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An Optimized Procedure for Determining the Amylase/ Amylopectin Ratio in Common Wheat Grains Based on the Dual Wavelength Iodine-Binding Method

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ABSTRACT

Wheat endosperm is mainly composed of starch consisting of amylose and amylopectin. The amylose/amylopectin ratio determines the starch properties. Thus, an appropriate method is essential to accurately measure this ratio. Here, we reported an optimized procedure based on the dual wavelength iodine-binding method that can accurately and conveniently determine the amylose/amylopectin ratio in wheat starch. This procedure is characterized by three aspects of advantages over the previously reported methods. Firstly, high purities of wheat starch standard can be easily isolated and thus replaces the commercial potato standard which is not suitable for determining wheat starch. Secondly, the comparison of wavelength pairs showed that the selection of wavelength pairs could be flexible. Finally, total starch of wheat grains can be extracted and used as measuring samples to eliminate the effects of non-starch impurities and obtain the amylose/amylopectin ratios with a single measurement. The amylose/amylopectin ratios of 28 wheat cultivars were analyzed using this improved procedure. Further, the data obtained from Megazyme assay kit confirmed the accuracies of this improved procedure. The results showed that the data generated by wheat standard were identical and were readily repeated. This optimized procedure allows efficient determination of amylose/amylopectin ratios in wheat starch.

Keywords: *amylose, amylopectin, dual wavelength, wheat*

INTRODUCTION

Wheat grains are mainly composed of starch, which accounts for 65–70% of the weight of the grain (Morell et al., 1995). Starch is composed of two components: amylose, the almost entirely linear form of α-(1, 4)-linked glucose polymers, and amylopectin, the highly branched form of α-(1, 4) glucan that has α-(1, 6) branch points. Generally, wheat starches contain 22–26% amylose and 74–78% amylopectin. The amount of amylose and the amylose/amylopectin ratio have critical effects on the properties of the starch, influencing the gelatinization, solubility, and loaf volume (Jane et al., 1999; Lee et al., 2001; Blazek and Copeland, 2008). Therefore, the amylose/amylopectin ratio is an important criterion for assessment of starch and wheat quality.

Numerous methods have been used to measure the content and composition of wheat starch. They include high-performance size-exclusion chromatography (HPSEC), iodine-binding capacity measurements, differential scanning calorimetry (DSC), and complexation with concanavalin A (Gibson et al., 1997; Gérard et al., 2001; Matveev et al., 2001; Grant et al., 2002; Herrero-Martínez et al., 2004; Zhu et al., 2008). All of these methods require costly equipment or considerable training. These shortcomings make it difficult to apply these methods widely in determining the content and composition of wheat starch. In addition, results generated by these methods are difficult to repeat and are easily affected by other components of wheat grains.

Given its advantages over other methods such as simplicity, low cost, and repeatability, colorimetric assay is widely employed to determine starch content. Colorimetry can be performed at single wavelength, dual wavelengths, or multiple wave lengths. The single wavelength method is easily operated (McGrance et al., 1998). However,

values measured by this method are not consistently accurate because a complex formed between iodine and amylopectin can interfere with amylose determination (Mahmood et al., 2007; Zhu et al., 2008). The multiple wavelength method can generate more accurate results than the single wavelength method. However, it is difficult to perform and the calculations involved are complicated (Jarvis and Walker, 1993; Séne et al., 1997; Wang et al., 2010). In addition to accurate results determined, the dual wavelength method can not only generate accurate results by eliminating the effects of the amylopectin–iodine complex but also can be easily to be operated with less time (Hovenkamp-Hermelink et al., 1988; Ma et al., 2014).

All the above-mentioned methods require a calibration standard curve with pure amylose. Amylose and amylopectin standards of high purity and standard curves need to be generated for samples from different sources (McGrance et al., 1998; Zhu et al., 2008). So far, commercial standards for wheat amylose and amylopectin are still unavailable.The commercial potato or maize standards were used in most previous studies on wheat starch (Blazek and Copeland, 2008).

Here, a procedure based on dual wavelength method, including isolation of wheat starch standards with high purity, was optimized to analyze the ratio of amylose/amylopectin in wheat grains. This method was successfully adopted to determine the ratios of amylose/ amylopectin of 28 wheat cultivars. Our results demonstrated that it could be a useful protocol for measurement of wheat starch.

