REVIEWS

Starch Determination by Perchloric Acid vs Enzymes: Evaluating the Accuracy and Precision of Six Colorimetric Methods

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Different methods of polymer extraction can markedly affect the accuracy of starch determination in plant tissues. This study compared starch values in pine root tissue extracted with either perchloric acid or starch-digesting enzymes. Three variations of the two extraction methods were tested. The accuracy of starch determination was assessed for five methods by comparing results to a standard method that employed perchloric acid and KI precipitation. Methods that employed commercial enzymes without additional purification yielded starch values slightly higher than the standard method. Commercial enzymes subjected to additional purification yielded starch values slightly lower than the standard method. Perchloric acid methods without KI precipitation yielded starch values 20–40% higher than the standard method. Also, enzyme methods had the highest precision for extraction and colorimetric steps and were more convenient than perchloric acid methods if additional enzyme purification was not performed. Detailed protocols for the laboratory procedures are provided.

INTRODUCTION

Starch is an important compound in plant physiology and consequently is of special interest to researchers in diverse disciplines such as agriculture, forestry, botany, and ecology. Often though, starch analysis is problematic for researchers with limited expertise in analytical chemistry. Difficulties usually are encountered in choosing and implementing an appropriate starch methodology. The profusion of different starch methods in the published literature complicates the task of method evaluation and selection. A lack of complete and detailed laboratory procedures in most methods papers also can be frustrating. Furthermore, variation in the accuracy of starch methods seriously confounds the interpretation and comparison of results among different studies.

Two popular methods of starch quantification use either perchloric acid or enzymatic digestion to extract the polymer. The extracted starch is then converted to glucose, which is quantified colorimetrically. The key processes in these methods include gelatinization, solubilization, and hydrolysis. Gelatinization refers to the swelling of the starch granule caused by polymer hydration when the granules are treated with boiling water or with a solution of hot ethanol (Manners, 1985). Gelatinization increases the accessibility of the starch polymer to either a solubilizing or hydrolyzing agent. Solubilization occurs when perchloric acid or dilute alkali split the polymer into smaller fragments of amylose and amylopectin which dissolve in aqueous solutions (McCready, 1970). Solubilization in effect extracts the starch polymer from the plant tissue and increases the exposure of starch polymers to a hydrolyzing agent. Hydrolysis is the splitting of amylose and amylopectin polymers into individual glucose units by either strong acid or enzymatic digestion (Loomis and Shull, 1937).

Both the perchloric acid and enzyme methods are subject to error in starch estimation due to the extraction of other polymers, mainly pectin and hemicellulose. Enzyme digestion is a preferred method of quantifying starch because, in theory, active, purified starch-degrading enzymes are the most specific for starch and should yield highly accurate values. However, the accuracy of enzyme methods can be compromised by plant compounds that interfere with analyses and by contamination with other enzymes. Most interfering substances, such as phenolics and lipids (Loomis and Battaile, 1966; Ebell, 1969; McCready, 1970), can be easily removed by extracting the tissue with alcohol or a mixture of methanol, chloroform, and water prior to enzymatic hydrolysis (Haissig and Dickson, 1979). The purity of commercial enzyme preparations is a more critical problem because the preparations are often contaminated by other enzymes such as cellulase and hemicellulase that can produce large overestimations in starch. Unfortunately, enzyme contamination problems may require elaborate and laborious purification procedures to correct (Davey and Kumar, 1983).

Perchloric acid methods are not subject to contamination problems, but may be less desirable than enzyme methods because of the extraction of pectic substances (Bennett, 1955; Ebell, 1969; Tetley, 1974; Hansen and Moller, 1975; Marshall, 1985). Interference by pectin in starch estimation can be reduced by precipitating starch fragments with iodine (Pucher et al., 1948; Hoffpauir, 1956; Hassid and Neufeld, 1964). However, this procedure is unreliable because perchloric acid can hydrolyze starch fragments to glucose, which will not precipitate with iodine. Hydrolysis can be decreased by reducing the exposure time of samples to the acid. This is done by quickly drip-percholating perchloric acid through the tissue sample and immediately diluting the eluate in water (Hansen and Moller, 1975). The acid percolation and iodine precipitation steps are quite time-consuming, though, and hence are not efficient for processing large numbers of samples.

Despite these problems, few studies have statistically compared the accuracy or precision of the different

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methods of starch analysis. The present study compared starch values obtained by colorimetric analysis of pine root tissue extracted with either perchloric acid or enzymes. The objectives were to (1) evaluate the accuracy of starch values obtained with six variations of the perchloric acid and enzyme methods, (2) examine the precision of extraction and colorimetric procedures, and (3) provide detailed and straightforward instructions on laboratory protocols.

Accuracy of the different starch methods evaluated by this study was determined by comparing starch values to results obtained with the standard method of the Association of Official Analytical Chemists (AOAC, 1984). The standard method uses perchloric acid to extract starch followed by KI precipitation to remove interfering polymers. The standard method is based upon the methods of Pucher et al. (1948) and Hoffpauir (1956). We modified the AOAC standard method slightly by using a percolation apparatus similar to that developed by Hansen and Moller (1975) to inhibit starch hydrolysis by perchloric acid.

The three perchloric acid methods we evaluated included (PA1) perchloric acid immersion, (PA2) perchloric acid percolation, and (PA3) the standard AOAC method using acid percolation and KI precipitation (Appendixes). The colorimetric reagent used for all three perchloric acid methods was anthrone.

The three enzyme methods we evaluated included (E1) purified α-amylase–amyloglucosidase with gelatinized starch, (E2) purified α-amylase–amyloglucosidase with solubilized starch, and (E3) unpurified amyloglucosidase with gelatinized starch (Appendixes). Enzymes were used in the unpurified form direct from the supplier or were purified according to procedures described in Haissig and Dickson (1979), Pazur et al. (1984), and Takagi et al. (1971). The colorimetric reagent used with the three enzyme methods was either *o*-toluidine or *o*-dianisidine.

MATERIALS AND METHODS

Sample Collection and Preparation. Root samples were collected from a study in which three different batches of 2-year-old ponderosa pine (*Pinus ponderosa* Laws.) seedlings were grown in six different blocks of a nursery bed (Gleason, 1989). The roots were dried for 48 h at 70 °C, ground in a Wiley mill to pass a 40-mesh sieve, homogenized in liquid nitrogen, placed in bags, and stored in a freezer at −20 °C. Liquid nitrogen treatment is recommended to ensure complete extraction of starch. Cell wall pores usually are too small to allow the passage of starch-digesting enzymes into the cell or the diffusion of acid-solubilized starch fragments out of the cell (Capita et al., 1979; Carpita, 1982; Sandstrom and Loomis, 1987). The simple grinding of dried plant tissue may not disrupt cell walls sufficiently for complete extraction of starch.

Next, the samples were treated to remove soluble sugars and other interfering substances that react colorimetrically and lead to the overestimation of starch values. Soluble sugars were removed by a number of liquid solvents such as water, acetone, and ethanol (AOAC, 1980) or by a solvent mixture of varying polarity such as methanol, chloroform, and water (MCW) (Dickson, 1979).

