Direct Starch Analysis Using DMSO Solubilization and Glucoamylase

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ABSTRACT

A new enzyme-based method for the direct analysis of corn, wheat, and potato starch and wheat flour is described which employs dimethylsulfoxide (DMSO) to dissolve the sample. Quantitative enzymatic hydrolysis is performed in the DMSO-citrate buffer solvent, and the glucose produced is measured either by an automated colorimetric technique or by a glucose oxidase technique. This solubilization method avoids starch degradation, and glucoamylase allows quantitative conversion to glucose. The sample in mg. quantities is dissolved in warm DMSO. The conditions for optimum glucoamylolysis in this solvent are described. A technique for measuring the reducing sugars or only glucose present before enzymatic hydrolysis is also described. Comparative data relating this technique to polarimetric and gravimetric methods of starch analysis are discussed.

Techniques for the quantitative analysis of starch can generally be classified as polarimetric or hydrolytic techniques. In either case, the starch polymer must be dissolved or dispersed in a manner that is compatible with subsequent reactions or measurements. Acidic gelatinization has been used extensively for polarimetric techniques, although calcium chloride solubilization is preferred. Acidic conditions are often used to degrade the starch polymer to reducing saccharides, but the concomitant hydrolysis of noncarbohydrate ingredients such as proteins often interferes with colorimetric, titrimetric, or gravimetric measurement of the reducing saccharides. Enzymatic techniques for starch liquefication and hydrolysis have also been evaluated. Enzymatic liquefication is hindered by the thermal instability of amylolytic enzymes at the temperatures of optimum liquefication (1). Enzymatic hydrolysis by alpha- or beta-amylase is incomplete, usually because terminal alpha

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[1]Other names for this enzyme are amyloglucosidase, glucamylase, and gamma-amylase. Glucoamylase is the recommended nomenclature by the Commission on Enzymes and will be used in this article.

(1 → 4) bonds or alpha (1 → 6) bonds at branch points resist hydrolysis. Furthermore, the maltose produced by alpha-amylolysis is a competitive inhibitor of alpha-amylase. Donelson and Yamazaki have adapted their damaged-starch technique to an analytical method for starch in wheat by gelatinizing the sample with heat prior to diastase hydrolysis (2). The conversion to reducing sugars is about 63% complete and the utility of the method requires strict adherence to the prescribed treatment of the sample. Their enzymatic results were converted by a correction factor of 1.58 and were slightly higher than polarimetric results.

Glucoamylase[1] hydrolyzes both the alpha (1 → 4) and alpha (1 → 6) glucosidic linkages in starch to form glucose as the exclusive product. Glucoamylase has been used after heat-gelatinization of starch for starch analysis in dietetic foods (3), in ruminant digesta (4), in a wide variety of other products (5), and to estimate starch gelatinization (6). It is now clear that glucoamylase is applicable to starch hydrolysis in a wide variety of matrices. The glucose produced after 3 hr. of incubation with glucoamylase at 50°C. was identified by thin-layer chromatography as the only saccharide produced, and was quantitatively measured by a glucose oxidase-peroxidase procedure (3). Glucoamylase was also shown to hydrolyze sugars of the maltose series present in flour, but did not decompose glucose (3).

Although the chemical specificity and the quantitative extent of glucoamylolysis has been demonstrated, the solubilization of starch has employed the time-consuming heat-gelatinization process. The extent and rate of dissolution of starch in dimethylsulfoxide (DMSO) at room temperature (7) and a description of the molecular interactions of this solvent with amylose and amylopectin (8) have been reported.

This paper describes a new method for quantitative starch analysis that employs rapid solubilization of starch in aqueous DMSO, quantitative hydrolysis with glucoamylase, and either automated colorimetric analysis of reducing sugars or enzymatic analysis of the glucose produced. Optimum conditions for analysis and comparisons to polarimetric and gravimetric techniques are discussed.

MATERIALS AND METHODS

Corn starch, potato starch, and glucoamylase (*Rhizopus*) were obtained from Sigma Chemical Co., St. Louis, Mo. Glucose oxidase-peroxidase was obtained from Worthington Biochemical Corp., Freehold, N.J., as the Glucostat Special kit. In this purity state, glucose oxidase is essentially free of carbohydrase activity. Aytex wheat starch was obtained from General Mills. All other chemicals were reagent grade and used without additional purification.