MATERIALS AND METHODS

Materials

Wheat cultivars developed in China were used in this study including ‘Chuannong16’ (CN16), ‘Guinong21’ (GN21), ‘Shumai110’ (SM110), ‘Shumai375’ (SM375), ‘Shumai482’ (SM482), ‘Shumai969’ (SM969), ‘Shumai6707’ (SM6707), ‘Changnmai26’ (CM26), Chuanmai27’ (CM27), ‘Chuanmai55’ (CM55), ‘Chuanmai104’ (CM104), ‘Fan07217’ (F07217), ‘Fan09048’ (F09048), ‘Neimai9’ (NM9), ‘Xichang18’ (XC18), ‘Xichang19’ (XC19), ‘Liangmai2’ (LM2), ‘Liangmai4’ (LM4), ‘Chuanyu20’ (CY20), ‘Neimai3416’ (NM3416), ‘Rongmai4’ (RM4), ‘Yumai1’ (YM1), ‘ Zhengmai9023’ (ZM9023), ‘Mianmai37’ (MM37), and ‘Mianmai51’ (MM51). Three cultivar of ‘Bobwhite’ (PI520330, PI520554, and PI614034) were kindly provided by USDA - ARS (http://www.ars-grin.gov). All these wheat cultivars were grown in a greenhouse, and the seeds were collected, preserved, and used. The potato amylose and amylopectin standards were purchased from Sigma (Shanghai, China)

Purification of Amylose and Amylopectin

Wheat grains were milled into flour using a Buhler experimental mill. Flour was mixed with water at a ratio of 2:1 (w/v) for 30 min with a stirrer. It was then placed at room temperature for 30 min. The paste was packed into six layers of cheesecloth and washed with water by kneading. The slurry was centrifuged at 4,000 × *g* for 3 min and the yellow gel-like layer on top of the white starch was carefully removed. The starch granule pellet was suspended in water. The centrifugation process and removal of the yellow gel-like layer were repeated two more times.

In order to isolate amylose, aqueous leaching and butanol precipitation were performed as reported previously, with some modifications (Mua and Jackson, 1998). A 500-mL volume of starch granule slurry (2% w/v) was heated on a hot plate to 65 °C with continuous magnetic stirring and then maintained at 65 °C for 1 h. The slurry was then centrifuged at 3,000 × *g* for 10 min and the precipitate was discarded. *N*-butanol was added to the supernatant at a ratio of 1:3 (v/v). The solution was mixed and maintained at room temperature overnight. The supernatant was collected after centrifugation and filtration and then mixed with *n*-butanol for 1 h. The precipitate was collected after centrifugation and washed with absolute ethanol four times, freeze-dried, and ground using a mortar and pestle.

The isolation of amylopectin was performed as described by Liu *et al.* (2009), with some modifications. A 500-mL volume of slurry (2% w/v) was heated on a hot plate to 100 °C with continuous magnetic stirring for 1 h. When the temperature of the slurry dropped to 55 °C, the pH was adjusted to 6.3 using phosphate buffer (40 g/L NaH_2PO_4, 10 g/L Na_2HPO_4). Then the slurry was boiled at 121 °C in an autoclave for 3 h and heated on a hot plate at 100 °C with continuous magnetic stirring for 1 h. *N*-butanol was added to the slurry at a ratio of 1:3 (v/v) and mixed with continuous magnetic stirring until the temperature dropped to 55 °C. The slurry was allowed to cool in a foam box overnight and

was then centrifuged at 8,700 × *g* for 30 min. After centrifugation, the top layer of *n*-butanol and the precipitate, which contained amylose and other impurities, were discarded; the middle layer was crude extract of amylopectin. In order to precipitate residues of amylose, *n*-butanol was added to the amylopectin crude extract at a ratio of 1:3 (v/v) and the solution was mixed and centrifuged at 8,700 × *g* for 30 min. This process was repeated at least four times. To isolate amylopectin from this solution, excess methanol was added, mixed, and centrifuged at 8,700 × *g* for 10 min. The precipitate was washed by absolute ethanol four times, freeze-dried, and ground using a mortar and pestle.

