An Alternative Method for Enzymatic Assay of Plant Invertases

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An alternative coupled method was developed for the enzymatic assay of plant invertases. In this method, ADP produced from the phosphorylation of glucose and fructose (hydrolysis products of invertases) is coupled to oxidation of NADH by the enzymes pyruvate kinase and lactate dehydrogenase in the presence of phosphoenolpyruvate and NADH. This method was compared with the glucose-6-phosphate dehydrogenase method for both continuous and discontinuous assay by using purified invertase from baker's yeast and protein preparations derived from plant materials of three different species. This method is applicable to filtered and concentrated crude extracts. Statistical analysis indicated that the alternative method was similar in accuracy to the glucose-6-phosphate dehydrogenase method.

Keywords: *Biochemistry; enzymes; physiology; sugars; invertase*

INTRODUCTION

The glucose-6-phosphate dehydrogenase (G-6-PDH) method and glucose oxidase method are widely used to determine reducing sugars present in biological samples (Sturgeon, 1990). These two methods are adapted for plant invertase assay, based on the utilization of reducing sugars released from sucrose by invertases. The G-6-PDH method was used to measure fruit invertase activities in muskmelon (*Cucumis melo* L.) (Hubbard et al., 1989), apple (Yamaki and Ishikawa, 1986), strawberry, kiwi, papaya, pineapple, and peach (Hubbard et al., 1991), and soybean cotyledons (Brown and Huber, 1987). A schematic diagram of the pathway is shown in Figure 1. A limitation of the G-6-PDH method is the relatively high cost of the coupling enzymes and cofactors. The glucose oxidase method was used to determine invertase activities of Marsh grapefruit (*Citrus paradisi* Macf) (Tomlinson et al., 1991), carrot root (*Daucus carota* L.) (Lee and Sturm, 1996), barley leaves (*Hordeum vulgare* L.) (Obenland et al., 1993), and blueberry (*Vaccinium ashei*) fruit (Darnell et al., 1994). An alternative and inexpensive method has been successfully developed for plant invertase assay in our laboratory. Details of the procedure are discussed in this paper.

MATERIALS AND METHODS

Materials. The following chemicals and enzyme reagents were purchased from Sigma Chemical Co. (St. Louis, MO): invertase from baker's yeast, hexokinase (HK) from yeast, pyruvate kinase (PK) from rabbit muscle, lactate dehydrogenase (LDH) from *Leuconostoc mesenteroides*, glucose-6-phosphate dehydrogenase (G-6-PDH) and phosphoglucose isomerase (PGI) from baker's yeast, adenosine *N*-(2-hydroxyethyl)piperazineethane-*N*′-(2-ethanesulfonic acid) potassium salt (HEPES), β-nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide phosphate ($NADP^+$), adenosine 5′-triphosphate (ATP), triethanolamine (TEA), *N*-(2-acetamido)iminodiacetic acid (ADA), poly(vinylpyrrolidone) (PVP), ethylenediaminetetraacetic acid (EDTA), Triton X-100, dithiothreitol (DTT), glycerol, ethylene glycol bis(β-aminoethyl ether) *N*,*N*′-tetraacetic acid (EGTA), and phenylmethanesulfonyl fluoride (PMSF). Phosphoenolpyruvic acid (PEP) was purchased from Fisher Scientific (Pittsburgh, PA). Sweetpotato roots [*Ipomoea batatas* (L.) Lam. cv. Beauregard] were provided by the Louisiana Sweetpotato Research Station, Chase, LA. Ripening blueberry fruit (*Vaccinium corymbosum* cv. Cooper) and peach floral buds at blooming (*Prunus persica* cv. La Feliciana) were obtained from the Idlewild Research Station, Clinton, LA.

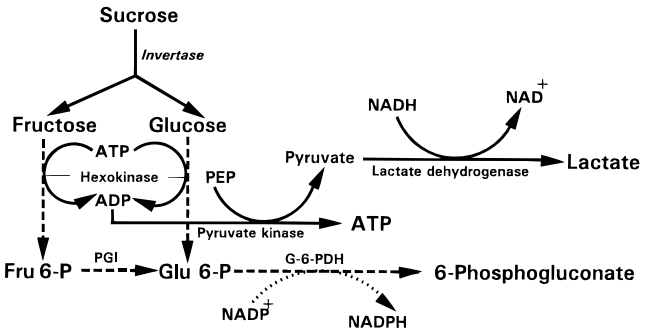

Figure 1. Schematic diagram of the coupling assay for plant invertases by the G-6-PDH method (dashed line) and the alternative method (solid line).