Statistical Design and Analysis. The experimental design was a randomized complete block (6 nursery blocks × 3 seedling batches, total = 18 tissue samples). The blocks represented different locations within the nursery bed, and the batch treatment consisted of seedlings grown under variable nutrient conditions. These data were subjected to a split plot analysis of variance. Analytical method was the main plot, and seedling batch was the subplot treatment. Because the analytical method times seedling batch interaction was highly significant ($F = 3.81$, $p < 0.01$), a least-squares means procedure (SAS Institute, Inc., 1985) was used to compare mean starch values among analytical methods by seedling batch. The colorimetric procedure used was assumed to have no effect on the final starch values.

Method precision was tested by making four extractions per batch of root tissue (samples) and running two or three colorimetric analyses (subsamples) per extract. Comparisons of analytical precision including sampling errors, subsampling errors, and experimental errors were made by using statistics described in Dixon and Massey (1983). This included calculating an overall F value and making specific pairwise comparisons by using the F ratio of variances. Error attributable to the starch extraction steps was estimated by removing error attributable to the colorimetric steps (i.e., averaging starch percent over aliquots per sample prior to analysis of variance), except for PA3 (missing data). Experimental error was determined by removing subsampling and sampling error (i.e., averaging over aliquots and samples). Experimental error was evaluated by calculating an F value to ascertain whether differences in starch values could be detected with equal probability for all methods.

An examination of the residuals confirmed that the data satisfied conditions of normality and constant variance. Percentage data were unaffected by transformations; therefore, statistical analyses were performed on untransformed data.

Method Overview. The analytical methods were evaluated in laboratories at Oregon State University, Corvallis, OR, and at Humboldt State University, Humboldt, CA.

The perchloric acid and enzyme methods of starch analysis that we examined in this study (Figure 1; Appendixes) differed in their techniques to (1) gelatinize or solubilize the starch, (2) hydrolyze the starch to glucose, and (3) colorimetically analyze the glucose.

In the perchloric acid method of starch determination (Figure 1), the prepared tissue was first extracted with boiling ethanol to remove interfering sugars and to gelatinize the starch granule. Next, the starch was solubilized by extracting the tissue in perchloric acid. This was accomplished in several ways, which included soaking or immersing the sample in acid (PA1), percolating acid through the sample (PA2), or percolating the sample and then precipitating starch with KI (PA3) (Pucher et al., 1948; McCready et al., 1950; Hoffpauir, 1956; Hassid and Neufeld, 1964). The solubilized starch solution was then reacted with a mixture of concentrated sulfuric acid and anthrone to hydrolyze starch to glucose and produce a color product that was quantified colorimetrically on a spectrophotometer (Viles and Silverman, 1949; Yemm and Willis, 1954).

In the enzymatic method of starch determination (Figure 1), the prepared tissue was first extracted with MCW (E1, E2) or ethanol (E3) to remove sugars and other interfering compounds. Next, hot ethanol (E1) or hot water (E3) was used to gelatinize the starch, or sodium hydroxide (E2) was used to solubilize the starch. The tissue sample was then incubated with buffered amyloglucosidase or a mixture of α-amylase–amyloglucosidase to enzymatically hydrolyze starch (Ebell, 1969; Dickson, 1979; Haissig and Dickson, 1979, 1982; Rowe, 1980). The glucose produced by enzymatic digestion was then colorimetrically assayed by using *o*-toluidine reagent or with a mixture of glucose oxidase/peroxidase-*o*-dianisidine (Ebell, 1969; Cooper and McDaniel, 1970; Haissig and Dickson, 1979). For each enzyme method used in this study, the enzyme activity required for complete starch extraction was determined by using standard procedures described in Haissig and Dickson (1979).

RESULTS AND DISCUSSION

Method Accuracy. Overall, the enzyme methods yielded lower starch values than the perchloric acid methods (Figure 2). The lowest starch values were obtained with purified enzymes (E1, E2). Starch values averaged 3% higher on a sample dry weight basis when unpurified enzymes (E3) were used compared to when purified enzymes (E1, E2) were used. The highest starch values were obtained with perchloric acid immersion (PA1) and percolation (PA2). The standard method (PA3) yielded starch values intermediate between the purified (E1, E2) and unpurified enzymes (E3).

With regard to the enzyme methods, different procedures to either gelatinize or solubilize starch apparently had little influence on the completeness of starch digestion