The sodium 2,4-dinitrophenolate reagent for automated reducing sugar analysis was prepared by dissolving with heat 4.0 g. of the reagent in 20 ml. of 50% sodium hydroxide and 200 ml. of water. One hundred grams of potassium sodium tartrate was dissolved in this solution and a few drops of Brij-35 (Technicon Corp., Chauncey, N.Y.) surfactant were added to improve the subsequent flow pattern in the AutoAnalyzer before dilution to 1 liter with distilled water. The citrate buffer was prepared as 0.05M citric acid with sodium hydroxide used to adjust the pH to 4.80 at 25°C. The DMSO solution was prepared just before each use by mixing 90 ml. anhydrous DMSO and 10 ml. water, and was kept in a glass-stoppered flask.

Thin-layer chromatograms employed a Kieselguhr G adsorbent, a butanol-ethanol-water (50-30-20) solvent, and an anisaldehyde spray reagent. Gas-chromatographic analysis of the starch hydrolysates used conventional trimethylsilylation techniques and a stainless-steel column 1/8 in. o.d. X 6 ft., packed with 10% UC-W98 (methylvinylsilicone) on Chromosorb AW (80- to 100-mesh) run at 210°C. with flame ionization detection.

The polarimetric experiments followed either AOAC Method 13.035 or AACC Method 76-20; a Rudolph polarimeter calibrated with National Bureau of Standards sucrose was used. The glucostat special reagent kit was prepared by dissolving one vial of Chromogen and one vial of Glucostat Special in 50 ml. of distilled water.

Dissolution

The starch and flour samples were Soxhlet-extracted for 16 hr. with an equivolume mixture of methanol, butanol, and benzene. The extract was dried and extracted with chloroform, and the lipid content was calculated directly as the percent soluble in chloroform. The final sample concentration of 0.03 to 0.04% (w./v.) was established by mixing an accurately weighed sample of starch or flour with 20 ml. of 90% DMSO preheated to 55°C. in a 50-ml. flask. This solution was covered, kept at 55°C. for 20 min., and diluted, with maceration, to 50-ml. total volume with citrate buffer (pH 4.80), also preheated to 55°C. This solution was placed in a 45°C. bath and after it had cooled to that temperature, the volume was readjusted to 50 ml. with a solution of 36% DMSO in the citrate buffer, also heated to 45°C. Under these conditions, the starch was completely dissolved and the solution had an apparent pH of 4.4.

Enzymatic Hydrolysis

The glucoamylase reagent was stored under refrigeration and was prepared by dissolving 200 to 300 mg. in citrate buffer to a total volume of 100 ml., filtering through Whatman GF/A glass-fiber paper, and rediluting to 100 ml. with citrate buffer. This solution was heated to 45°C. and mixed with an equal volume of starch solution for a 20-min. enzyme reaction at 45°C. At this point in the method, the solution is 18%, by volume, DMSO. A 0.04% solution of dextrose was treated in an identical manner to serve as a calibration point for the colorimetric measurement of glucose by the 2,4-dinitrophenolate method.

Initial Reducing Value

The quantity of glucose produced by glucoamylolysis was corrected for the reducing substances naturally present in starch and flour by a procedure identical with that above, except that glucoamylase was omitted from the system. At the concentrations used for hydrolysis, the absorbance due to the natural reducing substances was too low for accurate measurement on the AutoAnalyzer colorimeter. A standard addition technique was adopted to improve the accuracy of measuring the initial reducing value. The sample size was increased from 30 to 40 mg. to 100 mg. and a 0.04% glucose solution replaced the glucoamylase in the citrate buffer, giving a final concentration after mixing of 1 mg. per ml. starch and a 0.02% glucose solution. In this way, the absorbance differences between the starch solutions and a 0.02% glucose solution were measured in a more accurate range of the absolute absorbance scale.

Automated Glucose Analysis

The conventional Technicon AutoAnalyzer modules were used as shown in Fig. 1 for the thermochromic colorimetric procedure for reducing sugars based on sodium 2,4-dinitrophenolate (9). Samples of the starch and flour solutions and solutions for initial reducing value and for calibration were placed directly on the sampler module.

Enzymatic Glucose Analysis

Standard solutions of glucose were prepared in the concentration range of 0 to 10 mg. glucose per 100 ml. by a procedure identical with that for starch samples to compensate for any absorption at 425 mμ due to DMSO, citrate, or glucoamylase. The concentration of glucose produced by glucoamylase was reduced by one-half by dilution with distilled water before the glucose oxidase-peroxidase method was used.

The concentration of glucose present in samples before glucoamylolysis is measured similarly by replacing glucoamylase with an equivalent amount of DMSO and citrate buffer.

