications.

Genotype	I:KI	I:KI BGp	I:KI Lpa
Azhul	6.6 \pm 0.1	7.5 \pm 0.5	7.3 \pm 0.2
Baronesse	18.8 \pm 0.7	22.1 \pm 1.4	23.1 \pm 0.5
CDC Alamo	7.4 \pm 0.3	13.4 \pm 0.7	7.4 \pm 0.6
Glacier	19.4 \pm 0.5	29.6 \pm 1.3	28.2 \pm 0.8
Harrington	14.3 \pm 0.9	20.3 \pm 0.9	19.2 \pm 0.5
Lenetah	16.9 \pm 0.8	22.3 \pm 1.5	21.4 \pm 0.4
Morex	13.9 \pm 0.2	21.3 \pm 0.7	27.0 \pm 0.7
Steptoe	11.2 \pm 0.7	19.6 \pm 1.3	22.6 \pm 0.4
Tetonia	15.8 \pm 0.0	24.9 \pm 1.2	21.3 \pm 0.3
Waxbar	8.5 \pm 0.6	9.3 \pm 0.5	8.8 \pm 0.2
01AH2812	8.2 \pm 0.2	9.7 \pm 0.1	8.7 \pm 0.2
03AH2214	7.9 \pm 0.2	9.6 \pm 1.4	8.5 \pm 0.2
03AH3043	7.2 \pm 0.5	8.5 \pm 0.2	7.6 \pm 0.3
CM13	11.5 \pm 1.3	19.0 \pm 2.9	18.5 \pm 0.5
M38	8.0 \pm 0.3	15.1 \pm 0.4	11.8 \pm 0.4
M351	13.9 \pm 1.5	20.4 \pm 0.8	21.4 \pm 0.6
M436	14.5 \pm 0.6	24.8 \pm 2.2	25.8 \pm 0.3
Potato starch ck	23.4 \pm 0.2	23.8 \pm 1.1	28.9 \pm 0.4
Amylose ck	61.2 \pm 3.2	69.5 \pm 1.2	66.5 \pm 1.2

chemically enhanced the amylose binding to the I:KI complex. The significantly different results between I:KI and Megazyme in high amylose samples need to be investigated further.

3.3. Amylose measurements from free phosphate assay samples

Another important nutritional trait in barley is low phytic acid (Lpa). The *lpa* mutation in barley grains resulted in a high content of inorganic phosphorus that is beneficial for animal nutrition and for the environment (Leytem et al., 2004). Barley lines with *lpa* genes have been released (Bregitzer et al., 2008). Therefore measurement of free phosphate may become a routine laboratory test for barley nutrition trait improvement. The successful combination of amylose and phosphate tests using the same sample preparation is cost-effective in each biochemical assay. We compared the corresponding amylose levels from the *lpa* samples undergoing free phosphate testing to direct amylose measurements using the I:KI protocol. The overall patterns of amylose detection from free phosphate flour residues are similar to those of β -glucan residue precipitate and are higher than from direct flour measurement (Table 2). Comparisons of the amylose values generated from three sample sources for each line clearly demonstrated that comparable values were obtained between samples taken from the β -glucan and phosphate assays, even in the high amylose samples (Table 2). Amylose percentages calculated from I:KI assay of β -glucan and free phosphate residue precipitated flour compare favorably with amylose results from the Megazyme method (Fig. 1). The amylose measurement accuracy of I:KI technique using samples from both β -glucan and free phosphate assays are actually comparable to the Megazyme method in all low and normal amylose lines (below 25%) (Fig. 1). The only exception is the two high amylose lines of Glacier and M436. Results prove that colorimetric amylose level measurement can be combined with either β -glucan or free phosphate determinations, utilizing a single sample preparation, and that the results from dual assays approximate those from

commercially available enzyme-based assays (Fig. 1). The better amylose measurement accuracy from the dual assays is an interesting discovery. For the materials with low or regular amylose content, I:KI technique could replace the Megazyme method in combination with either of the β -glucan or phosphate assays.

3.4. Statistical analysis of amylose measurements between different methods

To test the statistical relationships in amylose detection techniques used in the studies, we analyzed correlation among methods and samples using 17 genotypes. The results showed that there are strong correlations between methods and among the sample preparations (Table 3). The best correlation with Megazyme methods is the I:KI using Lpa residue sample that gave correlation co-efficiency of 0.97–0.98 in two correlation analysis methods (Table 3). The next best followed by I:KI using β -glucan residue with 0.91 and I:KI using flour with 0.87–0.89 (Table 3). The results further confirmed that the I:KI using residue samples from β -glucan and *lpa* assays are better than I:KI using flour samples. The statistical analysis provided scientific basis for dual assays from a single sample preparation. To further test whether the variations from the I:KI method in higher amylose samples are actually due to the effect of different starch contents, we measured total starch in all samples (Table 1). Correlation analyses showed that correlation co-efficiency between amylose measurement using the I:KI method and total starch contents is 0.1574, indicating that there was no significant effect of starch content on the amylose measurements in the samples.