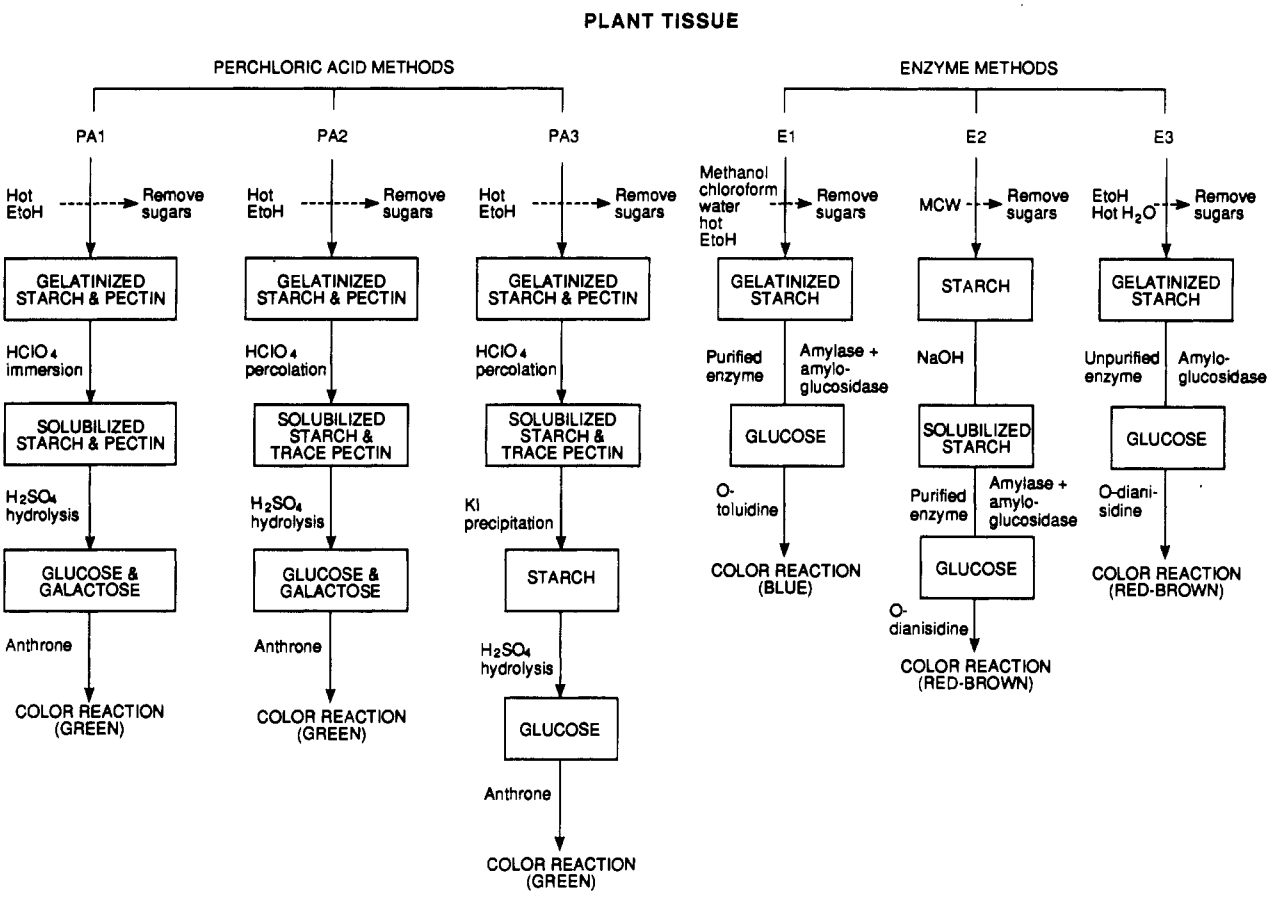

Figure 1. Analytical scheme for the six methods of quantifying starch. Method protocols are provided in the Appendixes.

in the root tissue because E1 and E2 starch estimates were nearly identical. Similarly, Haissig and Dickson (1979) found that gelatinization of starch had little effect on starch hydrolysis of red oak root tissue when adequate enzyme activity was present. The higher starch values obtained with the unpurified enzymes (E3) most likely reflected an overestimate of starch because this commercial preparation was cleansed of binders and fillers, but not enzyme contaminants.

With regard to the perchloric acid methods, the different extraction procedures yielded starch estimates ranging in average from 12 to 17% of tissue dry weight. Starch values obtained with acid percolation (PA2) were comparable to values obtained with acid immersion (PA1). However, the standard method (PA3) that used percolation with KI precipitation yielded nearly 30% lower starch estimates relative to PA1 and PA2. This would suggest that perchloric acid extracted pectic substances regardless of whether immersion or percolation techniques were used. Starch precipitation with KI (PA3) was apparently essential to reduce interference by pectin or other polymers.

Starch values varied among seedling batches extracted with perchloric acid. Specifically, starch values for batch 2 seedlings were significantly lower than values for batches 1 and 3. This finding was interpreted to signify the extraction of polymers other than starch by perchloric acid. This also implied that the relative proportions of starch and interfering polymers varied among the three seedling batches. As an alternative explanation, starch content may have varied among seedling batches. Yet this was not evident in the uniformity of starch values among batches for the three enzyme methods.

If batch variability in starch values was correctly interpreted to reflect lower specificity of the perchloric acid methods for starch, then starch values obtained with the standard method (PA3) probably were less accurate than with the purified enzymes (E1 and E2). Yet the small difference among starch values obtained with purified enzymes and the standard method indicated that E1, E2, and PA3 methods provided comparable accuracy. The overestimation of starch by the unpurified enzyme preparation (E3) was relatively minor and for most applications might not warrant additional purification.

Nonetheless, it is important to remember that when an extraction method is not 100% specific for starch, the accuracy of the starch value depends upon the relative proportions of starch and other interfering polymers in the plant tissue. Hence, it is possible that the accuracy of the less specific methods could vary across a much wider range than was found in this study.

Method Precision. Significant differences in precision were found for the six methods, both for the extraction of starch (sampling error) and for the colorimetric analysis of glucose (subsampling error). The precision of the colorimetric procedures in the different methods increased in the order PA2 < PA1 < PA3 < E1 < E2, E3 (Table I). That is, starch values obtained with anthrone were less precise than with *o*-toluidine, which were less precise than with *o*-dianisidine. The precision of colorimetry could not be determined for E3 and PA3 due to missing data, but was assumed to be comparable to other methods that used the same colorimetric reagents.

The precision of starch extraction increased in the order PA2 < PA1 < E3 < E1 < E2. The relative precision of extraction could not be determined for PA3 due to missing data. The low precision of starch extraction (high sampling error) observed in the PA2 method was interpreted to reflect difficulties in standardizing percolation rates of

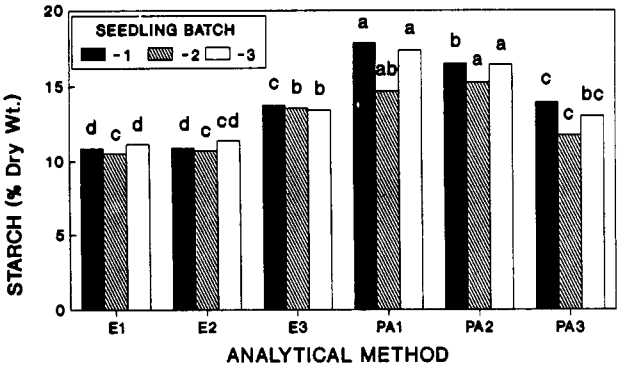

Figure 2. Mean starch concentrations in roots of three batches of ponderosa pine seedlings subjected to six analytical methods. Starch values were compared statistically among the analytical methods within each batch of seedlings. For each batch of seedlings, means with the same letter do not differ significantly as determined by the least-squares means procedure at $p < 0.05$.

Table I. Pairwise Comparison of Method Precision, Including Estimates of Subsampling Error (Colorimetry) and Sampling Error (Extraction)[a]

	error[b]			
	subsampling		sampling	
comparison	d_f	F	d_f	F
E1 vs E2	72, 71	2.19**	54, 53	2.53**
PA1 vs E1	144, 72	6.14**	54, 54	3.15**
PA1 vs E2	144, 71	13.46**	54, 53	7.94**
PA2 vs PA1	60, 144	1.87**	42, 54	1.88*
PA2 vs E1	60, 72	11.51**	42, 54	5.91**
PA2 vs E2	60, 71	25.24**	42, 53	14.92**
E3 vs E1	–[c]		54, 54	1.97*
E3 vs E2	–		54, 53	4.97**
PA1 vs E3	–		54, 54	1.60*
PA2 vs E3	–		42, 54	3.00**

[a] The F values are presented along with the associated degrees of freedom, for the hypothesis that the first variance is larger than the second. Pairwise comparisons were made by using the F ratio of variances (Dixon and Massey, 1983). [b] **, significant at $p < 0.01$; *, significant at $p < 0.05$. [c] Subsampling (aliquot error) could not be determined for the E3 or PA3 methods, and sampling error could not be determined for the PA3 methods due to missing data.

acid through the tissue sample caused by the compaction of glass wool fiber in the percolation column. Experimental error, that is, the amount of variation unaccounted for by the experimental design, was similar among all six analytical methods. Thus, the methods were similar to their ability to detect treatment effects.

