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A Continuous Spectrophotometric Screening Assay for Glucoamylase

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A continuous glucose oxidase–peroxidase based spectrophotometric assay suitable for the rapid screening of glucoamylase activity is described. The assay utilizes maltotetrose and the chromogens 3-(dimethylamino)benzoic acid (DMAB) and 3-methyl-2(3*H*)-benzothiazolinone hydrazone (MBTH). The assay was sensitive to glucoamylase levels of 0.05 μg/mL and was linear with respect to enzyme concentration to approximately 0.5 μg/mL.

Glucoamylase is a widely utilized exoenzyme that removes glucose units consecutively from the nonreducing ends of starch and oligosaccharides (Pazur and Ando, 1960; Reilly, 1979). Glucoamylase activity is defined as the quantity of glucose liberated from substrate per unit time. Methods commonly used for glucoamylase detection include reducing sugar determination (Somogyi, 1952; Bernfeld, 1955) and the enzymatic analysis of the glucose released by either the glucose oxidase–peroxidase method (Hugget and Nixon, 1957; Dahlqvist, 1961; Lloyd and Whelan, 1969) or the hexokinase–glucose-6-phosphate dehydrogenase (HK/GDH) coupled assay (Bergmeyer et al., 1974). Both analytical approaches have weaknesses. Reducing sugar methods are not valid indicators of glucoamylase levels when α-amylases or pullulanases are present. The glucose oxidase–peroxidase method is most often utilized as a fixed-point assay and frequently employs *o*-dianisidine, a carcinogen, as the chromogen. The HK/GDH method, also a fixed-point assay, can only be conducted at pH 7.0 or above. Since glucoamylase assays are conducted at pH 4.5, an adjustment of pH is required, thus necessitating a multistep assay. A continuous glucoamylase assay utilizing glucose oxidase–peroxidase with amylose as substrate has been reported (Pazur et al., 1971); however, this assay also employed *o*-dianisidine.

The development of a rapid and continuous spectrophotometric screening assay for detection of glucoamylase activity utilizing noncarcinogenic color reagents is highly desirable. Ngo and Lenhoff (1980) reported the use of 3-(dimethylamino)benzoic acid (DMAB) and 3-methyl-2-(3*H*)-benzothiazolinone hydrazone (MBTH) for measurement of peroxidase and peroxidase-coupled reactions. For glucose measurement, glucose oxidase is added to convert glucose and O_2 to gluconic acid and H_2O_2. Then, H_2O_2, MBTH, and DMAB reacted in the presence of peroxidase to form an indamine dye with an absorption peak of 590 nm. By either fixed-point or rate methods, this assay provided a sensitive measurement of glucose. The objective of this study was to adapt this reaction for a quick screening assay of glucoamylase-catalyzed glucose release from an oligosaccharide substrate. Since amylose is a substrate for interfering endo- and debranching α-amylases, its use was avoided and the purified maltooligosaccharide substrate maltotetrose was utilized instead.

MATERIALS AND METHODS

Materials. Purified *Rhizopus niveus* glucoamylases were obtained from Seikagaku Biochemicals (U.S. Distributor; Miles Biochemical Co., Elkhart, IN). DMAB and MBTH were purchased from Aldrich Chemical Co. (Milwaukee, WI). Maltotetrose, maltotriose, and maltose

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Table I. Residual α-Glucosidase Activity (Assays Conducted in the Presence of 6.8 and 2.0 Units/mL of Glucose Oxidase and HRP, Respectively)

substrate[a]	rate, ΔA/min	substrate[a]	rate, ΔA/min
maltose	0.026	maltotetrose	0.014
maltotriose	0.017		

[a] Substrate was added to a final concentration of 0.1%.

Table II. Effect of Glucose Oxidase and Horseradish Peroxidase Levels on Color Development

		glucoamylase act.[c]		
rel level glu ox/HRP reagent present in assay[a]	α-glucosidase act.,[b] ΔA/min	lag period, min	ΔA/min	glucoamylase/α-glucosidase ratio
1	0.007	8.0	0.177	25.3
1.5	0.021	7.2	0.189	9.0
2.0	0.031	6.5	0.208	6.7
6.0	0.083	3.0	0.273	3.3

[a] A relative level of 1 corresponds to glucose oxidase and horseradish peroxidase concentrations of 3.34 and 0.95 units/mL, respectively. The ratio of the two activities was held constant throughout. [b] In the presence of 0.1% maltotetrose. [c] Assayed with 0.667 μg of *R. niveus* glucoamylase.

were purchased from Sigma Chemical Co. (St. Louis, MO). Horseradish peroxidase (HRP) and glucose oxidase were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Maltotetrose, DMAB, MBTH, HRP, and glucose oxidase were prepared in 50 mM sodium acetate buffer, pH 4.5.

