A Continuous Method for Enzymatic Assay of Sucrose Synthase in the Synthetic Direction

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An appropriate method was developed for the continuous assay of sucrose synthase (SS) (EC 2.4.1.13) by spectrophotometry. The uridine 5′-diphosphate derived from sucrose synthesis was stoichiometrically coupled to oxidation of β-nicotinamide adenine dinucleotide by the enzymes nucleoside-5′-diphosphate kinase (NDPK), pyruvate kinase, and lactate dehydrogenase. Utilization of crude extracts led to a complete masking of SS assay by adenylate kinase, adenosine 5′-triphosphatase (ATPase), and phosphoenolpyruvate phosphatase found in the crude extracts. These interfering enzymes were mostly removed from the crude extracts by using a combination of gel filtration, centrifugation through a selectively permeable membrane (Biomax-100 Ultrafree centrifugal device), and inhibition by the addition of K_2HPO_4 to the assay buffer. Sensitivity of the SS assay was significantly increased by the inclusion of NDPK and ATP, which are essential to the reaction in the coupling system.

Keywords: *Sugars; enzyme assay; microfiltration; coupling reactions*

INTRODUCTION

Assays for sucrose synthase (SS) (EC 2.4.1.13) in the forward direction are typically based on the amount of sucrose or uridine 5′-diphosphate (UDP) formed. The most common methods utilized are colorimetric determination of sucrose formed using the thiobarbituric procedure (Pressey, 1969; Murata, 1971; Salerno and Pontis, 1978; Calderon and Pontis, 1985; Ajlouni and Hamdy, 1988; Nascimento et al., 1997), the resorcinol−HCl procedure (Fieuw and Willenbrink, 1987; Nguyen-Quoc et al., 1990; Willenbrink et al., 1998), or the H_2SO_4−anthrone procedure (Hubbard et al., 1989). The colorimetric methods are discontinuous and require utilization of a strong alkaline solution and boiling to destroy the unreacted fructose prior to sucrose measurement but are not considered reliable for use on crude extracts due to the presence of interfering substances (Pontis, 1977).

An alternative procedure for SS assay is to couple UDP production to β-nicotinamide adenine dinucleotide (NADH) oxidation by pyruvate kinase (PK) and lactate dehydrogenase (LDH) (Figure 1). This system (pathway II) has been used as a discontinuous procedure for citrus fruit (Lowell et al., 1989), pea seed coat (Déjardin et al., 1997), soybean nodules (Morell and Copeland, 1985), and wheat germ (Sigma Chemical Co., 1998). It has also been adapted for continuous assay of SS activities in Jerusalem artichoke tubers (Avigad, 1964), soybean nodules (Morell and Copeland, 1985), and maize kernel endosperm (Doehlert, 1987). Pathway II has been modified for continuous assay of sucrose phosphate synthetase (SPS) (Harbron et al., 1980) by including nucleoside-5′-diphosphate kinase (NDPK) and adenosine 5′-triphosphate (ATP) (hereafter termed pathway I).

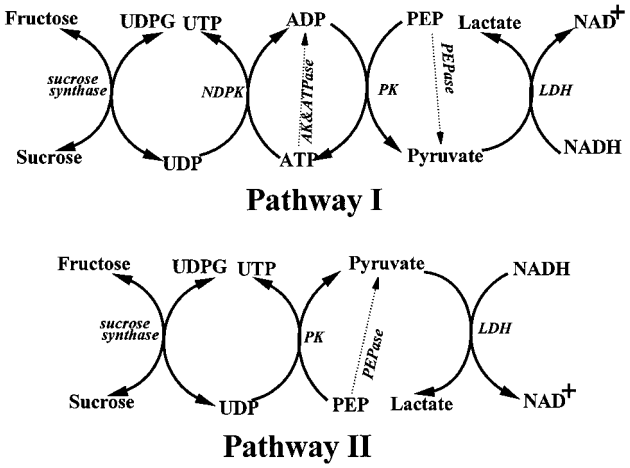

Figure 1. Schematic diagrams of the PK/LDH system for SS assay. Pathway I is the modified PK/LDH system by inclusion of ATP and NDPK. The broken lines indicate the interfering enzymes leading to blank rates of NADH oxidation.