Efficiency and Safety Considerations. The PA1 method was easiest to use in terms of technical simplicity and the equipment required. The PA1 method also was superior in terms of the efficiency of processing large numbers of samples. The PA2 and PA3 methods were the most labor intensive and required more elaborate laboratory equipment. All perchloric acid methods required protective clothing and equipment and special ventilation systems to remove acid fumes and reduce explosion hazards with perchloric acid.

The E1 and E2 methods were more labor intensive than PA1, PA2, and E3 due to the additional steps required to purify the enzymes. Even so, E1 and E2 were still less labor intensive than PA3. Commercial enzymes of high purity would greatly enhance the labor efficiency and convenience of the enzyme methods by precluding the need for additional purification steps.

Subsequent to the completion of this study, an investigation was conducted to assess the accuracy of starch extraction by numerous commercial enzyme preparations. One enzyme combination was identified that yielded highly accurate starch values without requiring additional purification of the commercial preparation. The enzymes included α-amylase (Sigma A-2643) and amyloglucosidase (Sigma A-3514) (S. Omi, personal communication). We recommend these products for enzymatic starch quantification. If purified enzymes are not available, and additional purification can not be carried out in the laboratory, then the PA3 method should be used to check starch values obtained with enzymes of unknown purity. Safety considerations for the enzyme methods included protective clothing and equipment and a standard ventilation hood, especially for colorimetry.

CONCLUSION

All six methods were similar in their ability to detect treatment differences (experimental error). However, methods using purified enzymes (E1, E2) were generally superior to perchloric acid methods in both accuracy and precision. New commercial preparations of highly purified enzymes also yield the enzyme methods more labor efficient than the perchloric acid methods. Although perchloric acid immersion (PA1) was superior for processing large numbers of samples, a larger sample size must be used to compensate for lower precision.

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APPENDIX: ENZYME METHOD 1

Enzyme method 1 was adapted from Dickson (1979) and Haissig and Dickson (1979, 1982).

General. This method uses MCW solution to remove sugars and other interfering compounds, hot ethanol to gelatinize the starch, a purified mixture of α-amylase–amyloglucosidase enzymes to hydrolyze the starch to glucose, and *o*-toluidine for colorimetric analysis. The color solution is stable for 30 min.

Solutions. *(1) MCW (Methanol/Chloroform/Water, 12:5:3 v/v/v).* Mix 1200 mL of reagent-grade methanol, 500 mL of chloroform, and 300 mL of distilled deionized water (dd H_2O).

(2) 47.5‰ Ethanol. Mix a 1:1 (v/v) ratio of dd H_2O and 95% ethanol.

(3) 30% Sodium Hydroxide. Dissolve 30.0 g of NaOH in 100 mL of dd H_2O.

(4) 0.05 M Sodium Acetate Buffer (NaOAc), pH 5.1. Add 2.84 mL of glacial acetic acid (HAc) to about 900 mL of dd H_2O. Adjust to pH 5.1 with addition of 30% NaOH (solution 3). Bring to total volume of 1000 mL with additional dd H_2O.

(5) α-Amylase/Amyloglucosidase Digestion Solution. This solution should contain 2 U/mL of amyloglucosidase (from *Aspergillus niger*, Sigma A3423) and 400 U/mL of α-amylase (from *A. oryzae*, Sigma A0273) in 0.05 M NaOAc buffer, pH 5.1. Use purified, assayed enzymes to prepare this solution. Test enzyme purity on cellulose, pectin, or hemicellulose, as described in Haissig and Dickson (1979). Enzyme units are in standard terms of micromoles of glucose liberated per milliliter of solution per minute.

(6) o-Toluidine. Add 1.5 g of thiourea to 940 mL of glacial acetic acid and stir to dissolve and mix. Add 60 mL of *o*-toluidine. Mix well and store in a dark bottle overnight in refrigerator before use. This reagent is also available commercially in premixed form.

(7) *Glucose Standards.* Make a stock solution of 0.1% glucose (0.5 g of benzoic acid and 0.5 g of glucose; bring to 500 mL of solution with dd H_2O). Prepare standards to concentrations of 0 (dd H_2O), 5, 10, 20, 40, and 80 mg of glucose/100 mL of solution. If necessary, adjust concentrations to match the range of concentrations in samples.

Procedure. *Initial Sample Preparation.* (1) See Materials and Methods for information on preparation of plant tissue.

(2) For additional information on purification and activity assay of commercial enzyme preparations, see procedures described in Haissig and Dickson (1979, 1982).

(3) Place clean 15-mL centrifuge tubes in a rack to hold samples and at least six standards.

(4) Weigh 100 mg of prepared plant sample into tubes and place rack in 100 °C oven for 1 h or in 50 °C oven overnight.

Remove Interfering Substances. (1) Pipet 5 mL of MCW solution into tubes containing samples, and shake, vortex, or place tubes briefly in a sonic bath. Allow the solution to sit for at least 10 min. Place the tubes into a centrifuge at 2200 rpm for 10 min and carefully pour off or aspirate the supernatant. Repeat the extractions at least two more times or until the supernatant is clear. On the final extraction use a glass micropipet to carefully and completely remove the supernatant.

(2) Evaporate the MCW completely from the samples by placing the rack of open tubes in an oven at 50 °C for 4 h. The oven must be in a well-ventilated area. Leave the tubes uncovered or the samples may explode.

(3) Remove the rack of tubes from the oven and allow to cool.

Gelatinization and Enzyme Reaction. (1) By use of a repipet-dilutor put 0.5 mL of 47.5% ethanol and 4 mL of dd H_2O into each tube and cap with a glass marble.

(2) Place the rack with tubes into a boiling water bath for 30 min, swirling tubes occasionally to rinse sample off the walls of the tubes. Cool tubes quickly to room temperature in a cold water bath.

(3) Add 1 mL of enzyme solution into each tube. Cover the tubes tightly with a rubber stopper and mix well on a vortex mixer.

(4) Immediately place the rack of samples into a 50 °C incubation oven for 48 h. Check periodically to ensure that the tubes remain tightly stoppered. Remove the rack from the oven after 48 h. The supernatant containing glucose is ready for colorimetric analysis.

Colorimetric Determination. (1) The sample extract may need to be diluted if the glucose concentration exceeds the range of the standards. The solutions are commonly diluted 1:9 (v/v, 1 mL of sample solution/9 mL of dd H_2O).

(2) Pipette 0.10-mL aliquots of glucose extract and glucose standards into another set of tubes or large scintillation vials (>15 mL). Preferably, run two or three replicates per sample.

(3) Under the fume hood (wear protective gloves, a face shield, lab coat, and rubber apron), pipet 5 mL of *o*-toluidine reagent into each tube and quickly cover with aluminum foil. Gently press the foil into the mouths of the tubes so as to make a slight dip in the foil.

(4) Place the rack of tubes into a shallow boiling water bath for 15 min to develop the color reaction. Remove the rack and allow it to rapidly cool in a cold water bath.

(5) Beginning with the standards, read absorbance values within 15–25 min for all of the samples on a spectrophotometer at 635 nm.