3.5. Amylose measurement from the crushed seed sample preparation

The crushed seed technique is popularly used to test barley for free phosphate (Bregitzer et al., 2008). The 96-well format provides a platform for high throughput assays. To align with this sample

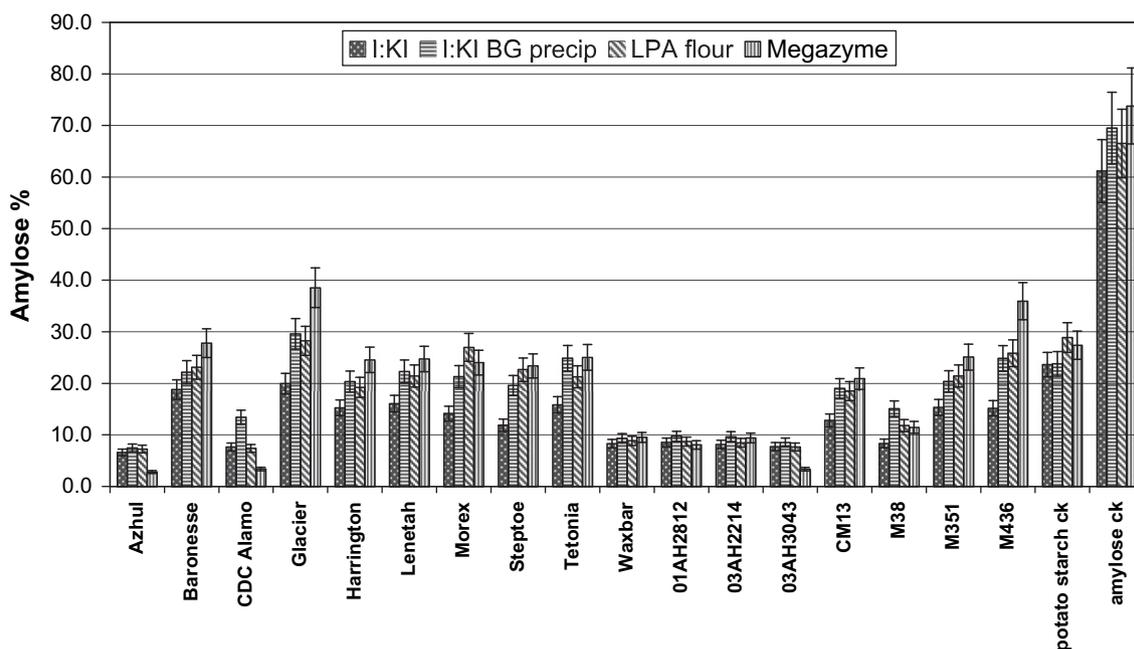


Fig. 1. Amylose percentage measurement comparison between the iodine–potassium iodide (I:KI) and the Megazyme amylose/amylopectin methods for 17 barley and two starch controls. I:KI represents the amylose measurements directly from flour samples using the iodine–potassium iodide protocol. I:KI BG precip. shows the percentage of amylose recovered from β -glucan assayed sample residues using the I:KI method. Lpa flour represents the I:KI amylose measurements using the residual extract from the free phosphate protocol. Megazyme indicates the percentage amylose detected with the Megazyme amylose/amylopectin assay kit. Values presented are the means of four test condition replications.

Table 3

Statistical analysis for amylose measurements between methods and sample preparations. The values reported in the table represent the correlation co-efficiency between two methods or sample preparations. I:KI represents the direct flour test. I:KI BGP represents the test using β -glucan assayed residue samples. I:KI Lpa represents the test using low phytate assayed samples.

Spearman's rank correlation	I:KI	I:KI BGP	Megazyme	I:KI Lpa
I:KI	1.00			
I:KI BGP	0.91	1.00		
Megazyme	0.87	0.91	1.00	
I:KI Lpa	0.84	0.87	0.97	1.00
Linear correlation				
I:KI	1.00			
I:KI BGP	0.93	1.00		
Megazyme	0.89	0.91	1.00	
I:KI Lpa	0.91	0.94	0.98	1.00

preparation pattern, we tested the possibility of using the residue crushed seed for amylose measurement after the extraction aliquots were removed for phosphate analysis. Eight genotypes, representing normal and low amylose lines, were used to compare crushed seed with flour samples in identical test setups. There was little difference between the two methods for the four low amylose genotypes, while greater variation between two methods was apparent with the four normal amylose genotypes (Fig. 2). We concluded that crushed seed may be substituted for flour in experiments that detect only relative differences between low and high amylose levels.

