Rapid Method for Estimation of Amylose in Maize Starches

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ABSTRACT

A rapid solubilization procedure for maize starch, using low-temperature gelatinization in 3*M* $CaCl_2$ in combination with 15–30 min of sonication in an iodine-dimethyl sulfoxide solution at a temperature of 65–70°C makes it possible to estimate amylose content of maize samples in less than 1 hr. Results obtained by this technique are equivalent to those obtained by conventional thermal dissolution of starch in iodine-dimethyl sulfoxide, which requires overnight heating to obtain complete solubilization.

Amylose content of starch is commonly determined by one of many variations of the classical reaction between amylose and iodine to form a blue complex, which is then measured either spectrophotometrically or by potentiometric titration. The reaction is not an absolute indicator of amylose content because of interference from amylopectin and the presence of intermediate materials and long chain amylopectins in numerous maize mutant genotypes. However, a 96% correlation between blue-value measurements and amylose content determined by size-exclusion chromatography (Wang et al 1993) has been demonstrated. Methods based on this reaction remain a convenient means for estimating amylose content. These methods give accurate and reproducible results, but they are generally time-consuming and therefore not well suited to use in large-scale quality control applications.

In previous work at this laboratory(Knutson 1986), a simplified method for amylose analysis was developed to take advantage of the fact that the triiodide (I_3^-) ion, which is necessary for initiation of amylose-iodine complex formation, forms spontaneously when iodine is dissolved in dimethyl sulfoxide (DMSO). Because DMSO is also an excellent solvent for starch, samples can be prepared for analysis simply by dissolving starch in DMSO which contains iodine, diluting this I_2-DMSO solution with water to form the amylose-iodine complex, and measuring the absorbance at 600 nm. This method greatly simplifies reagent and sample preparation, improves stability of reagents, and reduces analysis time. It has the further advantage that interference from lipids is minimal. The procedure is applicable to routine analysis of large numbers of samples. However, the time required for dissolution of starch samples (16–24 hr) precludes rapid completion of the assay. To overcome this limitation, methods for accelerating the dissolution of the starch were explored, with the goal of reducing total assay time to less than 1 hr, including grinding, dissolution, and spectrophotometric measurement. Specifically, the techniques that were examined were low-temperature gelatinization and heating in a sonicating bath.

MATERIALS AND METHODS

Standard Samples

Amylose was a highly purified laboratory preparation (Knutson et al 1982). A sample of waxy maize starch, previously determined by potentiometric titration to contain less than 0.5% amylose, was used for an amylopectin standard. This sample, as well as normal maize starch, amylomaize V, and amylomaize VII were obtained from American Maize Products (Hammond, IN).

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Maize Samples

Five cultivars of normal maize and five high-amylose maizes were used for evaluation. The normal cultivars were Alamo blue corn, A619×Oh43, B73, Oh43, and W64A. High-amylose cultivars were Vineyard Seed Company 11299, Oh43 *ae*, and three cultivars from Bear Hybrid Company. For the latter three samples, the supplier's sample designations and reported amylose contents were as follows: Class 5, lot 9645, 59% amylose (BH59); Class 7, lot 9721, 76% amylose (BH76); and class 8, lot 8L1634, 83% amylose (BH83). Actual amylose content of these three hybrids was determined by amylose assay in I_2-DMSO (Knutson 1986) and verified by the method of Wolf et al (1970); they were 46.7, 60.8, and 69.9%, respectively.

Equipment

Samples were ground in a Udy mill (Udy Corp., Fort Collins, CO) equipped with a 0.5-mm screen. The sonicating bath, equipped with a heater, was model TB9H manufactured by L & R Manufacturing Company (Kearney, NJ). A model 390 Sequoia-Turner (Mountain View, CA) spectrophotometer was used for absorbance measurements, using Coleman 19-mm cuvettes.

Analytical Procedures

All samples were analyzed by the conventional amylose-iodine-DMSO procedure (Knutson 1986) to provide a basis of reference for results obtained by rapid solubilization. Total carbohydrate content of the samples was determined by the phenol-sulfuric acid assay (Dubois et al 1956). For the purposes of this study, total carbohydrate content was used as a measure of starch content. At least three samples of each starch or corn were assayed, and means and standard deviations were calculated.