Calculations. (1) Determine regression equation relating glucose concentrations in standard solutions to absorbance readings on the spectrophotometer. The regression formula appears as

$$y_g = a + b(x)$$

where y_g is milligrams per 100 mL of glucose, a is the intercept, b is the slope, and x is absorbance units at 635 nm.

(2) Calculate glucose concentrations in the sample by substituting sample absorbance readings into the x variable in the regression equation above. The general equation for calculating the milligrams of starch in a sample is

$$\text{mg of starch/mg of sample } (y) = y_g d_f v h_f / \text{dw}$$

where y_g is the glucose concentration (mg/100 mL), d_f is the dilution factor (e.g., 10 for a 1:9 dilution), v is the original volume of starch extract (5.5 mL), dw is the original dry weight of the sample (mg), and h_f is the starch hydrolysis factor 0.9 (Volenec, 1986).

Abbreviations Used: dd H_2O, deionized distilled water; MCW, methanol/chloroform/water; rpm, revolutions per minute; U, units of enzyme activity as micromoles of glucose liberated per milliliter of enzyme solution; v/v/v, mixture of three components, each on a volume basis.

APPENDIX: ENZYME METHOD 2

Enzyme method 2 was adapted from Ebell (1969) and Haissig and Dickson (1979, 1982).

General. This method uses MCW solution to remove sugars and other interfering compounds, sodium hydroxide to solubilize the starch, a purified mixture of α-amylase–amyloglucosidase enzymes to hydrolyze the starch to glucose, and glucose oxidase/peroxidase–*o*-dianisidine for colorimetric analysis. The color solution is stable for several hours.

Solutions. (1) *MCW (Methanol/Chloroform/Water, 12:5:3 v/v/v).* Mix 1200 mL of reagent-grade methanol, 500 mL of chloroform, and 300 mL of distilled deionized water (dd H_2O).

(2) *0.1 N Sodium Hydroxide.* Dissolve 4.00 g of sodium hydroxide (NaOH) in 1000 mL of dd H_2O.

(3) *0.1 N Acetic Acid.* Add 5.75 mL of glacial acetic acid (HAc) to 950 mL of dd H_2O. Mix well and bring to a total volume of 1000 mL with dd H_2O.

(4) *30% Sodium Hydroxide.* Dissolve 30.0 g of NaOH in 100 mL of dd H_2O.

(5) *0.05 M Sodium Acetate Buffer (NaOAc), pH 5.1.* Add 2.84 mL of glacial acetic acid (HAc) to about 900 mL of dd H_2O. Adjust to pH 5.1 with addition of 30% NaOH (solution 4). Bring to a total volume of 1000 mL with dd H_2O.

(6) *0.1 M Sodium Phosphate Buffer, pH 7.0.* Dissolve 8.7 g of dibasic sodium phosphate (Na_2HPO_4) and 5.3 g of monobasic sodium phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$) in 1000 mL of dd H_2O.

(7) *α-Amylase/Amyloglucosidase Digestion Solution.* This solution should contain 2 U/mL of amyloglucosidase (from *A. niger*, Sigma A3423) and 400 U/mL of α-amylase (from *A. oryzae*, A0273) in 0.05 M NaOAc buffer, pH 5.1 (solution 5). Use purified, assayed enzymes to prepare this solution. Test enzyme purity on cellulose, pectin, or hemicellulose, as described in Haissig and Dickson (1979). Enzyme units are in standard terms of micromoles of glucose liberated per milliliter of solution per minute.

(8) *GOD/POD–o-Dianisidine.* Dissolve 50.0 mg of *o*-dianisidine dihydrochloride in 10.0 mL of dd H_2O (this step is needed to dissolve the dye). Mix the 10.0 mL of the

o-dianisidine solution with 990 mL of 0.1 M sodium phosphate buffer, pH 7.0 (solution 6). Add the appropriate amount of glucose oxidase (GOD) and peroxidase (POD) to yield a final concentration of 5 U of GOD/mL and 1 U of POD/mL and mix well. Refer to the Sigma catalog for the definitions of the GOD and POD units. [The approximate amounts of enzymes to add are 44–45 mg of glucose oxidase (from *A. niger*, type x, Sigma G-8135 or G-7141) and about 5 mg of peroxidase (horseradish, type II, Sigma P-8250).] The final solution is 0.16 mM o-dianisidine in 0.1 M sodium phosphate buffer, pH 7.0, containing about 5 U of GOD/mL and 1 U of POD/mL. (Note: Handle the o-dianisidine solution and the mixed enzyme solution with rubber gloves, lab coat, and other appropriate safety equipment. o-Dianisidine is carcinogenic.) This solution is stable for up to 1 month if stored in the refrigerator in a dark bottle.

(*9*) *75% Sulfuric Acid.* In a fume hood (wear protective gloves, face shield, lab coat, and rubber apron) add 250 mL of dd H_2O to a heavy glass stock bottle in an ice bath and very slowly (over an hour or two) add 750 mL of sulfuric acid (H_2SO_4) while stirring slowly with a magnetic stir bar.

(*10*) *Glucose Standards.* (*a*) *0.05 M Sodium Acetate Buffer, pH 5.1, with 0.1% Benzoic Acid.* Add 2.84 mL of glacial acetic acid to about 900 mL of dd H_2O. Adjust to pH 5.0 with 30% NaOH. Add 1.000 g of benzoic acid and mix until completely dissolved. Then add a few more drops of 30% NaOH to a final pH of 5.1. Adjust to 1000 mL with additional dd H_2O.

(*b*) *1% Glucose Solution.* Dissolve 0.100 g of anhydrous glucose in 10.0 mL of 0.05 M NaOAc buffer, pH 5.1, with 0.1% benzoic acid (solution 10a).

Using solutions 10a and 10b, prepare standards to concentrations of 0, 0.5, 1, 2, 4, 8, and 10 mg of glucose/100 mL of solution. The blank is made by using 0.5-mL aliquots of solution 10a. If necessary, adjust glucose concentrations of standards to match the range of glucose concentrations in samples. The glucose standards prepared with benzoic acid are stable for months if stored in the refrigerator.

Procedure. *Initial Sample Preparation.* (*1*) See Materials and Methods for information on preparation of plant tissue.

(*2*) For additional information on purification and activity assay of commercial enzyme preparations, see procedures described in Haissig and Dickson (1979, 1982), Pazur et al. (1984), and Takagi et al. (1971).

(*3*) Place clean 15-mL centrifuge tubes in a rack to hold samples and at least six standards.

(*4*) Weigh 100 mg of prepared plant sample into tubes and place rack in 100 °C oven for 1 h or in 50 °C oven overnight.

Remove Interfering Substances. (*1*) Pipet 5 mL of MCW solution into tubes containing samples and shake, vortex, or place tubes briefly in a sonic bath. Allow the solution to sit for at least 10 min. Place the tubes into a centrifuge at 2200 rpm for 10 min and carefully pour off or aspirate the supernatant. Repeat the extractions at least two more times or until the supernatant is clear. On the final extraction use a glass micropipet to carefully and completely remove the supernatant.

