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Short communication

Development of a 96-well plate iodine binding assay for amylose content determination☆

R.C. Kaufman[a], J.D. Wilson[a,*,1], S.R. Bean[a], T.J. Herald[a], Y.-C. Shi[b]

[a] USDA–ARS Center for Grain and Animal Health Research, 1515 College Ave, Manhattan, KS, United States
[b] Department of Grain Science and Industry, Kansas State University, Manhattan, KS, United States

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ABSTRACT

Cereal starch amylose/amylopectin (AM/AP) is critical in functional properties for food and industrial applications. Conventional methods of AM/AP are time consuming and labor intensive making it difficult to screen the large sample sets necessary for evaluating breeding samples and investigating environmental impact on starch development. The objective was to adapt and optimize the iodine binding assay in a 96-well plate format for measurement at both λ 620 nm and λ 510 nm. The standard curve for amylose content was scaled to a 96-well plate format and demonstrated R^2 values of 0.999 and 0.993 for single and dual wavelengths, respectively. The plate methods were applicable over large ranges of amylose contents: high amylose maize starch at 61.7 ± 2.3%, normal wheat starch at 29.0 ± 0.74%, and a waxy maize starch at 1.2 ± 0.9%. The method exhibited slightly greater amylose content values than the Concanavalin A method for normal type starches; but is consistent with cuvette scale iodine binding assays.

1. Introduction

Starch is synthesized and deposited in the endosperm of cereal grains to function as an energy reserve. In wild-type grains starch consists of two distinct polymers, amylose and amylopectin. Amylopectin is a large, highly branched polymer consisting of α-1,4 linked D-glucose units with branches linked by α-1,6 bonds. Amylose is a mostly linear polymer of α-1,4 linked D-glucose with a few α-1,6 branch points. In wild-type starches amylose content is usually in the 20–30% range, however mutants exist for several cereals that contain very high (>40%) and very low (0–15%) levels of amylose (Tester, Karkalas, & Qi, 2004).

The ratio of amylose to amylopectin is important to both the functionality and the nutritional properties of starch and starch based products. Amylose is important to the thermal characteristics of starch, such as gelatinization and pasting (Jane et al., 1999; Sasaki, Yasui, & Matsuki, 2000). The ratio of amylose:amylopectin also influences starch retrogradation, a major issue in the staling of food products (Hug-Iten, Escher, & Conde-Petit, 2003). Foods with a high amylose content have been shown to have a reduction in glycemic impact, which promotes many health benefits such as better control of diabetes and obesity (Behall & Scholfield, 2005).

There are currently several methods utilized for amylose content determination, ranging from high-performance size exclusion chromatographic techniques (Batey & Curtin, 1996; Chen & Bergman, 2007; Kennedy, Rivera, Lloyd, & Warner, 1992) to differential scanning calorimetry (Mestres, Matencio, Pons, Yajid, & Fliedel, 1996). The most commonly used methods are based on binding of either amylopectin or amylose with another compound. The method in which amylopectin is precipitated with Concanavalin A, developed by Yun and Matheson (1990) and modified by Gibson, Solah, and McCleary (1997), has recently increased in use due to advantages it possesses over other methods. The method can be commercially purchased as a kit and does not require a standard curve to quantify amylose. While effective, all the above methods are very labor intensive, time consuming and not conducive to screening large numbers of samples, such as is needed for evaluating breeders' samples.

Another widely used method has been the measurement of iodine binding of amylose producing a blue coloration. The iodine-binding method was introduced by McCready and Hassid (1943) for measurement of amylose in potato starch. Since the introduction many modifications have been made to the procedure, adjusting for sample preparation, standards, and measurement wavelength

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* Corresponding author. Tel.: +1 785 776 2763; fax: +1 785 537 5534.
E-mail address: jeff.d.wilson@ars.usda.gov (J.D. Wilson).

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(Juliano, 1971; Juliano et al., 1981; Knutson, 1986). The amylose content was commonly overestimated due to interference from the amylopectin–iodine complex, possibly due to the complexation of iodine and the amylopectin side chains. Recently, Zhu, Jackson, Wehling, and Geera (2008) evaluated many amylose content measurement techniques and developed a method utilizing a dual-wavelength approach. The dual-wavelength method had greater precision and accuracy than the single wavelength method due to a reduction in the effect of the amylopectin–iodine complex. Many of the amylose content measurements are capable of providing accurate and precise measurements; however the greatest disadvantage to all of the methods is the speed of measurement or the number of samples that can be analyzed in a day.

The measurement of quality traits in breeding populations of cereal grains is becoming more important. Plant breeders evaluate the end-product quality of their breeding lines at very early stages in the process, when populations number in the hundreds to thousands. Current amylose content measurements are very time consuming and low throughput, thus screening breeders' populations is very difficult and not commonly achieved. Therefore, the objectives of this study were to (1) develop a method capable of analyzing 50–100 samples of starch per day and (2) maintain a level of precision and accuracy needed for screening.