4. Discussion

Early generation of barley breeding selections and genetic mapping call for rapid, high throughput, and economical techniques for assaying important nutritional trait levels. Quantitative chemistry protocols, are considered accurate but not practical for rapidly screening a large number of samples. Colorimetric assay procedures, which do not have the specificity of enzyme-based methods, can nonetheless detect chemical levels that approximate the determinations from high cost quantitative assay kits. Even though the iodine–potassium iodide colorimetric method has been used (Haase, 1993; Hovenkamp-Hermelink et al., 1988; Swanston et al., 1995), systematic comparisons to the Megazyme protocol, using genotypes representing a wide range of amylose content, have not been reported previously. Washington et al. (2000) did

a direct comparison between two methods in barley, but only six low amylose genotypes were used in that study. In this report we have systematically compared the accuracy of amylose measurements between I:KI and the Megazyme enzyme-based assay methods. For low amylose samples ($\leq 10\%$ by our definition), measurements were the same using either method. For normal (approximately 20%) or high ($\geq 25\%$) amylose materials, the values obtained with the I:KI method were lower. We were able to achieve higher values for amylose percentage for the high amylose genotypes when samples were diluted 1:3 and assayed with I:KI (data not shown). Although the differences are statistically significant in some samples, the absolute values are not large enough to change the category of amylose contents from high to low. Amylose content in barley differs from other nutritional traits such as β -glucan, since only differences of 5% or bigger may be significant from a practical viewpoint, because small differences in amylose contents have negligible effect on nutritional value in food or feed and environmental factors can easily cause differences of 5% according to our field experiment record. Based on our results, caution is needed when testing high amylose materials with the I:KI method because of the lower values when compared to the Megazyme method. The chemical composition of high amylose grains may affect either the efficient extraction of starch or the binding efficiency of the iodine–potassium iodide with the amylose molecules. These hypotheses are supported by the data from β -glucan residue samples (Fig. 1). After β -glucan measurement, the amylose content from the same genotypes tended to be higher compared to direct measurements of flour (Fig. 1). This result indicates that the β -glucan detection process either helped to release amylose or removed components affecting the binding between amylose and I:KI. Physical changes might also be responsible for more efficient starch extraction. The sample suspensions seem more uniform for β -glucan residues than for flour that has not been previously assayed. Scientific evidence is required for the suspension differences in the two samples and their contribution to amylose detection. Regardless of the lower trend for amylose measurement, the iodine–potassium iodide method is still a practical and useful technique for the detection of amylose content in early generation selection from large number of samples because of the much lower cost when compared to commercially available kits, particularly combining with one of the two assays mentioned above. Enzyme-based quantitative assays such as the Megazyme protocol are useful for the confirmation of high amylose materials. Washington et al. (2000) concluded that the accuracy of determining amylose content for very low amylose types was limited in the enzyme-based procedure. During our use

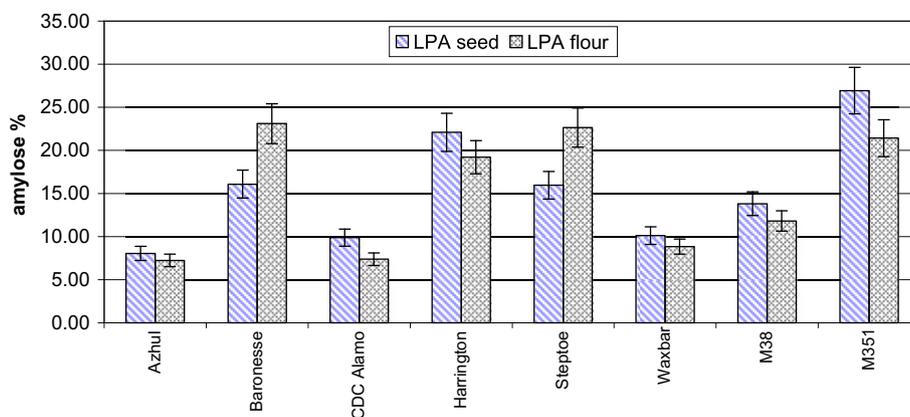


Fig. 2. Amylose percentage comparisons of sample sources in the free phosphate assay for eight barley lines. Lpa seed indicates that crushed single seeds were prepared for extraction and phosphate testing first, followed by amylose determination using the iodine–potassium iodide method (I:KI). Lpa flour represents the extraction of flour samples for phosphate testing, followed by amylose measurement using the I:KI method.

of the Megazyme procedure, we found that low amylose lines tended to form insoluble gel clumps that might hinder accurate amylose determination, causing the amylose content to be underestimated. In addition to supporting the previous results for I:KI in amylose measurement (Washington et al., 2000), a key point of our results was the discovery of apparent under estimation of amylose content with the I:KI method in genotypes with regular or high amylose content.