RESULTS AND DISCUSSION

DMSO Dissolution

The temperature of dissolution was selected to approximate that of maximum glucoamylase activity. Although the results of starch dissolution in 90% DMSO show that the percent solubility is minimal at neutral apparent pH values (8) and that about 16 to 20 hr. is required for greater than 90% solubility (7,8), our results show that at 55°C., the DMSO solubilization technique is essentially complete within 5 min. at an apparent pH value of 4.4. Under these conditions, the maximum solubility of these starches is about 0.1% by weight.

Prolonged soaking in anhydrous DMSO gave reduced enzymatic conversion of starch, which agrees with Leach and Schoch's observation that anhydrous DMSO was capable of suspending starch granules but did not swell or disperse the granule (7). Starch digestion in 90% DMSO at apparent pH values of 2 and 10 also reduces the susceptibility of the starch to glucoamylase hydrolysis.

The percent glucose recovered after solubilization and enzymatic hydrolysis was

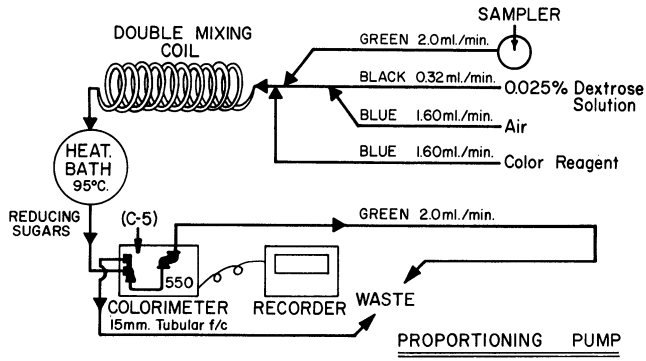

Fig. 1. Flow diagram of automated colorimetric analysis of reducing sugars.

measured after 5, 10, 15, 20, and 40 min. of dissolution in 90% DMSO at 55°C. These glucose data vary slightly with starch variety, but the conversion after 5 min. of dissolution is nearly complete. Twenty-minute dissolution time was selected as the most appropriate for the three starch varieties and two flour samples studied here.

Casu et al. (10) have shown that amylose maintains its helical configuration in DMSO solutions. There is substantial evidence that this is also the case for amylopectin (8). In addition, Leach and Schoch have reported, on the basis of specific viscosity data, that a corn-starch solution is virtually unchanged after standing 60 days in 90% DMSO at room temperature (7). They also report that potato starch is degraded by DMSO and discuss a DMSO scission of primary linkages in the branched portion of the polymer.

Differences between corn and potato starch were also observed in this study at 55°C. Phase-contrast microscopy of the starch solutions after 20 min. at 55°C. shows that the granules have lost their birefringence and that wheat and potato starch have lost their granular appearance entirely. Corn starch still displays some granular appearance after 20 min. at 55°C., but the subsequent enzymatic digestion serves to complement DMSO in completing the dissolution.

Thin-layer chromatography (TLC) of the starch solutions shows no evidence of starch degradation, although the specific viscosity data (7) are probably more diagnostic of degradation than TLC.

Glucoamylolysis

Glucoamylase was filtered prior to the final dilution in citrate buffer to remove an insoluble material. The infrared spectrum and microscopic morphology of the insoluble material indicates a siliceous material. The specific activity of glucoamylase was not determined at these solvent conditions of 18% (by volume) DMSO, but the enzymatic hydrolysis is complete in 10 min. or less. The glucoamylase activity, measured as mg. glucose produced after 20 min. of reaction, increased regularly with temperature over the range of 15° to 45°C. There were some varietal differences among the starches in the rate of activity change with temperature. Enzyme activity on Aytex starch appeared to be less sensitive to temperature changes; activity on potato starch was the most sensitive. At 45°C., all three starches were at or near the temperature of maximum glucoamylase activity. At 55°C., the enzyme lost 25% of the activity toward starch hydrolysis that it had at 45°C.

The ratio of enzyme:substrate in this study ranged from 2.5:1 to 5:1, and the extent of starch conversion was relatively insensitive to variations within this range. The starch sample weight was selected to give sufficient absorbance at 550 mμ for accurate measurement of the glucose produced. Excess glucoamylase was used routinely to ensure rapid and complete hydrolysis and to reduce the effect of variations in experimental technique. Gas-chromatographic analysis of the starch hydrolysates, after silylation, confirmed that glucose was the only product of glucoamylase hydrolysis.