(*2*) Evaporate the MCW completely from the samples by placing the rack of open tubes in an oven at 50 °C for 4 h. Leave the tubes uncovered or the samples may explode.

Starch Solubilization and Enzyme Reaction. (*1*) Remove the tubes from the oven and add 4.0 mL of 0.1 N NaOH. Stopper the tubes and then mix on a vortex mixer until the starch pellet is broken up and suspended within the solution. Incubate the tubes in a 50 °C oven for 30 min with occasional swirling to solubilize the starch. Neutralize and adjust the solution to pH 5.1 by adding 5.0 mL of 0.1 N acetic acid. The starch is now dissolved in a 0.05 M NaOAc buffer, pH 5.1, and ready for enzyme digestion.

(*2*) Add 1.0 mL of enzyme solution to each of the tubes. Cover the tubes tightly with a rubber stopper and mix well on a vortex mixer.

(*3*) Immediately place the rack of tubes into an incubation oven at 50–55 °C and incubate for 24 h. Check periodically to ensure that the tubes remain tightly stoppered. Remove the rack from the oven after 24 h. The supernatant containing glucose is ready for colorimetric analysis.

Colorimetric Determination. (*1*) After enzyme digestion, vortex the sample solution to mix well and centrifuge at 2200 rpm for 10 min. Transfer a 0.5-mL aliquot of the sample solution to small test tubes (approximately 10 mL). Preferably, run two or three replicates per sample.

(*2*) If dilution is necessary to adjust the sample solutions into the range of the glucose standard curve, use 0.05 M NaOAc buffer, pH 5.1, to prepare the dilutions. The appropriate dilution depends on the anticipated amount of starch in the sample: a 1:2 dilution is good for low starch tissue (up to 1.5% starch, i.e., needles); a 1:5 dilution is suitable for tissue with 1–4.5% starch; a 1:10 dilution is appropriate for tissues with 4–9% starch; and a 1:20 dilution is suitable for high starch tissues (9–18% starch). Some tissues with even higher starch content will require more than a 1:20 dilution. The weight of sample tissue can also be reduced to lower the final glucose solution in the extract.

(*3*) Add 5.0 mL of the GOD/POD/o-dianisidine solution to each 0.5-mL sample aliquot. Stopper, mix well, and incubate at 37 °C for 30 min. The temperature must be controlled carefully since the GOD solution is inactivated at 39 °C. An alternative procedure is incubation at room temperature for 45 min. The glucose standards should be treated in the same manner (two 0.5-mL aliquots of each concentration plus 5.0 mL of GOD/POD/o-dianisidine solution).

(*4*) Transfer the tubes to a cold water bath and rapidly add 1.0 mL of 75% sulfuric acid to each tube to stabilize the color. Pipetting of the viscous acid can be improved by cutting off 1 mm of the pipet tip.

(*5*) Once cooled, stopper the tubes and vortex to mix well. Read the absorbance at 525 nm versus a buffer–reagent blank.

Calculations. (*1*) Determine regression equation relating glucose concentrations in standard solutions to absorbance readings on the spectrophotometer. The regression formula appears as

$$y_g = a + b(x)$$

where y_g is milligrams per 100 mL of glucose, a is the intercept, b is the slope, and x is the absorbance units at 525 nm.

(*2*) Calculate glucose concentrations in the sample by substituting sample absorbance readings into the x variable in the regression equation above. The general equation for calculating the milligrams of starch in a sample is

$$\text{mg of starch/mg of sample } (y) = y_g d_f v h_f / \text{dw}$$

where y_g is the glucose concentration (mg/100 mL), d_f is the dilution factor (e.g., 10 for a 1:9 dilution), v is the

original volume of starch extract (10 mL), dw is the original dry weight of the sample (mg), and h_f is the starch hydrolysis factor 0.9 (Volenec, 1986).

Abbreviations Used: dd H_2O, deionized distilled water; GOD, glucose oxidase; MCW, methanol/chloroform/water; POD, peroxidase; rpm, revolutions per minute; U, units of enzyme activity as micromoles of glucose liberated per milliliter of enzyme solution; v/v/v, mixture of three components, each on a volume basis.

APPENDIX: ENZYME METHOD 3

Enzyme method 3 was adapted from Rowe (1980).

General. This technique uses hot ethanol to remove soluble sugars and other interfering compounds, boiling water to gelatinize the starch, unpurified amyloglucosidase enzyme to hydrolyze the starch to glucose, and glucose oxidase/peroxidase–*o*-dianisidine for colorimetric analysis. This color solution is stable for several hours.

Solutions. (*1*) *80% Ethanol.* Bring 843 mL of 95% ethanol to a volume of 1 L with distilled deionized water (dd H_2O).

(*2*) *30% Sodium Hydroxide.* Dissolve 30.0 g of sodium hydroxide in 100 mL of dd H_2O.

(*3*) *0.05 M Sodium Citrate Buffer, pH 4.5.* Dissolve 10.5 g of citric acid in about 900 mL of dd H_2O. Adjust the solution to pH 4.5 by using 30% sodium hydroxide (solution 2). Bring to a volume of 1 L with dd H_2O.

(*4*) *Amyloglucosidase Digestion Solution.* Mix 2 g of amyloglucosidase (Sigma A-7255) into 1 L of ice-cold 0.05 M sodium citrate buffer, pH 4.5 (solution 3). This solution provides enzyme activity in excess of that required to attain complete starch digestion for any plant tissue. As supplied by Sigma, this chemical contains approximately 40% diatomaceous earth, 33% protein, 5% water, and 22% starch/sugar. The starch and diatomaceous earth can be easily removed by centrifuging this chemical at 1500 rpm for 5 min immediately after mixing. This procedure will eliminate high blank values.

(*5*) *GOD/POD–o-Dianisidine (Sigma Kit 510A).* Dissolve the contents of the buffer/enzyme capsule in 100 mL of dd H_2O and mix slowly with a stirring rod. When dissolved, mix in 1.6 mL of *o*-dianisidine solution. (Note: Handle the *o*-dianisidine solution and the mixed enzyme solution with rubber gloves, lab coat, and other appropriate safety equipment. *o*-Dianisidine is carcinogenic.)

(*6*) *Glucose Standards.* Mix 50.0 mg of anhydrous glucose in dd H_2O to a volume of 50 mL. This stock solution has 1 mg of glucose/mL of solution. An alternative to this is to use the glucose standard supplied by Sigma with the 510 kit. This solution is also 1 mg of glucose/mL, but it has a preservative added that makes the stock solution stable. Dilute the stock solution to a concentration of 10 mg of glucose/100 mL of solution. Make a 0 blank by using sodium citrate buffer, and by serial dilution make standards with 0.3, 0.6, 1.25, 2.5, 5.0, and 10.0 mg of glucose/100 mL of solution. Absorbance readings for samples should fall within the range of the standards. If not, modify either the glucose concentration in the standards or the weight of sample.

