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Miniaturization of Three Carbohydrate Analyses Using a Microsample Plate Reader

Jeffrey D. Fox[1] and John F. Robyt[2]
Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa 50011

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Three carbohydrate analyses (reducing value by copper–bicinchoninate, total carbohydrate by phenol–sulfuric acid, and D-glucose by glucose oxidase) have been miniaturized using a microsample plate reader. The use of the reducing-value procedure to measure the hydrolysis of starch by α-amylase and the use of the glucose oxidase method to measure the hydrolysis of lactose by lactase are illustrated.

Several colorimetric analyses have been adapted for microassays using microtiter plates and a microsample plate reader (1–3). These microassays are convenient for measuring microvolume samples in ultralow concentrations, in relatively large numbers (96 per microplate), and in a relatively short time (e.g., ≤30 min). They particularly have applications in monitoring chromatographic column fractions, in enzyme assays, and in determining the initial velocities of enzyme-catalyzed reactions. These assays also lend themselves readily to use in triplicate analyses to improve the precision of the procedures.

We have adapted three analyses that are commonly used in performing many types of carbohydrate determinations: reducing-value determination by the copper–bicinchoninate method (4), total carbohydrate determination by the phenol–sulfuric acid method (5), and D-glucose determination by D-glucose oxidase (6). We also give some specific examples of their applications for the assay of enzymes.

MATERIALS AND METHODS

A Uniskan microsample plate reader (Cole-Parmer Instrument Co., Chicago, IL), with interference filters, was used to measure absorbance of samples in a 96-well microsample plate. The plates were mixed on a vortex mixer with a microplate adapter.

Reducing value by copper–bicinchoninate. Two stock solutions were prepared: Solution A consisted of 97.1 mg of disodium 2,2′-bicinchoninate (Sigma Chemical Co.) dissolved in 45 ml of water containing 3.2 g of sodium carbonate monohydrate and 1.2 g of sodium bicarbonate. The final volume was adjusted to 50 ml. Solution B consisted of 62 mg of copper sulfate pentahydrate and 63 mg of L-serine dissolved in 45 ml of water, and the final volume was diluted to 50 ml. The working reagent was prepared daily by mixing equal volumes of Solutions A and B. Reducing sugar standards consisted of 1–20 μg/ml of maltose.

The procedure consisted of adding 100 μl of reducing carbohydrate sample to 100 μl of working reagent in the wells of a 96-sample microtiter plate. The plate was covered with Saran wrap and incubated in a water bath at 80°C for 35 min. The plate was then cooled for 15 min and the absorbance measured at 560 nm. Triplicate analyses were performed for each sample.

α-Amylase assay using the copper–bicinchoninate reducing-value method. A solution of soluble starch (12.5 mg/ml) was prepared in 50 mM imidazole–HCl buffer (pH 6.5). *Bacillus amyloliquefaciens* α-amylase [crystallized from Miles Laboratories HT-concentrate (7)] with 0.025 IU/ml or less was prepared in 50 mM imidazole–HCl.

The procedure consisted of incubating 2.5 ml of the soluble starch solution at 37°C. The reaction was initiated by adding 0.6 ml of α-amylase; 100-μl aliquots of the enzyme digest were removed at various time intervals (e.g., 5, 10, 15, 20, and 30 min) and added to 0.5 ml of the copper–bicinchoninate working reagent plus 0.4 ml of water or buffer. The alkaline pH of the working reagent stops the reaction and gives a dilution factor of 1:5 for the enzyme digest. The dilution can be varied, depending on the amount of reaction, by adding variable

[1] Present address: Department of Biochemistry, University of Wisconsin, Madison, WI.
[2] To whom correspondence should be addressed.

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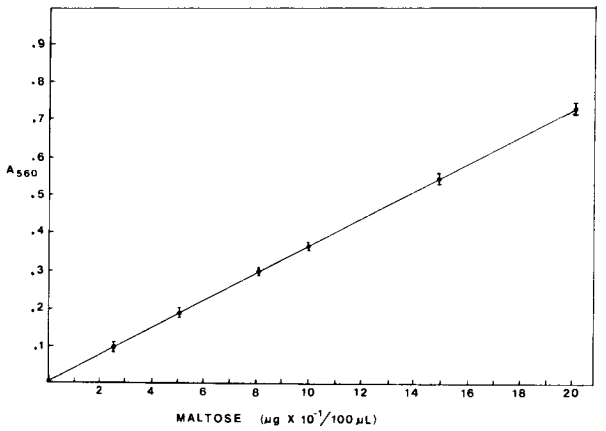

FIG. 1. Standard curve, using maltose, for the copper–bicinchoninate reducing-value procedure.