Solubilization Procedures

Low-temperature gelatinization was accomplished by introducing 50-mg starch samples into a volume of 1.0 ml of 3*M* $CaCl_2$ (Evans and Haisman 1982). To evaluate the effect of gelatinization on the rate of solubilization, gelatinized samples were diluted with 5 ml of a $6\times10^{-3}M$ solution of iodine in DMSO and heated at temperatures of 50 and 70°C. Samples were examined visually for loss of turbidity during heating as a preliminary estimate of dissolution.

A routine procedure was used to quantitatively determine the minimum sonication time required for complete solubilization of gelatinized samples. A sample containing ~50 mg of starch was introduced to 1 ml of 3*M* $CaCl_2$, shaken, and allowed to stand for 10 min. To the gelatinized starch was added 5 ml of $6\times10^{-3}M$ iodine in DMSO. This sample was treated in the sonicating bath at 65–70°C for 60 min; 100-μl aliquots were removed every 15 min. These aliquots were diluted with 2 ml of I_2-DMSO to reduce the concentration to a range in which absorbance measurement was possible. A 0.1–1.0 ml aliquot of this dilution, made to a total of 1 ml with additional I_2-DMSO to ensure sufficient I_3^- for complete reaction, was then diluted

with 8 ml of water to form the blue complex. Absorbance was measured at 600 nm to determine uncorrected amylose content. One milliliter of the water dilution was taken for determination of starch as total carbohydrate by the phenol-sulfuric acid procedure of Dubois et al (1956). The same sequence, substituting raw starch for gelatinized starch, was followed to evaluate the efficacy of $CaCl_2$ gelatinization. Three or more replicates were run for each starch or maize sample at each sonication interval, and from each solubilized sample, three individual aliquots were diluted for absorbance measurements.

After a standardized procedure combining low-temperature gelatinization and sonication was established using purified starch samples, ground maize samples were evaluated by the same procedure.

RESULTS AND DISCUSSION

Room temperature gelatinization of starch in 3*M* $CaCl_2$ (Evans and Haisman 1982) was nearly instantaneous. For all starch samples, a 5% starch solution formed a gel or a thick paste within minutes. Gelatinized samples were then treated with I_2-DMSO and heated at 50 and 70°C. Solubilization was judged by loss of turbidity of the sample. High-amylose starch samples appeared to dissolve rapidly, but normal dent corn starch remained turbid after an hour or more of heating. Therefore, sonication of starch samples was employed to attempt to reduce the heating time required. Starch samples heated in a sonicating bath at 65–70°C became clear within 15 min or less.

In preliminary experiments to determine the effect of $CaCl_2$ on the absorbance of the blue complex formed from starch solutions in I_2-DMSO, interference of $CaCl_2$ was observed, which resulted in higher absorbance values than were obtained with equivalent concentrations of starch with no $CaCl_2$ present. To determine the extent of the interference, amylose and amylopectin were treated with varying concentrations of $CaCl_2$ and their absorbance spectra measured. Absorptivity of pure amylose was unaffected by $CaCl_2$, but absorptivity of amylopectin increased significantly, accompanied by a shift in the wavelength of maximum absorbance (A_{max}). For amylopectin, A_{max} with no $CaCl_2$ was 545 nm, and absorptivity was 1.54. In 0.02–0.04*M* $CaCl_2$, A_{max} shifted to 555 nm, and absorptivity increased to 3.76. The absorptivity of amylopectin at 600 nm, the wavelength used for amylose assay in iodine-DMSO, was 3.14, equivalent to 13.8% that of amylose (22.68). In contrast, absorptivity of amylopectin in $CaCl_2$-free samples is 6.2% that of amylose (Knutson 1986). This difference required recalculation of the correction factor to be applied to raw amylose values in subsequent experiments.