Procedure. *Initial Sample Preparation.* (*1*) See Materials and Methods for information on preparation of plant tissue.

(*2*) Weigh 25 mg of sample into 15-mL centrifuge tubes.

Remove Interfering Substances. (*1*) In a fume hood, add 10 mL of 80% ethanol to each sample. Place in a water bath at 95 °C for 10 min. Cap each centrifuge tube with a glass marble to ensure adequate reflux of the boiling alcohol solution. The fume hood removes alcohol vapor and keeps the marbles cool enough to condense the boiling alcohol solution.

(*2*) Centrifuge the tubes at 1500 rpm for 5 min. Remove the supernatant by aspiration, being careful not to remove any of the tissue sample. Repeat the extraction at least two more times or until the supernatant is clear. Make sure the tissue is resuspended each time alcohol is added to the tube.

(*3*) Dry the tissue samples by placing the uncovered tubes in a hot water bath.

Gelatinization and Enzyme Reaction. (*1*) Add 5 mL of dd H_2O to each sample and cap the tubes with marbles.

(*2*) Place the tubes in a boiling water bath for 45 min. A standard laboratory autoclave can also be used in place of the water bath. The autoclave is run for 45 min at 15 psi to maintain a temperature of 121 °C.

(*3*) Cool the tubes in an ice–water bath. If left in the autoclave overnight, the tubes will remain sterile and will be ready for use the next morning.

(*4*) Add 5 mL of the amyloglucosidase solution to each tube and cover with paraffin film. Mix the tissue/enzyme mixture by gently inverting the tube. Make sure that any tissue fragments are uniformly suspended in the solution.

(*5*) Incubate the tubes overnight in a 30 °C water bath.

Colorimetric Determination. (*1*) Add approximately 5 mL of dd H_2O to bring each tube to a volume of exactly 10 mL. Mix well by inverting the tubes. Centrifuge the tubes at 1500 rpm for 5 min. Transfer aliquots of 0.25 mL to small test tubes.

(*2*) Add 2.5 mL of GOD/POD–*o*-dianisidine to each tube, mix well, and incubate in a 37 °C water bath for 30 min.

(*3*) After 30 min, read the absorbance values on a spectrophotometer at 450 nm.

Calculations. (*1*) Determine regression equation relating glucose concentrations in standard solutions to absorbance readings on the spectrophotometer. The regression formula appears as

$$y_g = a + b(x)$$

where y_g is milligrams per 100 mL of glucose, a is the intercept, b is the slope, and x is the absorbance units at 450 nm.

(*2*) Calculate glucose concentrations in the sample by substituting sample absorbance readings into the x variable in the regression equation above. The general equation for calculating the milligrams of starch in a sample is

$$\text{mg of starch/mg of sample } (y) = y_g d_f v h_f/\text{dw}$$

where y_g is the glucose concentration (mg/100 mL), d_f is the dilution factor (e.g., 10 for a 1:9 dilution), v is the original volume of starch extract (10 mL), dw is the original dry weight of the sample (mg), and h_f is the starch hydrolysis factor 0.9 (Volenec, 1986).

Abbreviations Used: dd H_2O, deionized distilled water; GOD, glucose oxidase; POD, peroxidase; rpm, revolutions per minute.

APPENDIX: PERCHLORIC ACID METHOD 1, IMMERSION

This method was adapted from Viles and Silverman (1949) and McCready et al. (1950).

General. This method uses hot ethanol to remove sugars and other interfering substances and to gelatinize the starch, immersion in 35% perchloric acid to solubilize starch, 75% sulfuric acid to hydrolyze starch, and anthrone reagent for colorimetric analysis. The color solution is stable for up to 6 h.

Solutions. (*1*) *100% Acetone.* Reagent grade.

(*2*) *80% Ethanol.* Dilute 843 mL of 95% ethanol to a volume of 1 L with distilled deionized water (dd H_2O).

(*3*) *35% Perchloric Acid.* Slowly add 500 mL of concentrated perchloric acid to 500 mL of dd H_2O in a heavy glass bottle on a magnetic stir plate, preferably in an ice–water bath to speed cooling. CAUTION: Do this under a ventilation hood specially designed for use with perchloric acid. Wear protective clothing including a face shield, gloves, lab coat, and rubber apron.

(*4*) *Anthrone Reagent.* Slowly add 500 mL of concentrated sulfuric acid to 200 mL of water in a heavy glass bottle on a magnetic stir plate, preferably in an ice–water bath to speed cooling. This makes 655 mL of 72% sulfuric acid. Add 1.146 g of anthrone powder to the acid solution (or for less solution, reduce acid and anthrone proportionally) and allow to mix for 2 or more h on a magnetic stir plate away from bright light. CAUTION: Do this under a ventilation hood and wear protective clothing including a face shield, gloves, lab coat, and rubber apron.

(*5*) *Glucose Standards.* Make a stock solution by mixing 500 mg of D-glucose in 1 L of dd H_2O. Then make standards ranging from 0 (dd H_2O + perchloric acid) to 50 mg of glucose/100 mL of solution by mixing (in 100 mL of volumetric flasks) 35 mL of 35% perchloric acid and varying proportions of dd H_2O and glucose stock solution.

Procedure. *Initial Sample Preparation.* (*1*) See Materials and Methods for information on preparation of plant tissue.

(*2*) Weigh 0.50 g of plant tissue onto a piece of Whatman No. 1 filter paper 9 cm in diameter. Fold the filter paper to form a tube with one narrow end and one wide end. Insert the narrow end into the wide end so as to seal the plant tissue inside. The sample is now ready for extraction.

Remove Interfering Substances. (*1*) Place the sample folded in filter paper into a 25-mL test tube with 10 mL of acetone and allow to sit several hours or overnight. Pour off the acetone and repeat this step until the extract is colorless.

(*2*) Place the acetone-extracted sample in a 100-mL test tube, cover with 80% ethanol, and place in a water bath to boil gently. Cap the tubes with a screw-top lid to ensure adequate reflux of the boiling alcohol solution, but do not tighten hard enough to seal the tube. After 1–2 h, pour off the supernatant and repeat the extraction until the solution is colorless. Perform three extractions over a 4-h period.

Starch Extraction and Solubilization. (*1*) Use three rinses, 5 mL each, of 35% perchloric acid to wash the sample off the filter paper into a 125-mL Erlenmeyer flask. Make two more rinses of 5 mL to remove the sample from the sides of the flask. Cover the flask with foil and shake at low speed on an orbital shaker for 0.5 h.

(*2*) Suction filter the solution in the flask through a Whatman No. 1 filter, rinsing the flask twice and the sample in the filter once. Transfer the solution to a 100-mL volumetric flask and bring to volume with dd H_2O. Cover the flask and shake solution to mix well.

Colorimetric Determination. (*1*) Add 0.5 mL of each starch solution to a 15-mL test tube in a rack immersed in ice water. Include a set of at least six glucose standards from 0 to 50 mg/100 mL with each batch of samples.