The activity of glucoamylase in a solvent containing 18% DMSO is remarkable and interesting. In this case, the properties of the solute and the solvent separately and collectively contribute to produce a durable and useful chemical system. No

significant change in glucoamylase activity was observed during 4 months' use, and no special precautions to avoid inhibitive contamination were taken. At 18% DMSO, by volume, DMSO will be in the dihydrate ($DMSO \cdot 2H_2O$) form. The water molecules hydrated to DMSO are more polar than normal water molecules and thus may serve to stabilize the stereochemical configuration of glucoamylase in the preferred active orientation. Collectively, the solvent properties for starch and compatibility with glucoamylase make DMSO a useful solvent for this and other applications.

Automated Reducing Sugar Analysis

The color developed by the reaction of sodium 2,4-dinitrophenolate was measured as indicated in Fig. 1. The details of this procedure will be described in a forthcoming publication[2]. A standard solution of dextrose served to calibrate the absorbance, and each series of samples contained two dextrose standards, as the first and last samples analyzed, to evaluate the magnitude of any instrumental drift.

The formula to calculate percent recovery data for the automated procedure shown in Table I is:

$$\% \text{ Starch recovery} = \frac{[\text{glucose}]\ 0.9 \times 10^4}{\text{sample weight}\ [100 - \Sigma(\% \text{ nonstarch components})]}$$

where the factor of 0.9 is the accepted factor (11) to account for the molecular weight difference between a starch monomeric unit in a polysaccharide and the glucose produced. The formula to calculate percent recovery by the enzymatic technique is:

$$\% \text{ Starch recovery} = \frac{[\text{glucose}]\ 0.9 \times 4 \times 10^4}{\text{sample weight}\ [100 - \Sigma(\% \text{ nonstarch components})]}$$

and differs only in the dilution factors necessitated by the more sensitive glucose oxidase-peroxidase method. The amount of starch on the "as-received" basis would not include the correction for nonstarch components and would be calculated as the product of glucose produced and the 0.9 factor.

In either formula, the glucose concentration can be corrected for the amount of reducing sugar or glucose initially present in the sample prior to glucoamylolysis. All starch and flour samples were fat-extracted. The sum of the nonstarch components includes water, protein, and ash, and these values were measured by conventional techniques on the fat-extracted sample.

The extraction of these samples with methanol, butanol, and benzene removes components of the sample other than lipids. For flour samples, these components represent about 1% of the original sample weight. This fraction, soluble in methanol-butanol-benzene but insoluble in chloroform, does not contain significant amounts of starch or glucose as measured by the DMSO-glucoamylase and the glucose-oxidase technique. The microscopic morphology suggests a mixture of protein and cellulosic materials. Although it is not clear that this

[2]Oborn, R. E., Libby, R. A., Ernst, J. M., and Henderson, J. C. (to be published).

chloroform-insoluble fraction contains all the cellulosic materials or any of the flour pentosans, the nonstarch components of the fat-extracted samples do not include the cellulose or pentosan levels.

Separate experiments to study the important variables in glucose analysis by reaction with 2,4-dinitrophenolate showed that pH, temperature, and the amounts of DMSO, glucoamylase, and citrate must be controlled. The pH of the reaction is controlled by the composition of the color reagent. The temperature is controlled precisely by the AutoAnalyzer. The three reagents react with the color reagent, DMSO having the most pronounced effect. DMSO-water solutions, without glucose, produced an absorbance increase at 550 mμ with 2,4-dinitrophenolate which increased as the % DMSO increased from 85 to 95%. Thus, it is very important to avoid conditions which allow DMSO to absorb water during the analysis. All operations were performed in closed flasks, and although no special precautions were taken to dry DMSO before use, all possible precautions were employed to maintain the DMSO composition at 90% during dissolution, 36% during hydrolysis, and 18% during analysis. DMSO-water mixtures were freshly prepared before use and were never used for more than 1 day. Glucoamylase and citrate buffer also enhanced the absorbance of 2,4-dinitrophenolate at 550 mμ, but to a lesser extent than DMSO. Glucoamylase and citrate buffer were present in all solutions at fixed concentrations and thus, with careful control of the DMSO-water composition, the absorbance increases for starch samples were related directly to the glucose formed by glucoamylase.

This work was performed to establish DMSO dissolution conditions for starch and to optimize conditions for glucoamylolysis that provide complete conversion of starch to glucose. The automated colorimetric technique and the glucose-oxidase technique for glucose analysis used here were entirely adequate as described, but other techniques for glucose analysis may also be compatible with this chemical system and may be used appropriately.

