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A Glucose Oxidase Reagent for Maltase Assay

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INTRODUCTION

Assay of chromatographic column fractions for maltase (α-glucosidase) activity requires a sensitive method adaptable to routine use and not unduly affected by varying salt concentrations in eluting solutions. The reducing nature of the substrate also limits the procedures available for this purpose. Dahlqvist (1) has used Tauber and Kleiner's acid copper reagent for reducing disaccharide substrates. The shortcomings of the various methods are noted by Dahlqvist; he used Caputto's modified acid copper reagent when the salt content of substrates interfered with the Tauber-Kleiner reagent. Later (2) he used glucose oxidase for determination of turanase, lactase, cellobiase, and gentiobiase activity, but since the glucose oxidase preparations hydrolyzed maltose and isomaltose, he could not use it with these substrates.

Whistler, Hough, and Hylin (3) removed amylase and maltase activities from commercial glucose oxidase with a 20% recovery of dehydrogenase activity. Dahlqvist (1) was not successful in preparing glucose oxidase free from maltase and isomaltase activity. Friedman (4) removed trehalase from glucose oxidase by diethylaminoethylcellulose (DEAE-cellulose) column chromatography, but was unable to remove maltase.

The inhibition of maltase and 1,6-oligoglucosidase by several amines, including tris(hydroxymethyl)aminomethane (Tris) has been noted by Larner and Gillespie (5) who found such inhibition much more pronounced at alkaline than at acid pH. Friedman (4) noted that Tris was inhibitory to trehalase and maltase contaminants in commercial glucose oxidase, but made no analytical use of this. Salomon and Johnson (6) have described a glucose oxidase–peroxidase–tolidine reagent for blood glucose. We have modified this reagent by substituting Tris buffer for the acetate, under which conditions it has negligible maltase activity

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but retains high glucose dehydrogenase action. Color developed by the reagent is a yellow–brown rather than blue; it must be stabilized after the reaction by adding acid, which produces a violet color. Acid stabilization of the glucose oxidase–peroxidase–dianisidine color has recently been reported (7).

EXPERIMENTAL

All developed colors were measured in 18 × 150 mm unmatched test tubes. Displacement spectrophotometry (8) eliminates the need for matching tubes, since each tube acts as its own blank. Although round tubes are used, results are essentially as accurate as though the solution were transferred to parallel-side cuvettes for measurement, and the procedure is much more suited to routine use. Tubes were read in a Lumetron[2] photometer with a 500-mμ interference filter.

The Salomon and Johnson reagent (6), which we had used for invertase assay, was tried for maltase assay, but considerable maltase activity was shown by all glucose oxidase preparations available. After unfruitful attempts to separate the two activities in practical yield by

TABLE 1
INHIBITION OF MALTASE CONTAMINANT IN GLUCOSE OXIDASE[a]

			Glucose		Maltose	
			100 μg	700 μg	1000 μg	7000 μg
Reagent comp.	pH	Min.	Absorbance		Absorbance	
Salomon-Johnson alone	4.1	30	0.054[b]	0.431	0.019	0.147
		60	0.100	0.635	0.106	0.432
With 0.02 *M* Tris	4.1	30	0.074	0.445	0.032	0.198
		60	0.116	0.650	0.102	0.445
With 0.2 *M* Tris	4.3	30	0.048	0.535	0.002	0.074
		60	0.076	0.830	0.044	0.276
With 0.2 *M* Tris buffer[c]	7.6	60	0.073	0.499	0.000	0.004
With 0.2 *M* phosphate buffer[c]	7.2	30	0.090	0.425	0.000	0.006
		60	0.136	0.463	0.000	0.044
With 0.2 *M* borate buffer[c]	7.4	30	0.023	0.314	0.000	0.000
		60	0.060	0.725	0.000	0.000

[a] Each tube contained indicated sugar in 2 ml with 5 ml reagent added, incubated 30 and 60 min at 26°C.

[b] Absorbance at 600 mμ of 7.3 mm thickness, by displacement photometry.

[c] Original acetate buffer omitted, replaced by indicated buffer, final concentration 0.2 *M*. Two drops HCl was added to each tube before reading Tris-buffered solutions.

[2] Mention of trade names does not constitute endorsement by the Department over others not named.

the procedure of Whistler *et al.* (3), selective inhibition of maltase by Tris was investigated.

Table 1 shows the results of the addition of Tris to the reagent, making it 0.02 and 0.2 *M* in Tris. No inhibition was shown at 0.02 *M*, but some was evident at 0.2 *M*. Elimination of the pH 4.1 acetate buffer from the original reagent and addition of a 0.2 *M* Tris buffer at pH 7.6 produced the results in the fourth entry of the table. In this case, as the reaction proceeded, the glucose solutions became yellow–brown, rather than blue. This yellow color appears proportional to glucose content at very low concentrations, but not above 50 μg/2 ml original solution. By adding acid subsequent to incubation, a violet color results which follows Beer's law between about 30 and 500 μg glucose/2 ml. Its visible spectrum has an absorption maximum at 530 mμ with minima at 442 and 740 mμ.