The PK/LDH system for continuous assay of SPS and SS activities is not compatible with the use of crude extracts, which contain appreciable amounts of interfering enzymes such as adenosine 5′-triphosphatase (ATPase), adenylate kinase (AK), and phosphoenolpyruvate phosphatase (PEPase) (Harbron et al., 1980; Figure 2). The SS activity determined by the PK/LDH system would not be accurate and reliable without separating the SS-catalyzed reaction from the interfering reactions. Earlier studies reported partial purification of SS using ammonium sulfate precipitation, Ultrogel columns, dialysis, fast protein liquid chromatography, Mono-Q anion exchange chromatography, Sephadex G-200 size exclusion chromatography, DEAE-cellulose ion exchange chromatography, and PBA-60 affinity chromatography (Morell and Copeland, 1985; Doehlert, 1987). The disadvantages of these purification procedures

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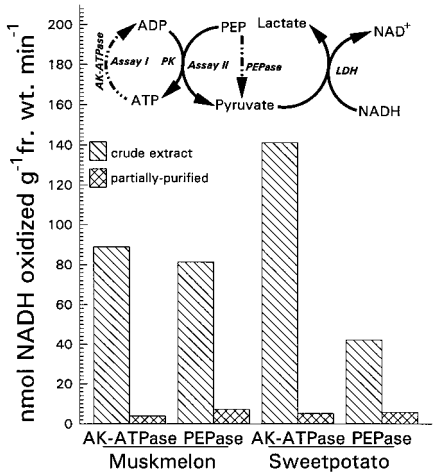

Figure 2. Activities of AK, ATPase, and PEPase in crude extracts and partially purified SS preparations from muskmelon fruit and sweetpotato roots. Schematic diagram of partitioning interferring enzyme activities is shown at the top of the bar graph. The reaction mixture contained PEP, NADH, and LDH for PEPase assay; ATP, PEP, NADH, PK, and LDH for assay I; and ADP, PEP, NADH, and LDH for assay II. Assays were performed in 50 mM HEPES/KOH buffer (pH 7.5) and initiated by adding 120 μL of SS preparation or crude extract. AK and ATPase activities were calculated by subtracting the rate of assay II from that of assay I. Each data point is the mean of four replications.

included severe loss of SS activity, laboriousness, and high cost. In this paper, we describe a simple and reliable method to obtain SS preparations suitable for a highly sensitive continuous assay by the modified PK/LDH system (pathway I).

MATERIALS AND METHODS

Materials. The following enzyme reagents and chemicals were purchased from Sigma Chemical Co. (St. Louis, MO): SS from wheat germ (5−20 units/mg of protein), NDPK from baker's yeast (1000−2000 units/mg of protein), PK from rabbit muscle (400−600 units/mg of protein), LDH from *Leuconostoc mesenteroides* (1000−3000 units/mg of protein), uridine 5′-diphosphoglucose (UDPG), uridine 5′-diphosphate (UDP), ATP, adenosine 5′-diphosphate (ADP), *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) potassium salt (HEPES), NADH, poly(vinylpyrrolidone) (PVP), ethylenediaminetetraacetic acid (EDTA), Triton X-100, ethylene glycol bis(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), phenylmethanesulfonyl fluoride (PMSF), dithiothreitol (DTT), and glycerol. Phosphoenolpyruvic acid (PEP) was purchased from Fisher Scientific (Pittsburgh, PA). Enzyme purity is essential when utilizing coupled reactions. The PK and LDH used were tested and found to be free of interfering enzyme activity. In addition, all of the reagents were subjected to a purity test.

Sweetpotato roots [*Ipomoea batatas* (L.) Lam.] cv. Beauregard were provided by the Louisiana Agriculture Center Sweetpotato Research Station, Chase, LA. Ripe muskmelon fruits (*Cucumis melo* L.) cv. Mission were obtained from the Lousiana Agriculture Center Burden Research Station, Baton Rouge, LA.

Extraction. All procedures were performed at 0−4 °C unless otherwise stated. Samples were taken from the cortex tissue of sweetpotato roots and from the inner mesocarp of muskmelon fruit. Portions (20 g each) of flesh were put in a prechilled mortar and ground into fine powder in liquid nitrogen using a pestle. The extraction buffer (ionic strength = 0.08, pH 7.5) was 100 mM HEPES/KOH containing 5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 5 mM DTT, 2% (w/v) insoluble PVP, 0.1% Triton X-100, and 1% glycerol. The powder was homogenized for 2 min in extraction buffer with a Virtis 45 homogenizer (Virtis Co., Gardiner, NY) 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 centrifuge (DuPont Instruments) at 23200*g*. The supernatant was collected and the pellet re-extracted in 8 mL of extraction buffer following the procedures mentioned above. The combined supernatants were vacuum-filtered four times through a double-layer of Whatman No. 1 filter paper, followed by filtering through two Nalgene filterwares (0.45 and 0.2 μm in pore size) (Nalgene Co., Rochester, NY). The resultant filtrate was termed the crude extract.