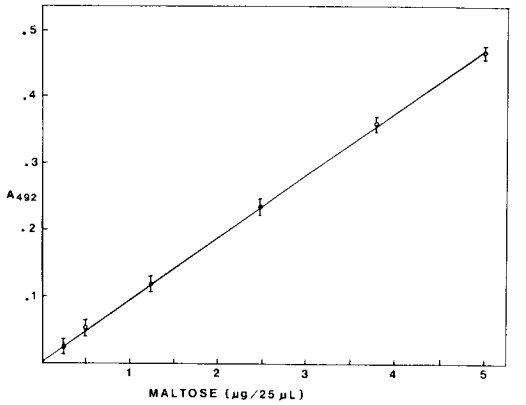

FIG. 3. Standard curve, using maltose, for total carbohydrate by the phenol–sulfuric acid procedure.

amounts of enzyme digest and water or buffer to the working reagent. For example, a 1:10 dilution could be obtained by adding 50 μl of the digest and 0.45 ml of water to 0.5 ml of the working reagent. Three 0.2-ml aliquots are then taken for each time period and added to the wells of a microsample plate. Maltose standards are also added to the same plate, the plate is heated and cooled, and the absorbances are measured as above for the reducing-value determination.

Phenol–sulfuric acid total carbohydrate determination. The procedure consisted of adding 25 μl of sample (containing 10–200 μg of carbohydrate/ml) and 25 μl of 5% (w/v) phenol to the wells of a microtiter plate. The plate was mixed at slow speed on a vortex mixer for 30 s. The plate was then placed onto a bed of ice and 125 μl of concentrated sulfuric acid was added to each well containing sample and phenol. The plate was again mixed at slow speed for 30 s incubated in a water bath at 80°C for 30 min and cooled, and the absorbance was measured at 492 nm. Standards of maltose or D-glucose (10–200 μg/ml) were used. Each sample was done in triplicate.

Glucose oxidase determination of D-glucose. Reagent A consisted of 30 mg of glucose oxidase (Sigma), 3 mg of peroxidase dissolved in 50 ml of Tris–phosphate–glycerol buffer [36.3 g of Tris and 56.5 g of sodium dihydrogen phosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$) dissolved in 400 ml of water plus 400 ml of glycerol and then diluted to 1 liter with water]. Reagent B consisted of 10 mg of *o*-dianisidine dissolved in 50 ml of the above-described buffer. The working Reagent consisted of equal volumes of Reagent A and Reagent B, prepared fresh daily.

The procedure involved the addition of 50 μl of sample (containing 10–100 μg of D-glucose/ml) to 200 μl of working glucose oxidase reagent in a microsample plate.

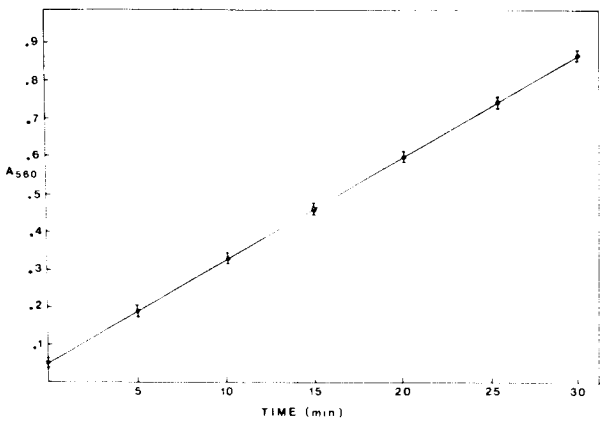

FIG. 2. Measurement of the hydrolysis of starch by α-amylase using the copper–bicinchoninate procedure.

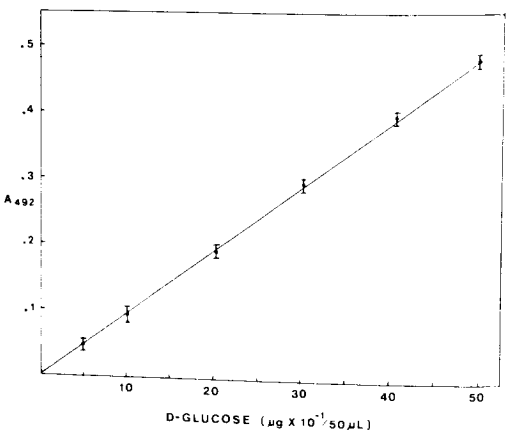

FIG. 4. Standard curve, using D-glucose, for the glucose oxidase procedure.

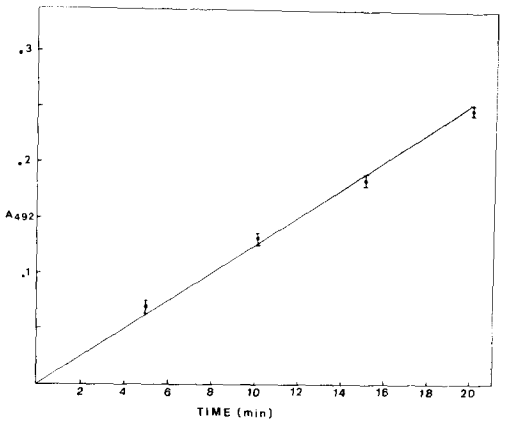

FIG. 5. Measurement of the hydrolysis of lactose by lactase using the glucose oxidase procedure.

The plate, with samples, was incubated at 37°C for 30 min; 50 μl of concentrated HCl was added and the absorbance was measured at 492 nm.