Extraction. All procedures were performed at 0−4 °C unless otherwise stated. Sweetpotato flesh, whole peach buds, and blueberry fruit were used for enzyme extraction. Portions (10 g each) of plant materials were ground into fine powder in liquid nitrogen, using a prechilled mortar and pestle. The extraction buffer (ionic strength = 0.04) was 50 mM HEPES−KOH, pH 7.4, containing 5% (w/v) insoluble PVP, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 5 mM DTT, 0.1% Triton X-100, and 1% glycerol. The powder was homogenized for 2 min in extraction buffer with a Virtis 45 homogenizer using a 1:2 ratio of tissue (in grams) to buffer (in milliliters). The homogenate was centrifuged for 20 min in a Sorvall RC-5B refrigerated superspeed centrifuge (DuPont Instruments) at 15000*g*. The supernatant was collected as the crude extract.

Invertase Preparation for Continuous Assay. The crude extract of sweetpotato was vacuum-filtered through two Nalgene filterwares (0.45 and 0.2 μm in pore size) (Nalgene

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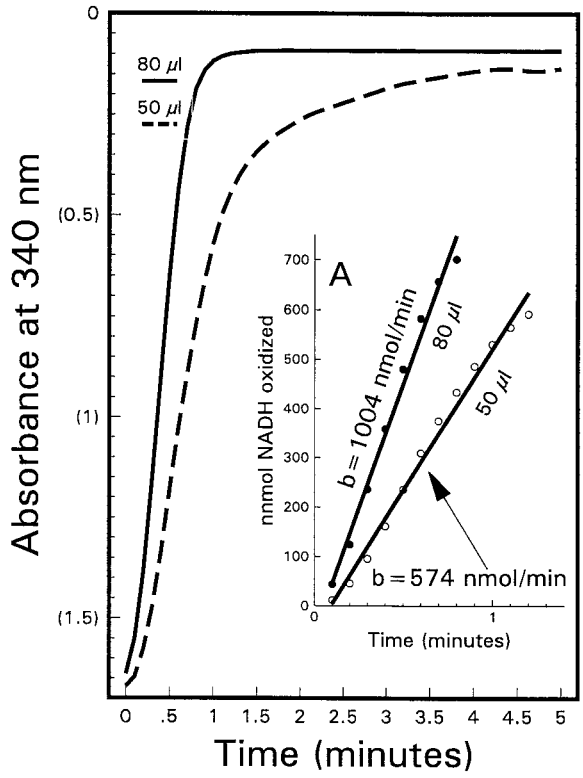

Figure 2. Examples of absorbance curves for the alternative method in 25 mM ADA−KOH buffer, pH 7.0. The invertase preparation was from sweetpotato root (cv. Beauregard). The linear correlation of NADH oxidized with time is shown in (A).

Table 1. Substrate Purity and Background Rates of NADH Oxidation[a]

1	sucrose purity	99.98% (Sigma: 99+%)
2	ATP hydrolysis by HK	1.56%
3	background rate I	6.23 nmol/min
4	background rate II	6.66 nmol/min
5	background rate III	6.72 nmol/min

[a] Test conditions were identical to the continuous assay for invertase, except invertase preparation was omitted: 1, reaction initiated by adding equimolar amount of sucrose; 2, sucrose omitted and reaction initiated by adding HK; 3, sucrose omitted and reaction initiated by adding ATP for the background rate due to ATP hydrolysis by HK; 4, sucrose omitted and reaction initiated by adding LDH for the background rate due to ATP hydrolysis by HK and impurity of PEP and PK; and 5, sucrose included and reaction initiated by adding LDH for the overall background rate. Each test was repeated six times.

Co., Rochester, NY). The filtrate was further filtered through a Biomax 50 Ultrafree 15 centrifugal filter device (Millipore Corp., Bedford, MA) by centrifugation (2000*g*). The filtrate was concentrated to about one-third of the volume in a Biomax 10 Ultrafree centrifugal device (Millipore) by centrifuging for 45 min at 2000*g*. Invertase of baker's yeast (100 mg) was reconstituted in 5 mL of double-deionized water, which was saved as a stock enzyme solution (52 units/μL).