Enzyme Preparation. Fourteen milliliters of the crude extract was concentrated to 2.5 mL in a Biomax-100 (molecular weight cutoff at 100 kDa) Ultrafree-15 centrifugal filter device (Millipore Corp., Bedford, MA) by centrifugation (650*g*). This step took ∼1 h. The concentrate was reconstituted to 8 mL using 50 mM HEPES/KOH (pH 7.5) containing 2 mM EDTA (hereafter called buffer A) and concentrated to 2.5 mL. After three 15-min cycles of reconstitution and concentration, the concentrate was loaded onto a 1.9 × 20 cm Sephadex G-25 (coarse) column (Supelco, Inc., Bellefonte, PA), previously equilibrated with buffer A. The enzyme protein was eluted off the column at a flow rate of 0.4 mL/min, using 10 mL of buffer A. The reasons for using the G-25 column were for desalting, buffer exchange, and cleaning up the concentrate. The 10-mL volume of buffer A was adequate to elute the SS protein. The effluent was concentrated to 2.5 mL using a Biomax-100 filter. Three additional cycles of reconstitution and concentration were performed. The final concentrate was considered a partially purified preparation. The entire series of six reconstitution and concentration cycles took ∼2.5 h.

SS obtained from wheat germ (Sigma Chemical Co.) was prepared by dilution to 50 units/mL with buffer A.

Enzyme Assay. The assay buffer (pH 7.5) consisted of 50 mM HEPES/KOH, 10 mM K_2HPO_4, 15 mM $MgCl_2$, and 20 mM KCl. The assay mixture (3 mL) contained 80 μmol of fructose, 5 μmol of UDPG, 1.02 μmol of ATP, 1.2 μmol of PEP, 0.8 μmol of NADH, 4 units of NDPK, and 8 units each of PK and LDH. Sucrose synthases in wheat germ, muskmelon fruit, and sweetpotato roots were assayed at 25 °C following the modified PK/LDH coupling system (Figure 1). NADH oxidation was measured at 340 nm using a Lambda 4B UV/VIS recording spectrophotometer (Perkin-Elmer, Norwalk, CT). Reactions were initiated by the addition of 120 μL of SS preparation. The observations at the linear part of the curve were used to calculate the slope, which is the rate of product formed versus time. SS activity was determined by regression from the initial slope of the curve (Huang et al., 1998).

RESULTS AND DISCUSSION

The rationale of assaying SS by the PK/LDH system is that the derived UDP from sucrose synthesis is coupled to NADH oxidation by the enzymes PK and LDH (Figure 1). There are many factors that contribute to NADH oxidation, such as AK, ATPase, and PEPase present in the SS preparations (Figure 2) and impurities of cosubstrates and auxiliary enzymes (Figure 3). The rates of NADH oxidation given by these factors are blank rates. To ensure that the assays of SS activity are valid and reliable, it is imperative to eliminate the blank rates from the SS assay.

Utilization of the PK/LDH system is not possible for measuring SPS activity in crude extracts from leaves of spinach, wheat, and maize, due to the presence of appreciable amounts of AK, ATPase, and PEPase (Harbron et al., 1980). Similarly, activities of AK, ATPase, and PEPase were very high in the crude extracts of muskmelon and sweetpotato (Figure 2). The high activities of these interfering enzymes resulted in a complete masking of the SS-catalyzed reaction. Therefore, gel filtration and microfilters were used to remove AK,

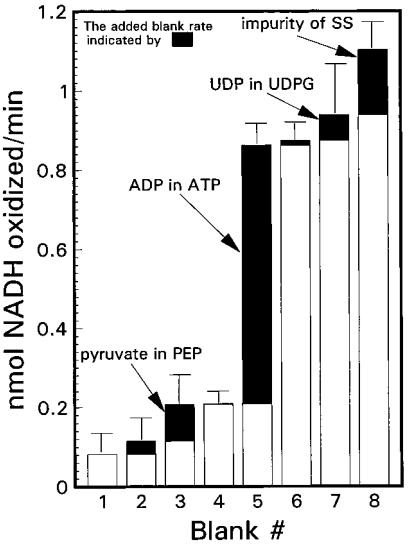

Figure 3. Impurities of cosubstrates, auxiliary enzymes, and SS preparation expressed as rate of NADH oxidation. Test conditions were identical with full assays for SS activity. Blank rates were obtained from a series of assays using incomplete reaction mixtures by including (1) NADH; (2) NADH and LDH; (3) NADH, LDH, and PEP; (4) NADH, LDH, PEP, and PK; (5) NADH, LDH, PEP, PK, and ATP; (6) NADH, LDH, PEP, PK, ATP, and NDPK; (7) NADH, LDH, PEP, PK, ATP, NDPK, and UDPG; and (8) NADH, LDH, PEP, PK, ATP, NDPK, UDPG, and heat-denatured wheat germ SS. Blank 1 was the nonenzymatic conversion of NADH to NAD^+. Subtract blank 1 from blank 2 for impurity of LDH, and so on for pyruvate in PEP; PEPase in PK; ADP in ATP; AK, ATPase, and UDP in NDPK; UDP in UDPG; and impurity in SS preparation. Each assay was the mean of four replications. Vertical bars represent the standard error.