Table I compares the calculated amylose values of starch samples dissolved by the conventional procedure to values obtained with samples of the same starches following rapid solubilization via sonication, with and without prior gelatinization with $CaCl_2$. Sonication of starch in iodine-DMSO without prior gelatinization with $CaCl_2$ was ineffective for dissolution of starch in 60 min or less. Calculated amylose values for the three starches sonicated 30–60 min averaged 80% of values obtained by conventional solubilization. Furthermore, high-amylose samples heated overnight after sonication still indicated lower amylose values than those obtained with the conventional procedure.

When $CaCl_2$ gelatinization was used before sonication, calculated amylose values indicated that all three starches dissolved completely within 15 min. Values after longer sonication were constant, within experimental error.

Absorbance data from gelatinized and sonicated samples were used to ascertain whether the effect of $CaCl_2$ on absorbance of amylopectin in starch was equivalent to the effect found with amylopectin alone. The uncorrected amylose values for these samples were compared to the actual amylose content as determined by the conventional method (Knutson 1986). The uncorrected amylose content of all three starches gelatinized with $CaCl_2$ shown in Table I averaged 12.96% higher than the actual amylose content, agreeing closely with the 13.8% value found in the preliminary experiments with amylopectin in $CaCl_2$ solution. Averaging data from amylopectin measurements and from those of the three starches resulted in an amylopectin-calcium correction factor of 13.29%.

The same experimental conditions used for the normal and high-amylose starches were then used to evaluate solubilization of maize samples. Results of analysis of five normal dent corn cultivars, compared to results by conventional analysis, are shown in Table II. Results for five high-amylose corn samples are shown in Table III. Results are calculated as percentage of starch, as determined by the phenol-sulfuric acid assay (Dubois et al 1956).

For normal dent corn and for amylomaize V corn, 15 min of sonication of the pregelatinized sample appeared to be sufficient for amylose analysis. Amylose content, measured as percent of starch, did not vary significantly between 15 and 60 min. However, starch content as percent of sample starting weight, determined

TABLE I
Amylose Content (% of starch) of Normal and High-Amylose (Amy) Starches, as Determined by Conventional Assay and by Rapid Solubilization Assay with and without $CaCl_2$ Gelatinization

	Sonication Time, min	Starch		
		Normal	Amy V	Amy VII
Conventional assay				
Mean		20.7	44.8	64.9
Standard deviation/%		1.9	3.4	5.0
Rapid solubilization				
No $CaCl_2$	15	14.3	31.6	45.7
	30	16.7	34.6	51.6
	45	18.4	35.4	52.4
	60	17.3	34.9	48.0
	ov[a]	19.4	38.4	53.5
$CaCl_2$	15	22.0	46.7	62.2
	30	21.9	49.2	65.2
	45	22.1	46.4	64.5
	60	20.9	48.1	69.2
Mean		21.7	47.6	65.3
Standard deviation/%		1.2	2.5	3.9

[a]Overnight heating at 70°C, no sonication.

TABLE II
Amylose Content of Starch in Normal Dent Corn Samples as Determined by Conventional and Rapid Solubilization Assay

	Sonication Time, min	Sample					
		A619 × Oh43	Alamo	B73	Oh43	W64A	Mean[a]
Conventional assay							
Mean		22.9	20.9	21.3	24.9	22.8	22.6
Standard deviation/%		1.5	1.2	0.8	0.9	2.4	1.4
Rapid solubilization	15	25.5	21.5	22.3	26.2	23.8	23.9
	30	26.3	22.8	21.9	23.1	22.2	23.2
	45	25.0	22.4	20.9	21.4	23.0	22.4
	60	25.9	24.1	22.0	24.2	22.6	24.2
Mean		25.7	22.7	21.8	23.7	22.9	23.4
Standard deviation/%		2.1	1.5	3.0	3.8	3.1	3.3

[a]Five cultivars.