(*2*) Add 5 mL of anthrone solution to each tube, cover the test tubes loosely with a plastic cap, and mix briefly on a vortex mixer. If the solution turns cloudy white, use a smaller aliquot of the sample solution (e.g., 0.25 mL of sample extract diluted to 0.5 mL). Correct for this dilution as described in the calculations.

(*3*) Place the rack of test tubes into a large container of vigorously boiling water 10 cm deep for 12 min and then set the rack in ice water and let the samples cool. Cover samples to prevent exposure to bright light.

(*4*) Read the samples at 625 nm on a spectrophotometer. The samples can be stored in a refrigerator for several hours if readings cannot be taken immediately.

Calculations. (*1*) Determine regression equation relating glucose concentrations in standard solutions to absorbance readings on the spectrophotometer. The regression formula appears as

$$y_g = a + b(x)$$

where y_g is milligrams per 100 mL of glucose, a is the intercept, b is the slope, and x is the absorbance units at 625 nm.

(*2*) Calculate glucose concentrations in the sample by substituting sample absorbance readings into the x variable in the regression equation above. The general equation for calculating the milligrams of starch in a sample is

$$\text{mg of starch/mg of sample } (y) = y_g d_f v h_f/\text{dw}$$

where y_g is the glucose concentration (mg/100 mL), d_f is the dilution factor (e.g., 10 for a 1:9 dilution), v is the original volume of starch extract (100 mL), dw is the original dry weight of the sample (mg), and h_f is the starch hydrolysis factor 0.9 (Volenec, 1986). If the sample material consists of fresh tissue homogenized in liquid nitrogen, then determine the fresh weight to dry weight ratio for the sample material to calculate dw.

Abbreviations Used: dd H_2O, deionized distilled water.

APPENDIX: PERCHLORIC ACID METHODS 2 AND 3, PERCOLATION AND IODINE PRECIPITATION

These methods were adapted from Pucher et al. (1948), Viles and Silverman (1949), Hoffpauir (1956), Hansen and Moller (1975), and Hassid and Neufeld (1964).

General. Perchloric acid method 2 is the same as perchloric acid method 1 except that in method 2 the acid is percolated through the sample and diluted in water to prevent starch hydrolysis. Method 3 is the same as method 2 except that starch fragments solubilized by acid percolation are precipitated with an iodine solution to remove interference by pectin and other polymers.

Solutions. (*1*) *100% Acetone.* Reagent grade.

(*2*) *80% Ethanol.* Dilute 843 mL of 95% ethanol to a volume of 1 L with distilled deionized water (dd H_2O).

(*3*) *35% Perchloric Acid.* Slowly add 500 mL of concentrated perchloric acid to 500 mL of dd H_2O in a heavy glass bottle on a magnetic stir plate, preferably in an ice–water bath to speed cooling. CAUTION: Do this under a ventilation hood specially designed for use with perchloric acid. Wear protective clothing including a face shield, gloves, lab coat, and rubber apron.

(*4*) *Anthrone Reagent.* Slowly add 500 mL of concentrated sulfuric acid to 200 mL of water in a heavy glass bottle on a magnetic stir plate, preferably in an ice–water bath to speed cooling. This makes 655 mL of 72% sulfuric acid. Add 1.146 g of anthrone powder to the acid solution (or for less solution, reduce acid and anthrone proportionally) and allow to mix for 2 or more h on a magnetic stir plate away from bright light. CAUTION: Do this under a ventilation hood and wear protective clothing including a face shield, gloves, lab coat, and rubber apron.

(5) *Glucose Standards.* Make a stock solution by mixing 500 mg of D-glucose in 1 L of dd H_2O. Then make standards ranging from 0 (dd H_2O + perchloric acid) to 50 mg of glucose/100 mL of solution by mixing (in 100-mL volumetric flasks) 35 mL of 35% perchloric acid and varying proportions of dd H_2O and glucose stock solution.

(6) *20% Sodium Chloride.* Add 20 g of NaCl to 80 mL of dd H_2O and mix. Bring to a volume of 100 mL with water and continue to mix until all of the NaCl dissolves.

(7) *Potassium Iodide (I–KI).* Add 7.5 g of I and 7.5 g of KI to a small amount of dd H_2O and grind in a mortar. Bring to 250 mL volume with dd H_2O. Filter to remove undissolved I.

(8) *EtOH–NaCl Solution.* Add 80 mL of dd H_2O and 50 mL of 20% NaCl (solution 6) to 350 mL of 95% EtOH for a total of 500 mL of solution.

(9) *EtOH–NaOH Solution.* Add 100 mL of dd H_2O and 25 mL of 5 N NaOH to 350 mL of 95% EtOH for a total of 500 mL of solution.

Procedure. Follow the steps for sample preparation and removal of interfering substances described under Perchloric Acid Method 1.

Starch Extraction and Solubilization—Method 2 + Method 3. (1) Rinse residue from the filter paper into a 5-cm section of Tygon tubing packed at the bottom with glass wool. Then pack the top of the tube with glass wool. Attach adaptors to each end of the sample holder and connect to 3-μm dialysis tubing. Attach one end of the 3-μm tubing to a dialysis pump to percolate 25 mL of 35% perchloric acid through the sample at a rate of 1.5 mL/h. The open end of the 3-μm tubing is suspended over a flask containing 75 mL of dd H_2O to collect the solubilized starch. This method is more accurate and easier to calibrate than the buret method described by Hansen and Moller (1975).

Colorimetric Determination. For perchloric acid method 2, colorimetric reaction with the anthrone reagent can now be performed as described under Perchloric Method 1. For perchloric acid method 3, KI precipitation should be carried out as described below.

Iodine Precipitation of Solubilized Starch. (1) Place 5 mL of solubilized starch solution in a 15-mL centrifuge tube. Add 5 mL of 20% NaCl. Add 2 mL of iodine solution, mix briefly on a vortex, and let stand overnight in a refrigerator.

(2) Centrifuge the tubes at high speed for 10 min. Decant the supernatant with a micropipet. Carefully wash and decant the pellet three times with 2 mL of EtOH–NaCl solution. Add 2 mL of EtOH–NaOH solution and mix on a vortex until the blue color vanishes.

(3) Centrifuge the tubes at high speed for 10 min, decant, and wash twice more with EtOH–NaCl to remove iodine and NaOH. Add 0.5 mL of 35% perchloric acid and mix. Add 2 mL of water and mix.

(4) The extract is now ready for colorimetric determination as described under Perchloric Acid Method 1. The standards for this method should range from 0 to 100 mg/100 mL of solution.

Calculations. (1) For method 2, follow calculations as described under Perchloric Acid Method 1. For method 3, multiply by a dilution factor (d_f) of 0.5. This accounts for the doubling of the starch concentration when the starch pellet from 5 mL of the sample extract is redissolved in 2.5 mL of acid and water (3 above).

If the sample material consists of fresh tissue homogenized in liquid nitrogen, then determine the fresh weight to dry weight ratio for the sample material to calculate dw.

Abbreviations Used: dd H_2O, distilled deionized water.

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