2. Materials and methods

2.1. Materials

Starches from waxy maize, high amylose maize, normal maize, high amylose barley, and rice were commercially produced. Sorghum hybrids and wheat varieties were laboratory scale milled into flour and starch was isolated by the sonication method of Park, Bean, Wilson, and Schober (2006). All chemicals used were reagent grade. Amylose from potato (product number 10130, Fluka, Sigma Aldrich) and amylopectin from maize (product number 10120, Fluka, Sigma-Aldrich) were used as controls for preparation of the standard curves.

2.2. Conventional amylose measurement

Amylose content was measured on starch from all samples in replicate using the Concanavalin A precipitation method (K-AMYL, Megazyme International, Wicklow, Ireland).

2.3. Development of 96-well plate method

The method reported here modified the starch suspension methodology used by Hogg et al. (2013) and combined with the analysis wavelengths reported by Zhu et al. (2008). First, 5 mg of starch sample or standards were weighed into 2 mL centrifuge tube. Next 1 mL of 90% DMSO in water was added and tubes heated to 95 °C for 60 min with vortexing every 10 min. After starch dispersion, samples are cooled for 5 min and 100 μL from each sample tube was added to a well on a 96-well plate. The standard curve for amylose content was prepared using both amylose and amylopectin, the ratios can be found in Table 1. After the samples were placed into the 96-well plates, 100 μL of 90% DMSO with 3.04 g/L iodine (due to I_2's solubility in DMSO the traditional I_2:KI solution was not necessary) was added to each well and plate was shaken for 2 min. The control blank, 100 μL of 90% DMSO plus 100 μL of 90% DMSO with 3.04 g/L iodine, was placed into duplicate wells. A subsample (20 μL) from each well was removed using a 96-well pipetting system and added to an empty plate, then 180 μL of deionized water was added to each well using the pipetting system and plate was shaken for 2 min. After agitation the 96-well plate was analyzed for absorbance at 620 nm and 510 nm. The absorbance was blanked with the control for a final ABS620 and ABS510 reading. A flow chart of the method can be found in Fig. 1.

Table 1
Standard curve preparation.

Amylose content (%)	Amount of 5 mg/mL amylose solution (μL)	Amount of 5 mg/mL amylopectin solution (μL)
0	0	100
5	5	95
10	10	90
15	15	85
20	20	80
25	25	75
30	30	70
50	50	50
75	75	25
100	100	0

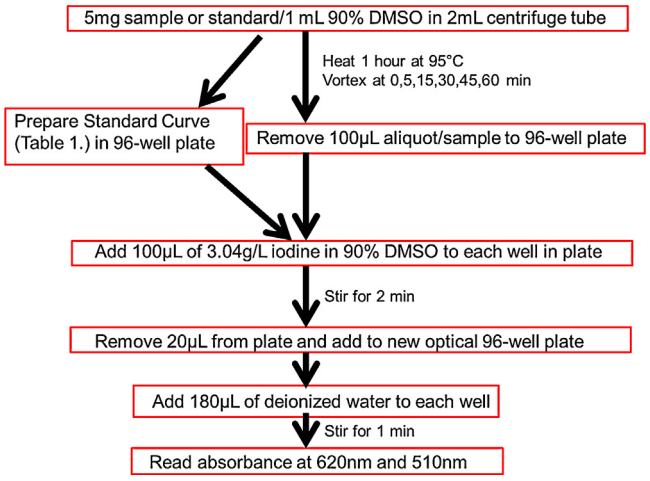

Fig. 1. Flow chart for 96-well plate method.

2.4. Amylose content calculation

A regression equation was determined for the standard curve on each plate analyzed using both the absorbance value at 620 nm and the Diff ABS (ABS620 − ABS510). The amylose content of the samples was calculated using these equations. Single wavelength amylose = (ABS620 − *y*-intercept of regression/slope of regression); dual wavelength amylose = (Diff ABS − *y*-intercept of regression/slope of regression).

2.5. Statistical analysis

All analyses were conducted in quadruplicate unless otherwise stated. The means, standard deviation, and coefficient of variation were calculated using an Excel spreadsheet (Microsoft Corp., Redmond, WA). The coefficient of determination for the standard curve was found using OriginPro8 software (OriginLab Corp., Northhampton, MA)

3. Results and discussion

3.1. Standard curve

Initial testing to produce a standard curve utilized only amylose as a standard. It was found that a combination of purified amylose and purified amylopectin produced a better standard curve, which was similar to many previous studies. The plate method was capable of producing a highly accurate standard curve with both the single (ABS620) and the dual wavelength approach. Fig. 2