TABLE III
Amylose Content of Starch in High-Amylose Corn Samples as Determined by Conventional and Rapid Solubilization Assay

	Sonication Time, min	Sample					
		Amylomaize V Starches				Amylose >50%	
		V11299	BH59	Oh43 *ae*	Mean	BH76	BH83
Conventional assay							
Mean		53.0	46.7	49.1	50.7	60.8	69.9
Standard deviation/%		3.0	1.1	1.3	3.4	1.6	2.2
Rapid solubilization	15	50.9	51.0	52.3	51.4	63.1	66.5
	30	50.2	46.1	46.7	49.9	59.2	67.1
	45	48.4	51.4	50.0	49.9	54.8	63.3
	60	53.8	50.5	50.5	51.6	58.8	65.6
Mean		50.8	49.8	49.9	50.2	59.0	65.6
Standard deviation/%		1.9	2.1	2.8	2.1	2.9	1.5

by phenol-sulfuric acid assay, was slightly lower for 15 min of sonication than it was for longer times. Average starch content calculated for normal starch samples sonicated 15 min was 71.2% of sample weight, vs. 74.9% found for samples prepared by conventional dissolution, indicating that 95% of the starch was dissolved in that time. The starch content for samples sonicated 30–60 min was 76.7%, equivalent to 102% of conventional dissolution. To assure complete solubilization, a 30-min sonication was used for the standardized procedure.

Phenol-sulfuric acid measurement of starch content of high-amylose maize samples also indicated a tendency for incomplete dissolution after 15 min. However, at all sonication times, total starch content was higher than it was with conventional dissolution. Average starch content by conventional dissolution was 66.7%. With 15 min of sonication, the value was 68.0%; after 30–60 min of sonication, starch content rose to 71.5%. This increase may be an indication of partial dissolution of cell-wall polysaccharides by the sonication procedure, which would result in a slight decrease in calculated amylose content.

Amylose values calculated for amylomaize V samples with rapid solubilization were equal to or slightly higher than those obtained with the conventional procedure. The two samples that had greater than 50% amylose gave somewhat lower results with rapid solubilization. The tendency toward lower values for rapid-solubilized high-amylose samples may or may not be significant in view of the fact that only two samples were available for examination. Part of the difference may be attributed to the higher starch values obtained from rapid solubilization of these samples, which would result in correspondingly lower amylose values. Also, it is possible that the higher lipid content of high-amylose starches requires more than a 60-min exposure to DMSO to completely dissociate complexed lipid from amylose, resulting in some lipid interference with formation of the amylose-iodine complex. In the earlier iodine-DMSO procedure, no significant variation was observed between assay values of defatted and nondefatted starches with 50% or less amylose (Knutson 1986), but there was a small discrepancy with nondefatted starch containing 70% amylose. With the purified starch samples used in testing the rapid solubilization procedure, no such difference was observed between conventional and rapid solubilization (Table I).

On the basis of these findings, the final procedure was standardized. Volumes and dilutions used during evaluation of the procedure were modified slightly to further simplify the operations. Grind sample of corn in a mill fitted with a 0.5-mm screen. Accurately weigh ~50 mg of ground corn, add to 0.5 ml of $3M$ $CaCl_2$. Shake sample thoroughly. Allow to stand 10 min. Add 5 ml of I_2-DMSO solution, containing $6\times10^{-3}M$ iodine. Stir mixture and place in sonicating bath at 65–70°C for 30 min. Remove a 100-μl aliquot. Dilute with 1 ml of I_2-DMSO. Add 8 ml of water to form the blue amylose-iodine complex. Measure absorbance at 600 nm, and determine uncorrected amylose content, in μg/ml, from an amylose standard curve. If starch content is to be determined, remove 1 ml of blue complex solution. Determine total carbohydrate by the phenol-sulfuric acid assay (iodine caused no interference with the assay at this concentration). From uncorrected amylose content and total carbohydrate content, determine uncorrected amylose as percent of starch content. Apply the amylopectin-calcium correction factor to this value according to the following equation, giving corrected amylose content as percent of starch:

$$\%\text{Amylose}_{corr} = (\%\text{amylose}_{uncorr} - 13.29/100 - 13.29) \times 100$$

In summary, rapid solubilization of starch by a combination of low-temperature gelatinization and sonication at 60–70°C provided samples for the iodine-DMSO amylose assay that yielded results with ground whole grains that were equivalent to those obtained using the conventional solubilization procedure. Although some variation between the two techniques was observed for maize samples with >50% amylose, the difference was minimal and does not preclude use of the procedure for rapid estimation of amylose content of high-amylose maize.

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