Invertase Preparation for Discontinuous Assay. About 30 mL of supernatant was collected, from which an 8 mL aliquot was taken and brought to 60% saturation by titrating with a 90% saturated ammonium sulfate solution at a flow rate of 1 mL min^{-1} while being constantly stirred at a moderate speed with a magnetic stir bar in an ice bath. The homogenate was centrifuged for 20 min at 13000*g* at the end of titration. The precipitate was resuspended in 3 mL of 25 mM HEPES−KOH buffer containing 5 mM EDTA, pH 7.4 (hereafter called buffer A), and centrifuged for 20 min at 13000*g*. The supernatant was passed through a 1.9 × 20 cm Sephadex G-25 (coarse) column (Supelco, Inc., Bellefonte, PA), previously washed using buffer A, to remove low molecular

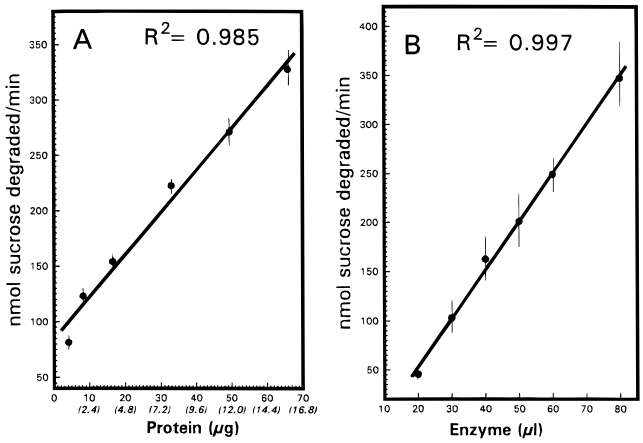

Figure 3. Enzyme concentration dependence of invertase assay in 25 mM ADA−KOH buffer, pH 7.0: (A) Baker's yeast invertase purchased from Sigma. Various concentrations of enzyme solutions were diluted from the stock solution as mentioned under Materials and Methods. Protein content and unit definition were provided by Sigma. The corresponding enzyme units are indicated with italic numbers at the *X*-axis. (B) Invertase preparation derived from sweetpotato roots. For both preparations, reaction mixtures were as described under Materials and Methods, except that various concentrations or volumes of invertase were used in the assay. Each data point is the mean of six separate measurements. Vertical bars represent the standard error.

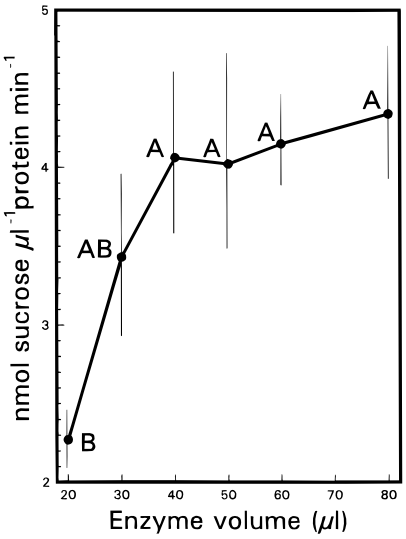

Figure 4. Comparison of invertase specific activity for the alternative method at various volumes of sweetpotato enzyme preparations. Each data point is the mean of six individual measurements. A different letter means a difference in specific activity. Vertical bars represent the standard error.

weight compounds such as salt ions, phenolic compounds, and sugars (Ruffner et al., 1992). The invertase protein was eluted off the column using 7 mL of buffer A. The effluent was collected and concentrated using a Centricon 10 (Amicon Corp., Beverley, Mass.) with a tabletop centrifuge (IEC HN-SII, Needham Heights, MA) until a final volume of ≈3 mL was obtained (Obenland et al., 1993). The concentrated extract was used for discontinuous assay.

Continuous Assay by Alternative Method. The rationale of enzymatic assay is shown in Figure 1. Invertases in baker's yeast and sweetpotato roots were assayed in 25 mM ADA−KOH buffer, pH 7.0, containing 10 mM $MgCl_2$ and 20 mM KCl. The assay mixture (3 mL) contained 100 μmol of sucrose, 1.53 μmol of ATP, 1.2 μmol of PEP, 0.9 μmol of NADH, 8 units of HK, 8 units of PK, and 8 units of LDH. Invertase preparation was used to initiate the reaction. The change in absorbance at 340 nm verus time was recorded at 25 °C using