Gel Permeation Column Chromatography

The purity levels of amylose and amylopectin obtained from various wheat cultivars were monitored using gel permeation column chromatography (Jane and Chen, 1992). To prepare each sample working solution, 100 mg of amylose or amylopectin was dispersed using 1 mL absolute ethanol, and 9 mL of 1 M NaOH was added. The solution was placed in a boiling water bath for 15 min with frequent shaking to obtain a clear solution. Distilled water (60 mL) was then added and the pH was adjusted to 3.6 using 0.5 M HCl. The total volume was adjusted to 100 mL using distilled water. A 1-mL working solution was loaded into a chromatographic column filled with Bio-gel P 100 gel and connected with the Bio-Gel chromatography system. The eluant was NaCl solution (0.02%, pH 3.6) with a flow rate of 9 mL/h. Each tube collected 2 mL of solution and the elution curves of amylose or amylopectin were generated using iodine-binding spectrophotometry.

Absorption Spectra and Standard Curves

One hundred milligrams of amylose or amylopectin was dispersed using 1 mL absolute ethanol, and 9 mL 1 M NaOH was added to the solution. The solution was placed in a boiling water bath for 15 min with frequent shaking to obtain a clear solution; then the total volume was adjusted to 100 mL using distilled water to obtain the stock solution. To generate absorption spectra, 4 mL amylose stock solution or 2.6 mL amylopectin stock solution was added to 30 mL distilled water and the pH was adjusted to 3.6 using 0.5 M HCl. Then, 0.5 mL potassium iodide–iodine (I_2-KI) solution (2 g/L I_2, 20 g/L KI) was added, and the total volume was adjusted to 50 mL using distilled water. This reaction solution was mixed and placed at room temperature for 30 min, after which it was scanned using a spectrophotometer at the 400–900 nm wavelengths. To generate the amylose standard curve, the amylose stock solution was diluted to obtain reaction solutions with final concentrations of 10, 18, 26, 34, 42 and 50 μg/mL, respectively. Wheat amylose reaction solutions were measured at the wavelengths of 632, 620, 455, and 443 nm, using the colored blank control. A potato amylose reaction solution was measured at the wavelengths of 599 and 471 nm, using the colored blank control. Standard curves were created by plotting the concentration of amylose on the x-axis and the difference of absorbance (ΔA) on the y-axis.

Determination of Amylose / Amylopectin Ratios in Wheat Samples

To confirm the accuracy of the modified method, amylose/amylopectin ratios of 28 common wheat cultivars from China and the North America were analyzed. Total starch was considered to be 100%; the percentage of amylopectin was defined as the difference between 100% and the percentage of amylose. The isolation of starch from samples was performed as described by Peng *et al.* (1999), with some modifications. The sample stock solution was prepared in the same manner as the amylose and amylopectin standard. A 5-mL sample stock solution was added to 30 mL distilled water, and the pH was adjusted to 3.6 using 0.5 M HCl. Then, 0.5 mL I_2-KI solution was added, and the total volume was adjusted to 50 mL using distilled water. This reaction solution was measured after the same period of time and at the same temperature as the amylose and amylopectin standards. In addition, all these samples were analyzed by using Megazyme amylose/amylopectin assay kit, the measurement and calculation were strictly followed the assay procedure.

Results And Discussion

Purification of Amylose and Amylopectin Standards

In order to remove as much impurity as possible, the supernatant of a starch granule slurry made by different methods was collected and the amylose or amylopectin was purified from the supernatant. Extra steps were added to the subsequent purification to create starch of a higher purity. The purity levels of the amylose

and amylopectin were analyzed by iodine-binding spectrophotometry and gel permeation column chromatography. The coloration results showed that the iodide-amylose solution clearly appeared blue, and the iodide-amylopectin solution appeared reddish brown (Fig. 1A, C). The absorption spectra of starch components from various common wheat cultivars showed maximum values of around 630 nm for amylose and 540 nm for amylopectin (Fig. 2A, B). The results of gel permeation column chromatography showed that amylose and amylopectin had unimodal curves (Fig. 1B, D). All these results demonstrated that this modified methods allowed effective isolation of amylose and amylopectin with high levels of purity from wheat grains.