When the pH 4.1 acetate buffer in the original reagent was replaced by borate or phosphate at slight alkalinity, most of the tolidine was precipitated. Sufficient remained in solution, however, to give the results in the last two entries in the table. Absorbance values in the glucose tubes show that low values for maltose are not due to insufficient tolidine. Reduction of maltase activity was complete in borate and substantial with phosphate at the indicated pH values, both in the absence of Tris. The tolidine in the reagent appears to be inhibitory toward maltase at these pH values, though not under the original acid conditions of Salomon and Johnson, as noted for other amines by Larner and Gillespie. This was confirmed by adding *o*-tolidine to Huggett and Nixon's (8) glucose oxidase–dianisidine reagent, which is phosphate-buffered at pH 7. Their reagent shows maltase activity, but addition of *o*-tolidine dihydrochloride, filtration, and readjustment of the pH to 7 reduces maltase activity to one-third. A more stable reagent, in which no precipitation takes place, can be made with Tris and tolidine; the color obtained after acidification is much more suitable for measurement. The color becomes quite stable 1 hr after acid addition, remaining unchanged for 90 min thereafter. However, if a timed routine is used, reproducible results can be obtained by reading the tubes 1 min after acid addition. This eliminates an additional hour's wait in the procedure, so that 60 tubes can be analyzed for glucose in 2 hr.

The sensitivity of the reagent varies with the particular lot of glucose oxidase. A calibration curve must be established with each lot to determine the upper limit of reliability of the reagent. For example, one lot of enzyme gave a reagent with a range of 30–500 μg glucose in 2 ml; that prepared with another lot was reliable only between 30 and 350 μg

in 2 ml. Where the upper limit is exceeded, color after acidification is a brownish violet and shows completely unreliable absorbance values, unrelated to the glucose concentration. Thus, a maximum absorbance value should be determined for the procedure as it is to be used. We have found a value of 0.400–0.425 to be the limit under our conditions (7.2 mm depth), corresponding to the upper limits given above with different lots of glucose oxidase.

Interference by salt from gradient elution is negligible from 0 to 0.5 *M* NaCl in the substrate. Color is increased about 5% at 0.5 *M* NaCl, 7% at 1 *M* NaCl, and at 2 *M* NaCl about 15% more color is produced. However, if tubes are not measured until an hour after acidification, salt interference is negligible (<5%) to at least 2 *M* NaCl.

Reagent and Procedure

Tris Buffer. A volume of 384 ml of 0.8 *M* HCl is added to 500 ml of 0.8 *M* Tris ("Sigma 7–9", Sigma Chemical Company, St. Louis, Mo.) solution, pH adjusted if necessary to 7.6, and the solution is made to 1 l with water.

A solution containing 800 mg glucose oxidase (crude, Sigma Chemical Company) and 16 mg peroxidase (horse-radish, type I, Sigma Chemical Company) in 200 ml of Tris buffer (above) and 200 ml water is filtered into an amber glass bottle. A solution of 180 mg *o*-tolidine dihydrochloride in 260 ml water is also filtered into the bottle. The contents are mixed and stored under refrigeration. The reagent should stand overnight before use and is stable in cold storage for at least 6 weeks.

For routine measurement of maltase or invertase activity of column filtrates, aliquots are incubated with the appropriate substrate in 2 ml volume. The buffer used must not be strong enough to affect materially the pH of the reagent added later. After incubation, 5 ml of the glucose oxidase–Tris reagent (at room temperature for accurate work) is added to tubes at exactly 1-min intervals, to allow time to acidify and read them. After 60 min at room temperature (25°C) 0.1 ml of 4 *N* HCl is added to each of the first two tubes, each being immediately mixed (a rod with a flat tip or touching the tube to a rubber stopper revolving on a stirring motor mixes rapidly). The first tube is read, using a displacement bar,[3] following Hamilton's (8) procedure. Then acid is added to the third tube; it is mixed, replaced in the rack, and the second tube is read, and so on. About 1 min is required for acidification and mixing

[3] The bar is a 220 × 12 mm piece of plate glass, 7.3 mm thick, polished on all six surfaces.

of a tube and reading the preceding tube. The color should be a clear violet, with no brown cast. Two tubes containing 300 μg glucose in 2 ml water are included in each lot for calibration.

If a blank with zero absorbance is desired, it is necessary to use substrates containing less than 0.005% glucose when 0.139 *M* saccharide substrates are employed. The glucose content of 0.4% found in a commercial maltose sample was reduced by one charcoal column treatment to less than 0.005%. Commercial sucrose gives no color with this reagent at this concentration.

SUMMARY

Inhibition of maltase contamination of glucose oxidase used for glucose assay is described, which provides a glucose oxidase reagent suitable for routine maltase assay of chromatographic fractions.

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