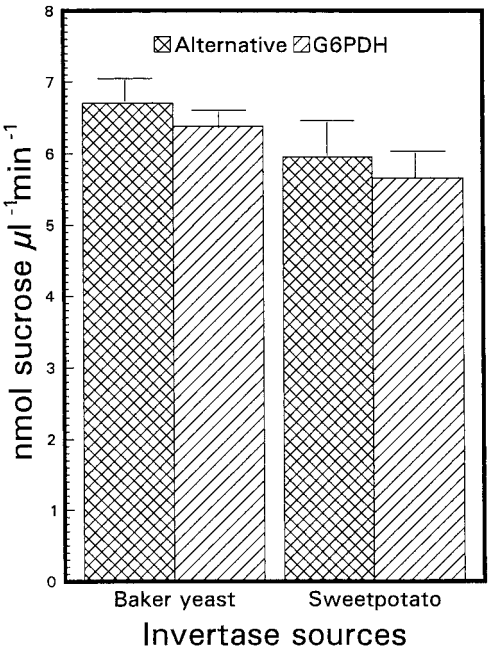

Figure 5. Comparison of invertase specific activity between the alternative and G-6-PDH methods using the continuous assay. Conditions were identical, except the coupling enzymes and cofactors were different. The assays were performed in 50 mM HEPES−KOH buffer, pH 7.6. Fifty microliters of enzyme preparation was used in each assay. Each number represents the mean of six separate assays. Vertical bars represent the standard error.

Table 2. Comparison of the Specific Activities of Acid and Alkaline Invertases Derived from Three Different Plant Materials Using Two Assay Methods

	specific acitvities[a] [nmol of sucrose (g of dry wt)$^{-1}$ min^{-1}]	
assay method	acid invertase, pH 4.6	alkaline invertase, pH 7.6
Blueberry Fruit (Cv. Cooper)		
G-6-PDH method	592.3 ± 12.0 a	97.1 ± 7.7 a
alternative method	579.6± 3.9 a	83.5 ± 8.7 a
Sweetpotato Roots (Cv. Beauregard)		
G-6-PDH method	625.9 ± 49.8 b	873.4 ± 36.3 a
alternative method	782.9 ± 66.9 a	880.6 ± 10.4 a
Peach Floral Buds (Cv. La Feliciana)		
G-6-PDH method	122.1 ± 11.9 a	120.5 ± 6.6 a
alternative method	128.3± 4.7 a	128.4 ± 5.2 a

[a] Mean comparison between the two assay methods by least significant difference test at $P \leq 0.01$; means marked by the same letter indicate no difference in activity between the two assay methods. Each mean was taken from four separate assays.

spectrophotometry (Figure 2). The observations at the linear part of the curve were used to calculate the slope (Figure 2A). Invertase activity was determined from the slope of the regression line (Bracho and Whitaker, 1990). To compare the accuracy of the alternative method with the G-6-PDH method, invertase in baker's yeast and sweetpotato roots was assayed under identical conditions, except for the coenzymes and coupling enzymes as well as their specific requirements for metal ions.

Discontinuous Assay. All incubations were carried out at 25 °C for 30 min. The incubation media included 20 μL of concentrated extract and either 980 μL of 50 mM potassium acetate buffer (pH 4.6, for acid invertase) or 980 μL of 50 mM HEPES−KOH buffer (pH 7.6, for alkaline invertase) containing 100 mM sucrose. Controls were prepared by utilizing heat-denatured extract. In the case of acid invertase, the medium was neutralized using 24 μL of 3 M KOH at the end of incubation to prevent acid hydrolysis of sucrose under heat. Enzymatic reactions were terminated by heating for 5 min in a boiling water bath, a procedure that effectively stopped the enzymatic reactions as determined by trial assays.

Invertase activity was determined using the alternative method in 25 mM ADA−KOH buffer, pH 6.9, containing 10 mM $MgCl_2$ and 20 mM KCl (Harbron et al., 1980) and using the G-6-PDH method in 50 mM TEA buffer, pH 7.6, containing 70 mM $MgCl_2$ (Sturgeon, 1990). For the alternative method, the incubation medium was brought to 3 mL by adding buffer, coenzymes, cosubstrates, and coupling enzymes as described previously for the continuous assay. For the G-6-PDH method, the mixture contained 1.53 μmol of ATP, 8 units of HK, 0.88 μmol of $NADP^+$, 8 units of G6PDH, and 8 units of PGI in a final volume of 3 mL. Absorbance readings were taken at 340 nm using a spectrophotometer (Lambda 4B Perkin-Elmer). Invertase activity was calculated from the differences in absorbance before and after LDH or G6PDH and PGI were added. Enzyme activity was expressed on a dry weight basis (nanomoles of sucrose per gram of dry weight per minute). Four replications or more were run for each assay. Data were analyzed by the general linear models procedure, and comparison of means was performed by least significant differences (SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Continuous Assay. The principle of the alternative method is the phosphorylation of fructose and glucose by hexokinase concomitant with production of ADP, which is coupled to oxidation of NADH in the presence of PEP, PK, NADH, and LDH (Figure 1). Since several factors can contribute to the background rate of NADH oxidation, that is, sucrose purity, ATP hydrolysis by hexokinase (Creighton, 1993), and the presence of phosphoenolpyruvate phosphatase, ATPase, and adenylate kinase in the auxiliary enzymes (Harbron et al., 1980), it is important to evaluate sucrose purity and background rate of NADH oxidation caused by the factors mentioned above. The results (Table 1) indicate that the background rate of NADH oxidation was mainly due to ATP hydrolysis by HK (6.23 nmol/min), since the total background rate (III) was 6.72 nmol/min, which was only 0.67% of the initial velocity of invertase (Figure 2A). This indicated that the substrate, cosubstrates, and auxiliary enzymes are of high purity, due to their minor contribution to the background rate of NADH oxidation. The invertase activity measured with the continuous assay was directly proportional to the amount of purified invertase from baker's yeast (Figure