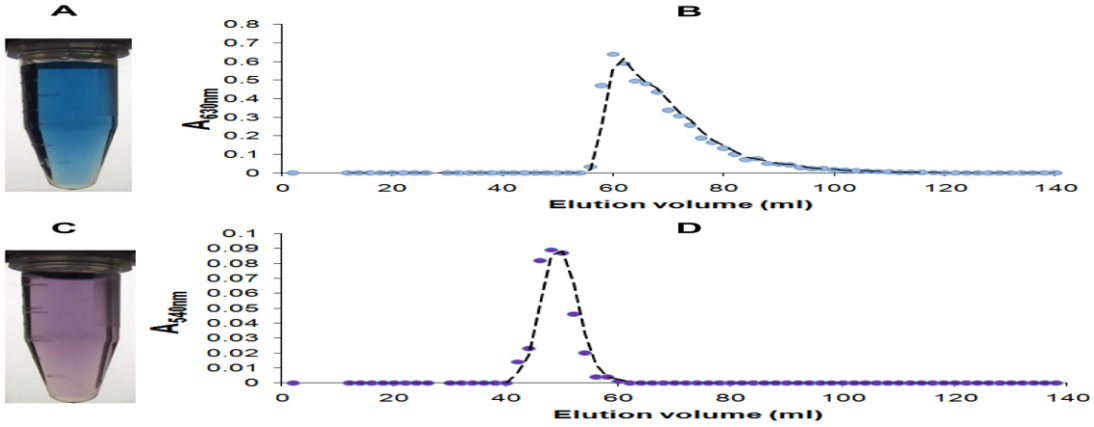

Figure1.*Iodine-binding staining and analysis of gel permeation column chromatography.(A) The staining of amylose with I_2-KI solution.(B) The elution curves of amylose using gel permeation column chromatography. (C) The staining of amylopectin with I_2-KI solution. (D) The elution curves of amylopectin using gel permeation column chromatography*

Analysis of Absorption Spectra

To verify the similarity of amylose or amylopectin obtained from various wheat cultivars, the absorption spectra of amylose or amylopectin were tested and compared. Amylose of all common wheat cultivars showed maximum values of near 630 nm and potato amylose showed the maximum value of near 600 nm (Fig. 2A). In addition, the peak value of each wheat amylose was noticeably higher than that of potato amylose at the same concentration. In contrast to amylose, both of wheat amylopectin and potato amylopectin showed maximum of near 545 nm and their waveforms were similar, though their peak values of absorption spectrum were slightly different (Fig. 2B). The absorption spectrum of amylose (Fig. 2A) showed that scan curves of amylose obtained from various common wheat cultivars were similar to one another and significantly differed from those of potato amylose. These obvious differences in absorption spectra indicated that the structure of wheat amylose differs from that of potato amylose. In addition, wheat amylopectin and potato amylopectin showed maxima near 545nm and their waveforms were similar (Fig. 2B). According to the principle of the dual wavelength spectrophotometry, the obvious differences between wheat and potato amylose may cause errors when the latter is used as the standard to estimate the former (Shibata, 1976; Jain,2010).

Generation of Standard Curves

In order to apply the dual wavelength method, it is necessary to select two proper wavelengths to measure the absorption of the amylose–iodine complex. To generate a credible amylose standard curve, there should be a sufficiently large difference between the amounts of amylose absorbed at two selected wavelengths. At the same time, the same absorbance of amylopectin should exist at the selected wavelengths (Shibata, 1976; Jain, 2010). In this study, two wavelength pairs of 632 nm/443 nm and 620 nm/455 nm, both conforming to these rules, were selected to create standard curves.

Three standard curves for wheat amylose were highly similar to one another, regardless of whether the 632 nm/443 nm or 620 nm/455 nm wavelengths were used (Fig. 2C, D). It illustrated that the selection of measurement wavelengths could be flexible and should be adjusted based on absorption spectrum of starch standards. In addition, standard curves of wheat

were obviously different from the curves for potato amylose. The R^2 values of all the standard curves were 0.999 or higher, indicating that these standard curves were highly credible. The differences between the standard curves of wheat and potato demonstrated that it is not appropriate to use a standard curve generated by potato starch to measure wheat starch.