Assay of enzyme reactions producing D-*glucose.* One milliliter of substrate (e.g., lactose), buffered at the appropriate pH, was used and the reaction was started by addition of 0.1 ml of enzyme (e.g., β-galactosidase). At various time intervals, 150-μl aliquots of the reaction digest were added to 25 μl of 2 M HCl. After standing for a minimum of 15 min, three 50-μl aliquots were then taken for each timed sample and added to 200 μl of glucose oxidase working reagent in a microsample plate well. Standards of D-glucose were also added to plate wells containing 200 μl of glucose oxidase. The plate was mixed at slow speed for 30 s on a vortex mixer and then incubated at 37°C for 30 min. Fifty microliters of concentrated HCl was added to each sample well and the plate was mixed at slow speed for 10 s on a vortex mixer. The absorbance was measured at 492 nm.

Each of the values in Figs. 1–5 is the average of triplicate determinations. The lines generated are the results of a linear least-squares fit.

RESULTS AND DISCUSSION

Figure 1 illustrates a maltose standard curve for the copper–bicinchoninate reducing-value method. We found that covering the microsample plate with Saran wrap was effective in preventing losses by evaporation during the heating step. Other authors (8) have added a high-boiling hydrocarbon, such as pristane, to the sample wells to prevent evaporation and projection of the sample and reagent out of the wells. The addition of pristane adds another pipeting step that we have found to be unnecessary when using Saran wrap and mixing the samples on a vortex mixer at slow speeds.

This is a micromethod with determinations of maltose in the range 0.2–2 μg or 0.6–6 nmol. A normalized comparison of the copper–bicinchoninate procedure and the Somogyi–Nelson procedure (3), using identical amounts of reagents and carbohydrate concentrations, shows that the copper–bicinchoninate method is two to three times more sensitive than the Somogyi–Nelson procedure. Further, this method requires the addition of only one reagent, whereas the Somogyi–Nelson method requires the addition of two reagents with the evolution of carbon dioxide to give maximum color. Because of the increased sensitivity and the simplicity of the procedure, the copper–bicinchoninate micromethod should become the preferred method for the determination of reducing values. It should be especially useful for the determination of reducing values of polysaccharides that in conjunction with a total carbohydrate analysis by the phenol–sulfuric acid microprocedure can be used to determine the degree of polymerization or molecular weight of polysaccharides (9) and for the measurement of initial velocities of enzymes that produce reducing sugars from polysaccharides or nonreducing sugars, such as starch, cellulose, and sucrose. Figure 2 illustrates the kinetic analysis of the production of reducing sugars from the hydrolysis of starch by *Bacillus amyloliquefaciens* α-amylase.

Figure 3 illustrates a maltose standard curve for the determination of total carbohydrate by the phenol–sulfuric acid method (5). This is also a micromethod with determinations in the range 0.5–5 μg. The procedure has been modified slightly from the original method of Dubois *et al.* (5) by adding the sulfuric acid to samples cooled in an ice bath. This is followed by mixing and heating at 80°C for 30 min. This modification eliminates the problem of obtaining results that are dependent on the rate of addition of sulfuric acid (8) and the variable and uneven heating that results from the hydration of sulfuric acid. The dehydration of the carbohydrate by the sulfuric acid and the full development of color, however, require heat, which is obtained by a controlled, uniform heating step in an 80°C water bath. The standard curve using maltose can be extrapolated to all D-glucose-containing carbohydrates such as maltodextrins, starch, dextran, and cellulose. When materials containing other kinds of sugars such as D-galactose, D-mannose, or D-xylose found in galactans, mannans, or xylans are to be determined, standards with these sugars should be used. As discussed above, this total carbohydrate microanalysis can be combined with the reducing-value microanalysis to obtain the average degree of polymerization of oligosaccharides and polysaccharides. The method is also very useful for monitoring the elution of carbohydrates from chromatographic columns.

Figure 4 illustrates a D-glucose standard curve using the glucose oxidase method. Like the other two analyses, this procedure is also an ultra-micromethod with a range of 0.5–5 μg or 2.8–28 nmol of D-glucose. Glucose oxidase is highly specific for the oxidation of β-D-glucopyranose (10). It will not give a reaction with any other carbohydrate. The method, then, can be used to specifically determine the initial velocities of a wide variety of enzymes that produce D-glucose as a product, e.g., α-glucosidase, β-glucosidase, glucoamylase, β-galactosidase (lactase), and invertase (sucrase). A specific example for the assay of the hydrolysis of lactose by lactase is illustrated in Fig. 5. Thus, a wide variety of enzymes that hydrolyze reducing and nonreducing sugars to release D-glucose can be assayed. With reducing sugar substrates, the method can be used particularly for those cases in which the reducing value method could not be used because of the high blanks that the substrates would give.

The use of a microsample plate reader to measure absorbance has several advantages involving ease of sample handling, uniform treatment of many samples, direct transfer of data to a computer, and the speed and convenience of obtaining measurements of many samples in a relatively short time. The latter can improve the precision of the analyses by easily allowing triplicate determinations of each sample.

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