Table 3. Detection of Invertase Activities in Sigma PGO Enzyme Formula[a]

				activity (nmol capsule^{-1} min^{-1})	
lot	purchase date	expiration date	assay date[b]	acid invertase	alkaline invertase
096H6053	Nov 19, 1996	Oct 97	July 1, 1997	807	345
047h6058	Sept 29, 1997	Feb 99	Oct 1, 1997	638	342

[a] The critical component of glucose kit 510-A. Each kit contains 10 capsules of PGO enzymes. [b] In the case of invertase assay, one capsule of PGO enzymes was dissolved in 10 mL of deionized water. Three capsules were checked for each lot number, and three measurements were taken from each capsule. Deionized water was substituted for PGO solution in controls. Invertase activity was expressed as the amount of sucrose (nmol) hydrolyzed by one capsule of PGO enzymes per minute.

3A) and to the volume of concentrate from sweetpotato roots as well (Figure 3B), indicating that it is suitable to use the concentrated crude extract for invertase assay in the alternative method. Specific activity was calculated and compared at various volumes of enzyme preparation (Figure 4). The results showed that specific activity was significantly lower at 20 and 30 μL, and there was no difference between 40 and 80 μL of enzyme preparation, indicating that at least 40 μL of enzyme preparation was needed for optimum specific activity in the case of sweetpotato. However, this may not be the same for different plant materials. It is important to determine the appropriate enzyme volume for optimum specific activity. The results of specific activities revealed no significant difference between the two invertase assay methods from preparations of baker's yeast and sweetpotato roots (Figure 5). Therefore, the invertase specific activity obtained with the alternative method was comparable to that with the G-6-PDH method.

Discontinuous Assay. Plant tissue from three different species was tested to compare the accuracy of the alternative method for both acid and alkaline invertases with the G-6-PDH method (Table 2). Statistical analysis indicated the alternative method had the same accuracy as the G-6-PDH method in acid invertase activity for Cooper blueberry fruit and La Feliciana peach floral buds and in alkaline invertase activity for all three plant tissues. However, the alternative method showed a 20% higher acid invertase activity for Beauregard sweetpotato roots. Therefore, the data indicate in the discontinuous assay of plant invertase the alternative method is also reliable.

The rationale of the glucose oxidase method for plant invertase is that glucose released from sucrose by invertase is oxidized to gluconic acid by glucose oxidase with concomitant production of equimolar amounts of hydrogen peroxide. The oxidation of the peroxide by peroxidase is coupled to colored dye formation. The absorbance is measured at 425−475 nm (Sturgeon, 1990). In the case of the glucose oxidase method, a glucose kit (Sigma, 510-A) is routinely used for plant invertase assay (Lee et al., 1996; Darnell et al, 1994; Tomlinson et al., 1991). However, our data (Table 3) indicated that the glucose kit is not suitable for invertase assay, due to the presence of high activities of acid invertase and alkaline invertase in the peroxidase and glucose oxidase (PGO) enzyme formula, which gives a falsely high invertase activity value.

The discontinuous assay for plant invertases is commonly used in invertase-related research (Zhu et al., 1997; Zrenner et al., 1996; Obenland et al., 1993; Tomlinson et al., 1991; Miron and Schaffer, 1991; Hubbard et al., 1989). However, it has obvious disadavantages. It is time-consuming and tedious, requiring a length of time for incubation and either alkaline reagents or thermal denaturation to stop enzymatic reactions prior to assay.

This investigation has demonstrated a suitable, reliable, and effective alternative method is available for continuous and discontinuous assays of plant invertases.

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