Glucoamylase Assay. Reaction mixtures in a final volume of 1.0 mL were prepared by combining 333 μL of 4 mM DMAB containing 0.3% maltotetrose, 333 μL of 80 μM MBTH, and 167 μL of a solution containing 11.4 units/mL HRP and 40 units/mL glucose oxidase in a 1-cm path length cuvette. Reactions were initiated by the addition of 167 μL of glucoamylase at levels between 0 and 10 μg/assay. Absorbance at 590 nm was continuously monitored on a Gilford 2600 recording spectrophotometer. The control for each assay consisted of a cuvette containing all reagents except glucoamylase, which was replaced with 167 μL of water. Background absorbance due to the presence of traces of α-glucosidase activity invariably present in glucose oxidase preparations was automatically subtracted at each time point.

RESULTS

Color Development at pH 4.5. It was shown by Ngo and Lenhoff (1980) that DMAB, MBTH, and H_2O_2 combine at pH 6.5 in the presence of peroxidase. To establish the feasibility of using this system for glucoamylase, it was necessary to establish that the system was operational at pH 4.5. In the presence of 0.003% glucose, color development was maximal between pH 5.5 and 7.0. At pH 4.5, color development proceeded at 80% of the maximal rate.

Substrate Selection. α-Glucosidase is a known trace contaminant of highly purified glucose oxidase solutions, and therefore its effect on color development must be minimized. Background color development in the presence of the three substrates maltose, maltotriose, and maltotetrose was quantified (Table I). The lowest level of background was found with maltotetrose. Maltotetrose was therefore used for all subsequent assays.

The effect of varying the amount of the glucose oxidase/HRP reagent was then investigated (Table II). With a 6-fold increase of enzyme reagent, background activity increased by almost 12-fold. However over this same range, glucoamylase activity increased by only 1.5-fold. In this experiment, the lowest ratio of background to glucoamylase-catalyzed color development was obtained with glucose oxidase and HRP levels of 3.34 and 0.95 units/mL, respectively.

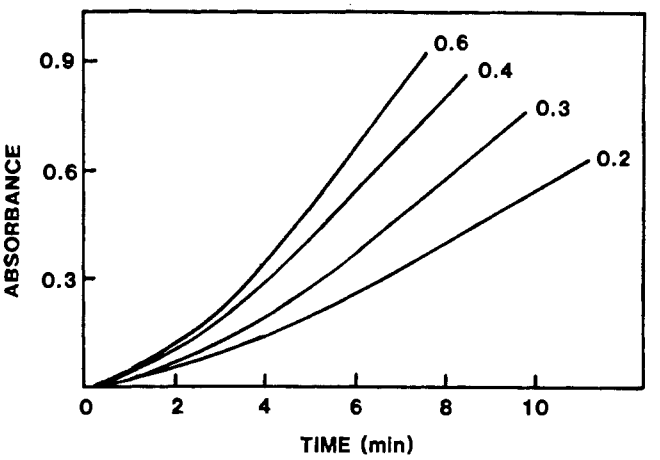

Figure 1. Spectrophotometric time course of color development. Assays were conducted as described in Materials and Methods. Glucoamylase levels (μg/assay) are indicated by each time course.

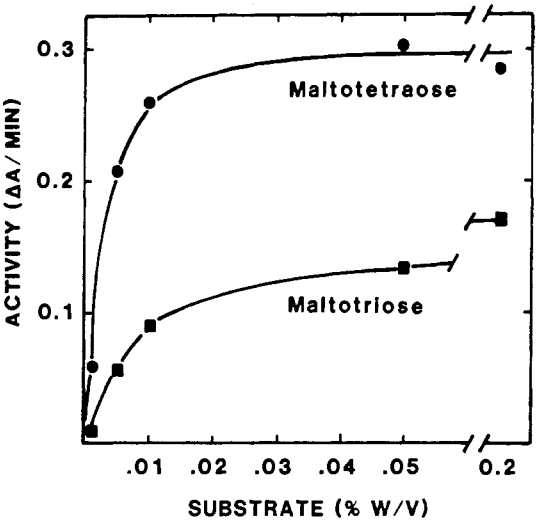

Figure 2. Effect of substrate concentration on color development.