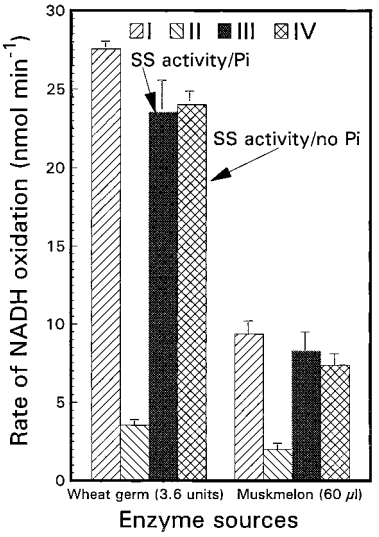

Figure 4. Effect of K_2HPO_4 on SS activity. Experimental conditions were identical with those mentioned for SS assay under Materials and Methods, except (I) K_2HPO_4 was excluded, (II) UDPG and K_2HPO_4 were omitted, and (III) 10 mM K_2HPO_4 was included in the HEPES/KOH buffer. Data for IV were obtained by subtracting II from I. Amounts of enzyme for each assay were 3.6 units of purified SS [Sigma unit definition: 1 unit converts 1.0 μmol each of UDPG and D-fructose to UDP and sucrose in 30 min at pH 7.5 at 37 °C, assayed in coupled assay system with PK/LDH (pathway II)] from wheat germ provided by Sigma Chemical Co. (lot 46H9508) and 60 μL of partially purified SS preparation from muskmelon. Each assay was the mean of four replications. Vertical bars represent the standard error.

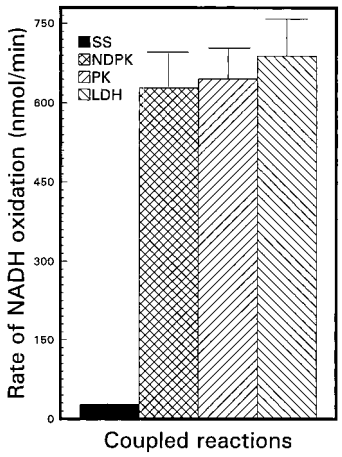

Figure 5. Reaction rates of the SS-catalyzed and coupling reactions in the modified PK/LDH system (pathway I) in terms of NADH oxidation. Assay conditions were identical with those described under Materials and Methods, except 1.2 μmol of pyruvate and 8 units of LDH were included in the LDH-coupled reaction; 1.2 μmol of ADP, 1.2 μmol of PEP, 8 units each of PK and LDH were included in the PK-coupled reaction; and 1.2 μmol of UDP, 1.02 μmol of ATP, 1.2 μmol of PEP, 4 units of NDPK, and 8 units each of PK and LDH were included in the NDPK-coupled reaction. The SS-catalyzed reaction rate was determined with 4 units of sucrose synthase from wheat germ. Assays for each coupled reaction were replicated at least four times. Vertical bars represent the standard error.

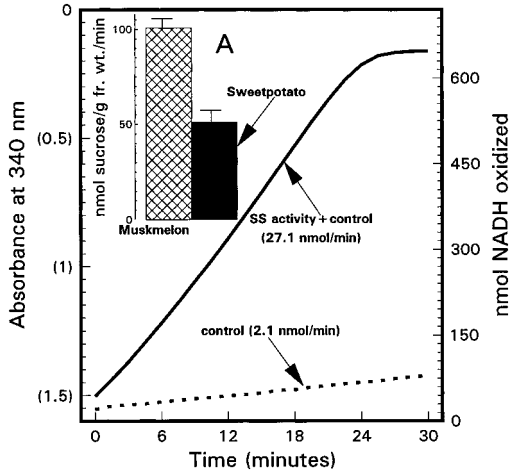

Figure 6. SS assay in a coupled assay system with PK/LDH (pathway I). The absorbance curve was obtained from partially purified SS preparation of muskmelon fruit (cv. Mission). Experimental conditions in the controls were identical with full assays except UDPG and NDPK were omitted. Assays for SS-specific activities of muskmelon fruit and sweetpotato roots were replicated four times and shown in (A).