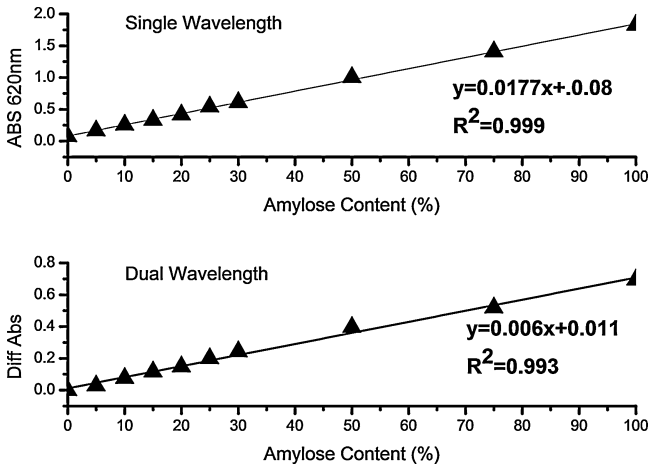

Fig. 2. Regression equations for amylose standard curve prepared in 96-well plate.

shows a standard curve for both the single and dual wavelength methodologies. Since there may be slight variations in the plates, a standard curve was generated with every plate analyzed. The regression equation on the single wavelength assay had an R^2 of 0.999 on many plates and the values for the slope and y-intercept were very repeatable. The dual wavelength method also exhibited a very high coefficient of determination ($R^2 = 0.993$) with plate to plate repeatability on slope and intercept values. The coefficients of determination were very similar to values reported for standard curves produced in larger cuvette scale methods (McGrance, Cornell, & Rix, 1998; Zhu et al., 2008). The level of accuracy and repeatability of the standard curves allows for quantification of amylose in starch samples.

3.2. Measurement of amylose in cereal starches

The amylose content of the cereal starches analyzed can be found in Table 2. The values found for amylose by the Concanavalin A method and the 96-well plate methods developed all were within the range normally expected for the respective sample. The singe wavelength (ABS 620) prediction of amylose content tended to be slightly higher than the Concanavalin A values. The overestimation of amylose on a single wavelength iodine binding had been previously reported by Zhu et al. (2008). Recently, a study showed that increases in amylopectin chain length lead to a greater disparity between the iodine binding amylose content and Concanavalin A values (Park, Kim, Chung, & Shoemaker, 2013), suggesting that amylopectin interferes with the single wavelength detection.

The dual wavelength detection of the iodine binding used in this methodology appears to reduce the overestimation and improve the precision and accuracy when compared to the single wavelength approach. The dual wavelength method produced values for amylose content very similar to the Concanavalin A measurement.

Table 2
Comparison of amylose content estimates for cereal starches.

Cereal starch	Amylose content (%)		
	Concanavalin A assay	Single λ 96-well plate	Dual λ 96-well plate
Rice	18.5 ± 0.88	21.5 ± 1.16	19.6 ± 0.54
High amylose barley	36.5 ± 0.05	42.9 ± 1.99	39.4 ± 0.96
HYLON V maize	49.7 ± 3.06	68.1 ± 3.43	49.2 ± 1.45
Wheat	28.9 ± 0.28	30.9 ± 0.65	29.0 ± 0.74
Sorghum	29.1 ± 0.92	28.1 ± 1.34	30.4 ± 1.26
High amylose maize	59.5 ± 0.31	78.5 ± 1.15	61.7 ± 2.30
Waxy maize	2.4 ± 0.70	0.24 ± 0.73	1.2 ± 0.90

The 96-well plate dual wavelength method is capable of analyzing amylose in waxy to high-amylose types of starch (Table 2). Previous attempts at high-throughput plate methods experienced difficulty achieving similar Concanavalin A values and iodine binding values for normal starches (Hu, Burton, & Yang, 2010) or produced values with high coefficients of variation (Hogg et al., 2013). These variations may be due to difficulties with interference due to amylopectin–iodine interactions or standard curve preparation techniques.

The dual wavelength method's precision was tested on wheat starch with 10 replicate samples. The mean amylose content of the starch was found to be 29.0% with a standard deviation of 0.74%. The coefficient of variation for these 10 replicates was 2.55%, which is slightly lower than the common 5% value of the Concanavalin A measurement. The coefficient of variations found with four replicates of the other starches showed a range from 2.44% to 4.14% in normal and high amylose starches. The waxy maize starch had very large coefficient of variation due to mean values being close to zero. This suggests that there may be some difficulty in statistical separation of waxy starches from other waxy starches; however this is not a concern for normal and high-amylose starches.

Normal and high-amylose starches contain lipids which can form complexes with amylose and affect the intensity of the blue color formed by the amylose–iodine complex (Morrison & Laignelet, 1983). Lipids in those cereal starches need to be removed to obtain absolute amylose content, thus amylose measurements in this study are apparent amylose values.

4. Conclusions

The adaptation of several iodine binding methodologies allowed for an accurate and precise amylose content determination. The 96-well plate method is capable of analyzing over 50 samples in replicates on a daily basis. This speed of analysis is a drastic improvement over current methodologies and will allow for timely screening of large breeder populations. Since the accuracy of amylose measurement is not effected by the speed of measurement this method could become an effective tool for early generation quality testing using relatively small quantities of isolated starch.

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