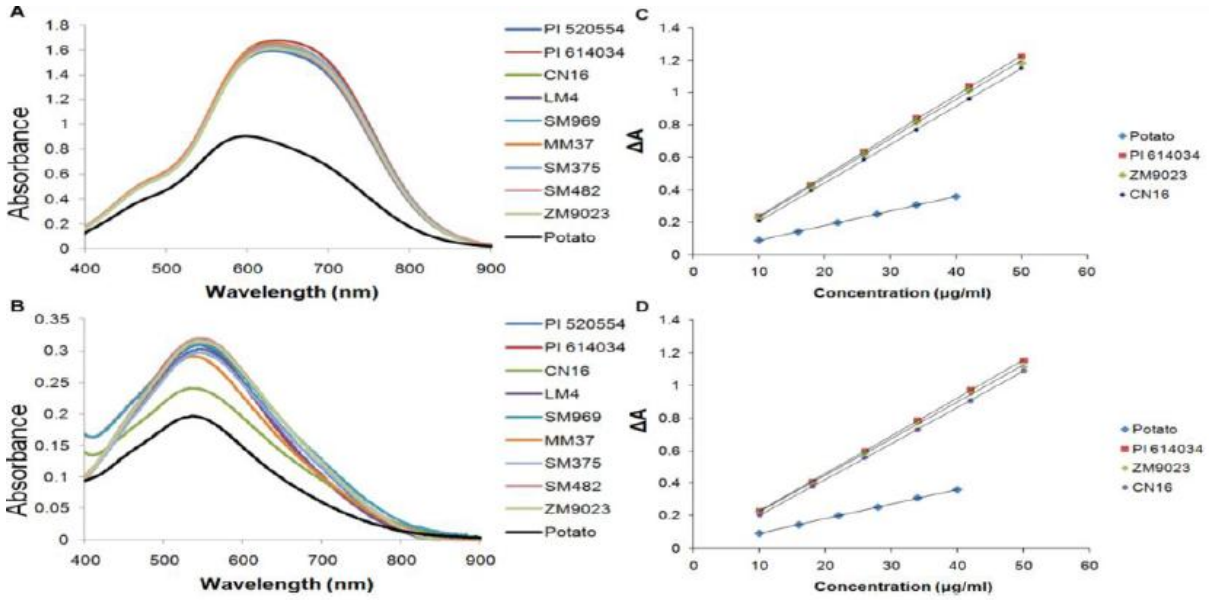

Figure 2. *Absorption spectra and standard curves obtained from various wheat cultivars and from potato. (A) Comparison of absorption spectra of amylose. (B) Comparison of absorption spectra of amylopectin. (C) Wheat amylose standard curves (632/443 nm). PI 614034 y = 0.0248x - 0.01 (0.9999); Z9023 y = 0.0241x - 0.0072 (0.9994); CN16 y = 0.0235x - 0.0246 (0.9999). (D) Wheat amylase standard curves (under 620/455 nm). PI 614034 y = 0.0233x - 0.0068 (0.9999); Z9023 y = 0.0226x - 0.0049 (0.9994); CN16 y = 0.0211x + 0.0003 (0.9999). The potato amylose standard curve was generated under the wavelengths of 599/471 nm. Potato y = 0.009x + 0.0015 (R^2 = 0.9998).*

Determination of Amylase / Amylopectin Ratios in Wheat Samples

Amylose contents of samples could vary noticeably by using different methods (Vilaplana et al., 2012). Using colorimetric methods, to some extent, the non-starch impurities such as lipid influenced the amylose determination (Mahmood et al., 2007; Ma et al., 2014). In this study, the total starch of wheat grains was extracted and used as the measuring samples. This improvement effectively eliminated the effects of non-starch impurities. Meanwhile, it allowed the amylase /amylopectin ratio to be determined with a single measurement.

In this study, standard curves with high linear correlation coefficient were obtained from wheat starch standards ($R^2 \geq 0.999$). The amylose contents derived from different standards and wavelengths were compared (Table 1) and the amylose/amylopectin ratios of these samples were showed in Figure 3. The data measured at the wavelengths of 632/443 nm and 620/455 nm were similar (Table 1). It demonstrated that the selection of wavelength pairs could be flexible and should be adjusted based on absorption spectrum of starch standards(Jain, 2010). Differences among the amylose contents derived from three wheat starch standard curves were not significant in each sample ($p > 0.05$). The concanavalin A related assay need high cost and precise experimental control, but it could directly produceaccurate results without standard curve (Gérard et al., 2001; Vilaplana et al., 2012). Thus, the data from this optimized procedure were compared with those produced by Megazyme assay kit (Table 1). The differences between the data derived from these two methods ranged from -2.6% to +5.1%. Relative to previous reports, the results derived from CN16 standard under 632nm/443nm showed more dependable (Gérard et al., 2001; Zhu et al., 2008), when the accuracies were confirmed using Magezyme assay kit (differences ranged from -2.5% to +4%). Moreover, the data generated by each wheat starch standard were more repeatable (RSDr ≤ 2.0%), compared with Megazyme assay kit (RSDr ≤ 5 % for pure starches). However, the results generated by potato amylose and amylopectin showed that the quantity of amylose was significantly overestimated. All these results showed that this optimized procedure was accurate

and could provide a universal determination of the amylose/amylopectin ratio in the starch of wheat grains.