Comparative Results

The gravimetric technique for starch analysis did not produce theoretical (100% recovery) results and for this reason, the polarimetric results for starch recovery shown in Table I were used to assess the validity of this enzymatic technique. The samples were previously extracted by an equivolume mixture of methanol, butanol, and benzene for enzymatic hydrolysis or by alcoholic mercuric chloride in the AACC procedure or ethanol in the AOAC procedure. In the three polarimetric methods, the initial polarimetric results were corrected for the sum of the other nonstarch components (namely, moisture, protein, and ash) with the same data as were used in calculating the percent starch recovery for the enzymatic technique. In this form, the results are compared on the same basis, and the agreement between polarimetric and enzymatic results is satisfactory. Without the correction for nonstarch components, the enzymatic and polarimetric results in Table I for corn, wheat, and potato starch will be lower by 10.8, 10.0, and 12.3% respectively. Regardless of the form of the data, the enzymatic results agree with polarimetric results and the percent recovery value of 100%. Ruttloff et al. (3) support this agreement by reporting that their system and two polarimetric methods gave nearly theoretical results for starch. The standard deviation for five determinations of

TABLE I. COMPARATIVE DATA OF STARCH ANALYSES EXPRESSED AS PERCENT RECOVERY OF STARCH

	Type and Analytical Method DMSO/Glucoamylase[a]			
	Sodium 2,4-Dinitrophenolate		Glucose Oxidase-Peroxidase	
	%	%	%	%
Starches				
Corn (Sigma)	100.0	(100.0)[b]	100.8	(99.6)
Wheat (Aytex)	102.0	(97.0)	101.0	(99.8)
Potato (Sigma)	100.0	(101.0)	101.7	(100.5)
Short patent wheat flours				
Soft red winter	99.8	(95.7)	94.4	(94.4)
White	101.0	(92.0)	93.1	(93.1)

	Polarimetric[c]			Gravimetric[c]
	AOAC	AACC	Ewers[d]	(Munson-Walker)
	%	%	%	%
Starches				
Corn (Sigma)	99.3	100.0	96.6	78.5
Wheat (Aytex)	98.1	98.6	97.0	77.8
Potato (Sigma)	100.0	101.0	88.0	74.6
Short patent wheat flours				
Soft red winter	...	...	...	...
White	79.5	...	101.0	...

[a] Percent starch recovery = $\frac{[\text{glucose}] \times 0.9 \times 10^4}{\text{sample weight } [100 - \Sigma(\%\ H_2O,\ \%\ \text{protein},\ \%\ \text{ash})]}$

[b] All values in parentheses corrected for initial reducing sugar content or initial glucose content.

[c] Data corrected for nonstarch components.

[d] Data by commercial laboratory.

corn, wheat, and potato starches on successive days at two sample sizes was 3.1, 2.7, and 2.6% respectively. Our technique is inherently faster than that described in reference 3, because the 1-hr gelatinization at 130°C. is replaced by a 20-min. dissolution in DMSO at 55°C. and the enzymatic reaction time is reduced by a factor of 9. In a routine application for the determination of starch and initial reducing groups, about 15 samples per day can be handled by the automated colorimetric method and about eight per day by the manual glucose oxidase-peroxidase method.

The superiority of the DMSO-glucoamylase technique compared to polarimetry is demonstrated by the two flour samples described in Table I. The nature and level of nonstarch components in these two flour samples introduce error in both the DMSO-glucoamylase and the polarimetric technique. In polarimetry, proteins and nonstarch carbohydrates may introduce error by altering the optical rotation of the dispersion and by producing dispersions which are difficult to filter. In the DMSO-glucoamylase technique, these materials do not appear to alter significantly the specificity of glucoamylase for glucosidic linkages, but may introduce

cumulative errors in the result because of the inaccuracies involved in accounting for their presence in the percent recovery calculation.

The 2,4-dinitrophenolate technique and the glucose oxidase-peroxidase technique give nearly identical results on corn, wheat, and potato starch. These two measurement techniques differ to some extent on flour samples and this is probably due to differences in specificity. Maltose and other reducing sugars present at significant levels in flour prior to glucoamylolysis respond differently to 2,4-dinitrophenolate than does the glucose used to calibrate the absorbance-concentration graph. For this reason, the recovery data differ by as much as 9% for 2,4-dinitrophenolate data if the data include correction for the initial reducing sugar content. The glucose oxidase-peroxidase technique is the more specific measurement. Thus, there is no disparity in the correction for initial glucose content.

The extensive application of this basic technique should consider the varietal and processing differences in flour samples, and the conditions for extraction, dissolution, and glucoamylase hydrolysis can then be adjusted, if necessary, to the characteristics of the sample.

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