Another purpose of this study was to find possible ways to reduce the cost of amylose assays for barley. The I:KI method makes use of chemicals commonly found in the lab and costs only a few cents per sample to perform or only about 5% of the enzyme method. Since nutritional traits have to be analyzed from the grain seed, sample preparation, including cleaning, milling, and weighing, is very labor intensive. Using a single sample for more than one analysis is an economically advantageous way to reduce the costs. In this report, we have confirmed that amylose measurements can be consolidated with either β -glucan or free phosphate analysis. Beta-glucan is becoming more important for human health due its cholesterol-lowering role in food (Brown et al., 1999; Keenan et al., 2007) as well as unfavorable traits for malting and feed utilizations (McNab and Smithard, 1992; Vis and Lorenz, 1997). Low phytic acid has been introduced into barley cultivars for better nutritional value and more environment-friendly effects (Bregitzer et al., 2008). Barley amylose content may become more important in terms of human health benefit and recent emphasis on alternatives to corn for biofuel production. Improving the β -glucan and amylose content and reducing phytate contents is attracting more attention in both barley genetic and breeding programs. Therefore, development of economically advantageous methods for those assays will greatly assist future research.

5. Conclusion

In this study, we compared the colorimetric iodine-potassium iodide and the enzyme-based Megazyme methods for amylose detection. Results showed that amylose percentages determined by iodine-potassium iodide (I:KI) are comparable to those from Megazyme in low amylose samples, but significantly lower for the regular or high amylose samples. Amylose measurement from β -glucan or free phosphate assayed barley grain samples using I:KI is comparable to the enzyme-based method in most cases. The successful adaptation of dual assays with other nutritional trait analyses, such as β -glucan and phosphate, will significantly reduce the labor costs associated with sample preparation. New protocols with lower costs for both chemicals and labor should make the analysis of large numbers of samples for those nutritional traits more affordable and practical. It should be a good supplement protocol to the existing ones with high accuracy, high cost, and low throughput features for early generation selection of amylose in breeding program.