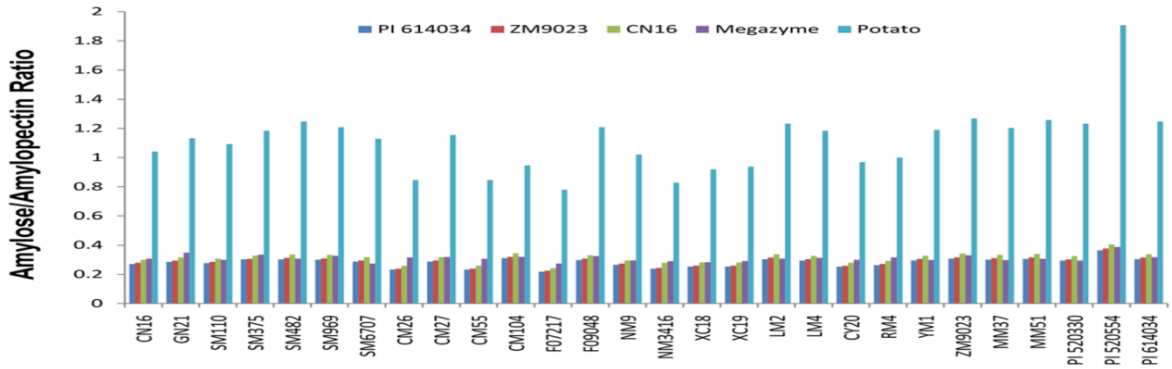

Figure3. *Amylose/amylopectin ratios of samples. The values derived from wheat standards were calculated using mean values under two wavelength pairs of 632/443 nm and 632/443 nm. The values derived from potato standards were calculated using mean values measured under the wavelengths of 599/471 nm. The values derived from Megazyme assay kit were calculated using the mean values of each sampl*

Table1. *Comparison of amylose contents (% of starch) from different methods*[ab]