As is typical in many enzyme assays involving multiple coupled reactions, color development in the presence of glucoamylase was accompanied by a lag period (Figure 1). The length of the lag period increases as the level of the GOP reagent is decreased. Use of glucose oxidase and HRP at levels of 6.68 and 1.9 units/mL represents a compromise between high background color development rates and excessively long lag periods.

Figure 2 diagrams the effect of varying maltotriose and maltotetrose concentrations on color development at pH 4.5 in the presence of 0.67 μg of glucoamylase. Maltotetrose gave considerably faster color development than maltotriose. This is consistent with the model proposed by Hiromi et al. (1973), who demonstrated that K_m values for maltooligosaccharides decline and V_{max} values increase with increasing chain length. With maltotetrose, saturation was achieved at a concentration of 0.01%.

Linearity of Glucoamylase Reaction. In the experiment shown in Figure 3 color development was linear with glucoamylase concentration between 0 and 0.5 μg of glucoamylase. The range of linearity over the course of months in which these experiments were conducted showed some variation. With some trials, linearity as high as 1.1 μg was observed.

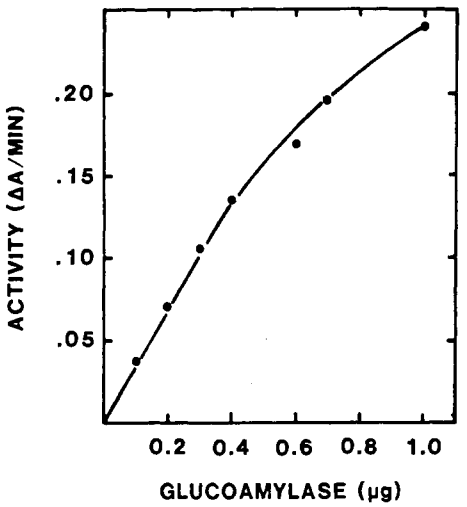

Figure 3. Effect of glucoamylase levels on color development.

DISCUSSION

DMAB and MBTH offer an alternative for *o*-dianisidine in continuous glucoamylase assays. This assay has the advantage of taking less time than conventional fixed-point assays that require at least three steps: digestion of substrate, termination of the reaction, and detection of glucose. By the continuous assay with a four-cell spectrophotometer, three assays can be conducted in 10 min.

The large number of samples that can be quickly screened makes this assay attractive for applications such as monitoring the distribution of activity during fractionation or thermal inactivation studies. It is not recommended that this assay system be depended upon for pH or effector studies, unless the effects of these parameters on glucose oxidase and peroxidase are taken into account. Another point to be aware of is that the presence of catalase or other H_2O_2-requiring or -producing enzymes could result in anomalous results.

The continuous assay was directly compared to the enzymatic fixed-point HK/GDH assay to determine the amount of glucose detected during a reaction time course (data not shown). Detectible glucose levels after 5 min of reaction time ranged anywhere between 20 to 50% lower with the continuous assay than with the HK/GDH method. This may be accounted for by the fact that in the continuous assay a lag period was invariably observed. Although the lag period could be shortened by increasing the amount of GOP reagent, unacceptable levels of background α-glucosidase activity were introduced. To our knowledge, all commercially available purified glucose oxidase preparations contain this trace activity. A procedure for removing this background activity would enable the level of enzyme reagent to be increased. Another possible reason for the discrepancy between the continuous and fixed-point assays is that when aliquots are withdrawn for the HK/GDH fixed-point assay, the reaction may continue for a brief period before inactivation by heat and the TEA buffer. For these reasons, conversions of absorbance values to molarities at any given time during the reaction always resulted in an underestimation of the true quantity of glucose released as measured by the HK/GDH fixed-point assay. Thus, quantitative estimates of glucose release from absorbance values were not possible. Problems remaining to be solved with the development of efficient coupled enzyme assay systems have been rigorously defined (Brooks et al., 1984a,b).

In summary, with DMAB, MBTH, and maltotetrose, a linear relationship between glucoamylase levels and color development was obtained. The continuous assay therefore may be used as a rapid semiquantitative screening method for detecting glucoamylase.

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Registry No. DMAB, 99-64-9; MBTH, 1128-67-2; peroxidase, 9003-99-0; glucose oxidase, 9001-37-0; maltotetrose, 34612-38-9; glucoamylase, 9032-08-0.

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