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Short communication

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

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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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(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

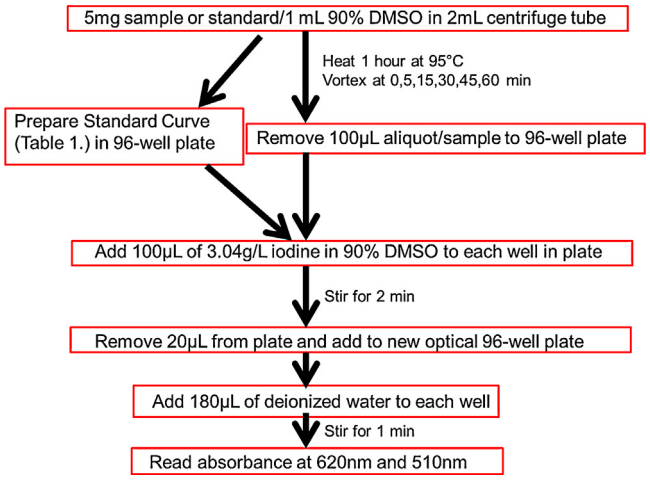

Fig. 1. Flow chart for 96-well plate method.

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