Sample	Iodine(632nm/443nm)[c]			Iodine(620nm/455nm)[c]				
	PI614034[d]	Zm9023[d]	CN16[d]	PI614034[d]	Zm9023[d]	CN16[d]	Megazyme[e]	Potato[cd]
CN16	21.3±0.3	21.8±0.4	23.0±0.4	21.3±0.4	21.9±0.4	23.2±0.4	23.5±1.1	51.0±0.8
GN21	22.2±0.4	22.7±0.4	24.0±0.4	22.3±0.4	22.9±0.4	24.2±0.4	25.8±1.9	53.1±1.0
SM110	21.7±0.4	22.2±0.3	23.5±0.3	21.8±0.4	22.4±0.3	23.7±0.3	23.0±2.4	52.2±0.9
SM375	23.2±0.4	23.4±0.4	24.7±0.4	22.9±0.4	23.5±0.4	25.0±0.4	25.1±2.0	54.2±1.0
SM482	23.2±0.4	23.8±0.5	25.1±0.5	23.4±0.4	24.0±0.4	25.5±0.4	23.5±1.7	55.5±1.1
SM969	23.1±0.4	23.6±0.4	25.0±0.4	23.2±0.4	23.8±0.4	25.3±0.4	24.6±0.8	54.7±1.0
SM6707	22.3±0.4	22.8±0.4	24.1±0.4	22.4±0.4	23.0±0.4	24.4±0.4	21.5±1.8	53.0±1.0
CM26	18.9±0.5	19.3±0.5	20.5±0.5	19.0±0.5	19.5±0.5	20.6±0.5	24.0±1.8	45.8±1.3
CM27	22.3±0.2	22.8±0.2	24.1±0.2	22.4±0.2	23.0±0.2	24.4±0.3	24.2±2.0	53.6±0.8
CM55	18.9±0.4	19.3±0.4	20.6±0.4	19.0±0.4	19.6±0.4	20.7±0.4	23.4±2.4	45.8±1.0
CM104	23.7±0.4	24.2±0.4	25.6±0.4	23.7±0.5	24.4±0.4	25.7±0.4	24.2±1.8	48.6±1.2
F07217	17.9±0.4	18.3±0.4	19.5±0.4	18.0±0.4	18.5±0.4	19.6±0.4	21.5±2.3	43.8±1.0
F09048	22.9±0.5	23.5±0.5	24.8±0.5	23.0±0.5	23.7±0.5	25.1±0.4	24.5±2.3	54.7±1.3
NM9	21.0±0.4	21.5±0.4	22.8±0.4	21.2±0.4	21.8±0.4	23.0±0.4	22.8±1.8	50.5±1.0
NM3416	19.3±0.5	19.6±0.4	21.8±0.5	19.4±0.5	19.7±0.4	21.9±0.5	22.5±2.4	45.3±1.3
XC18	20.2±0.4	20.6±0.4	21.9±0.4	20.2±0.4	20.8±0.4	22.0±0.4	22.1±2.0	47.9±1.9
XC19	20.2±0.4	20.6±0.4	21.9±0.4	20.2±0.4	20.8±0.4	22.0±0.4	22.6±1.7	48.4±1.1
LM2	23.3±0.5	23.9±0.5	25.2±0.5	23.5±0.6	24.1±0.5	25.6±0.6	23.5±1.1	55.2±1.5
LM4	22.7±0.5	23.3±0.5	24.6±0.5	22.9±0.5	23.5±0.5	25.0±0.5	23.8±1.6	54.2±1.3
CY20	20.1±0.4	20.5±0.4	21.8±0.4	20.3±0.4	20.8±0.4	22.1±0.4	23.0±2.1	49.2±1.0
RM4	20.8±0.5	21.3±0.5	22.6±0.5	21.0±0.5	21.5±0.5	22.8±0.5	24.0±2.2	50.0±1.3
YM1	22.8±0.5	23.4±0.5	24.7±0.5	23.0±0.6	23.6±0.5	25.0±0.5	22.9±0.8	54.3±1.4
ZM9023	23.5±0.4	24.0±0.4	25.5±0.4	23.7±0.4	24.2±0.4	25.8±0.4	24.8±2.1	55.9±1.0
MM37	23.1±0.4	23.7±0.4	25.0±0.4	23.2±0.4	23.8±0.4	25.2±0.4	22.9±1.6	54.6±1.0
MM51	23.4±0.5	24.0±0.5	25.3±0.5	23.5±0.5	24.1±0.5	25.6±0.5	23.5±2.0	55.7±1.5
PI 520330	22.7±0.5	23.2±0.5	24.5±0.5	22.9±0.5	23.5±0.5	24.9±0.5	22.7±1.3	55.2±1.3
PI 520554	26.7±0.6	27.4±0.6	28.8±0.6	27.1±0.6	27.8±0.6	29.6±0.6	27.9±2.0	65.6±1.6
PI 614034	23.3±0.5	23.9±0.5	25.2±0.5	23.4±0.5	24.1±0.6	25.5±0.6	24.1±2.0	55.5±1.5

- *All % amylose values are based on starches extracted from 28 wheat cultivars.*
- *95% confidence intervals were based on 3 replicates for each sample; ± represent standard deviation.*
- *Samples were measured under selected wavelength pairs according to absorption spectrum of wheat starch standards and potato starch standards.*
- *Values were calculated based on the standard curves derived from 3 wheat cultivars and commercial potato standards.*

- *Values were calculated using the formula for the Megazyme amylose/amylopectin assay kit.*

CONCLUSIONS

In this study, the methods for wheat starch extraction were modified and the dual wavelength iodine-binding method was optimized to determine the amylase / amylopectin ratio in wheat starch. In addition, the feasibilities of these techniques were verified for use in wheat starch analysis. The measurement of 28 wheat cultivars using this optimized procedure (based on three wheat standard curves and two wavelength pairs) and Megazyme assay kit showed that the results generated by this optimized procedure were replicable and reliable (and significantly different from those derived from potato amylose). Thus, this study can give an accurate and efficient approach to the measurement of the amylose/ amylopectin ratio in wheat seeds.

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