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References

- Behall, K.M., Scholfield, D.J., 2005. Food amylose content affects postprandial glucose and insulin responses. *Cereal Chem* 82, 654–659.
- Bhatty, R.S., Rosnagel, B.G., 1997. Zero amylose lines of hullless barley. *Cereal Chem* 74, 190–191.
- Bregitzer, P.P., Raboy, V., Obert, D.E., Windes, J., Whitmore, J., 2008. Registration of 'Clearwater' low-phytate hullless spring barley. *J. Plant Regist* 2, 1–4.
- Brown, L., Rosner, B., Willett, W.W., Sacks, F.S., 1999. Cholesterol-lowering effects of dietary fiber: a meta-analysis. *Am. J. Clin. Nutr* 69, 30–42.
- Gao, M., Wanat, J., Stinard, P.S., James, M.G., Myers, A.M., 1998. Characterization of dull1, a maize gene coding for a novel starch synthase. *Plant Cell* 10, 399–412.
- Gibson, T.S., Solah, V.A., McCleary, B.V., 1997. A procedure to measure amylose in cereal starches and flours with concanavalin A. *J. Cereal Sci* 25, 111–119.
- Haase, N.U., 1993. A rapid test procedure for estimating the amylose content of pea starch. *Plant Breed* 111, 325–329.
- Higgins, J.A., Higbee, D.R., Donahoo, W.T., Brown, I.L., Bell, M.L., Bessesen, D.H., 2004. Resistant starch consumption promotes lipid oxidation. *Nutr. Metabol* 1, 8.
- Hovenkamp-Hermelink, J.H.M., DeVries, J.N., Adamse, P., Jacobsen, E., Witholt, B., Feenstra, W.J., 1988. Rapid estimation of the amylose/amylopectin ratio in small amounts of tuber and leaf tissue of the potato. *Potato Res* 31, 241–246.
- Ishikawa, N., Ishihara, J., Itoh, M., 1995. Artificial induction and characterization of amylose-free mutants of barley. *Barley Genet. Newsl* 24, 49–53.
- Keenan, J.M., Goulson, M., Shamlivan, T., Knutson, N., Kolberg, L., Curry, L., 2007. The effects of concentrated barley beta-glucan on blood lipids in a population of hypercholesterolaemic men and women. *Br. J. Nutr* 97, 1162–1168.
- Leytem, A.B., Turner, B.L., Thacker, P.A., 2004. Phosphorus composition of manure from swine fed low-phytate grains: evidence for hydrolysis in the animal. *J. Environ. Qual* 33, 2380–2383.
- McCleary, B.V., Codd, R., 1991. Measurement of (1 → 3, 1 → 4)- β -D-glucan in barley and oats: a streamlined enzymatic procedure. *J. Sci. Food Agric* 55, 303–312.
- McNab, J.M., Smithard, R.R., 1992. Barley β -glucan: an antinutritional factor in poultry feeding. *Nutr. Res. Rev* 5, 45–60.
- Morell, M.K., Kosar-Hashemi, B., Cmiel, M., Samuel, M.S., Chandler, P., Rahman, S., Buleon, A., Batey, I.L., Li, Z., 2003. Barley sex6 mutants lack starch synthase IIa activity and contain a starch with novel properties. *Plant J* 34, 173–185.
- Murata, T., Sugiyama, T., Akazawa, T., 1965. Enzymatic mechanism of starch synthesis in glutinous rice grains. *Biochem. Biophys. Res. Commun* 18, 371–376.
- Nakamura, T., Yamamori, M., Hirano, H., Hidaka, S., Nagamine, T., 1995. Production of waxy (amylose free) wheats. *Mol. Gen. Genet* 248, 253–259.
- Patron, N.J., Smith, A.M., Fahy, B.F., Hylton, C.M., Naldrett, M.J., Rosnagel, B.G., Denyer, K., 2002. The altered pattern of amylose accumulation in the endosperm of low-amylose barley cultivars is attributable to a single mutant allele of granule-bound starch synthase I with a deletion in the 5'-non-coding region. *Plant Physiol* 130, 190–198.
- Raboy, V., Gerbasí, P.F., Young, K.A., Stoneberg, S.D., Pickett, S.G., Bauman, A.T., Murphy, P.P.N., Sheridan, W.F., Ertl, D.S., 2000. Origin and seed phenotype of maize *low phytic acid 1-1* and *low phytic acid 2-1*. *Plant Physiol* 124, 355–368.
- Regina, A., Bird, A., Topping, D., Bowden, S., Freeman, J., Barsby, T., Kosar-Hashemi, B., Li, Z., Rahman, S., Morell, M., 2006. High-amylose wheat generated by RNA interference improves indices of large-bowel health in rats. *Proc. Natl. Acad. Sci. USA* 103, 3546–3551.
- Schondelmaier, J., Jacobi, A., Fischbeck, G., Jahoor, A., 1992. Genetical studies on the mode on inheritance and localization of the amo1 (high amylose) gene in barley. *Plant Breed* 109, 274–280.
- Swanston, J.S., Ellis, R.P., Stark, J.R., 1995. Effects on grain and malting quality of genes altering barley starch composition. *J. Cereal Sci* 22, 265–273.
- Vis, R.B., Lorenz, K., 1997. Importance in brewing and methods of analysis. *Lebensm. Wiss. Technol* 30, 331–336.
- Vrinten, P.L., Nakamura, T., 2000. Wheat granule-bound starch synthase I and II are encoded by separate genes that are expressed in different tissues. *Plant Physiol* 122, 255–264.
- Washington, J.M., Box, A., Karakousis, A., Barr, A.R., 2000. Developing waxy barley cultivars for food, feed and malt. *Barley Genet.* VIII, 303–306.
- Weatherwax, P., 1922. A rare carbohydrate in waxy maize. *Genetics* 7, 568–572.
- Wessler, S.R., Varagona, M.J., 1985. Molecular basis of mutations at the waxy locus of maize: correlation with the fine structure genetic map. *Proc. Natl. Acad. Sci. USA* 82, 4177–4181.
- Yamamori, M., Fujita, S., Hayakawa, K., Metsuki, J., Yasui, T., 2000. Genetic elimination of a starch granule protein, SGP-1, of wheat generate an altered starch with apparent high amylose. *Theor. Appl. Genet* 101, 21–29.
- Yasui, T., Sasaki, T., Matsuki, J., Yamamori, M., 1997. Waxy endosperm mutants of bread wheat (*Triticum aestivum* L.) and their starch properties. *Breed. Sci* 47, 161–163.