ATPase, and PEPase from crude extracts. Over 95% of AK and ATPase and 88−91% of PEPase activities (Figure 2) were removed from the crude extracts of muskmelon fruit and sweetpotato roots by using the Biomax-100 Ultrafree centrifugal device and Sephadex G-25 columns.

The cosubstrates and auxiliary enzymes used for SS assay are of high purity, as indicated from their low contribution to NADH oxidation. The negligible blank rates of NADH oxidation were mainly due to the presence of ADP in ATP, pyruvate in PEP, UDP in UDPG, and impurities in the purified SS from wheat germ (Figure 3).

Molecules >100 kDa are retained by the Biomax-100 centrifugal device. SS molecular weight is ~400 kDa

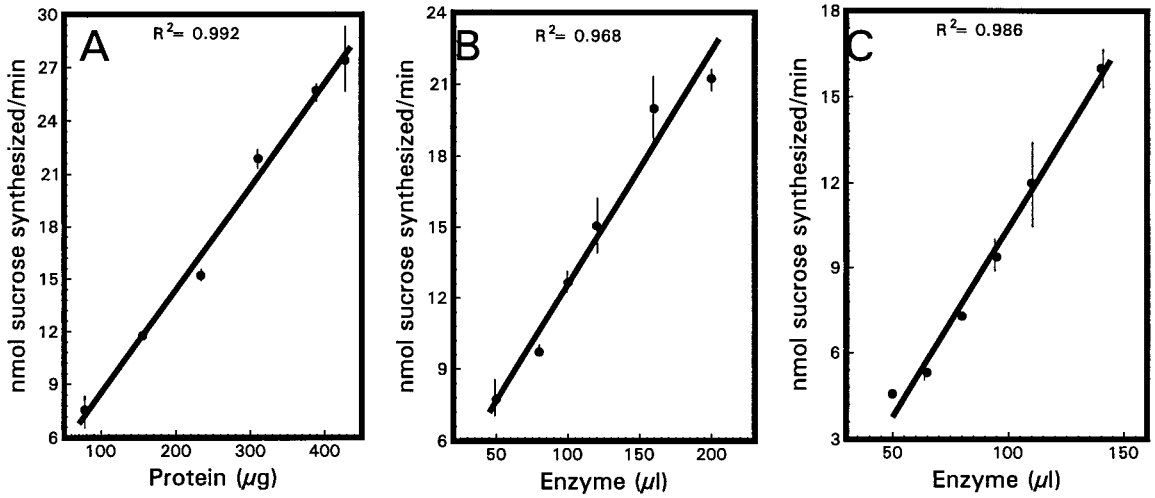

Figure 7. Enzyme concentration dependence of SS assay in 50 mM HEPES/KOH/10 mM K_2HPO_4 buffer: (A) purified sucrose synthase from wheat germ; (B and C) partially purified SS preparations from muskmelon fruit and sweetpotato roots, respectively. Each data point was the mean of four replications. Vertical bars represent the standard error.

with a range from 280 to 1000 kDa (Copeland, 1990). The SS activities recovered from Biomax-100 concentrates were 87.9% for muskmelon and 89.5% for sweetpotato (data not shown). Only a small percentage of SS activity was found in the lower part of the Biomax centrifuge tube containing the filtrate. The interfering enzymes AK, PEPase, and some ATPase polypeptides were easily removed from the SS preparations with the Biomax-100 centrifugal filter devices. Adenylate kinases are a group of small molecular weight proteins with a range from 21.5 to 27.5 kDa (Noda, 1973). The molecular weight for PEPase is 56 kDa (Duff et al., 1989). Purified preparations of plant plasma ATPases contain multiple polypeptides (15, 30, 50, 60, and 100 kDa) (Serrano, 1985). The interfering activities (AK, ATPase, and ATPase) were 12.7% in the purified SS from wheat germ and 21.4% in the SS preparation of muskmelon (Figure 4).

It is laborious and expensive to completely remove the interfering enzymes from the SS preparations. This is not practical when large amounts of samples have to be analyzed. Therefore, an alternative was used to inhibit the remaining interfering enzymes in the SS preparations by including 10 mM K_2HPO_4 in the assay buffer (50 mM HEPES/KOH). This strategy takes advantage of inorganic phosphate (P_i) as a potent competitive inhibitor of ATPase and PEPase (Podestá and Plaxton, 1991). The inclusion of HPO_4^{2-} in the assay buffer decreased the interfering activities for muskmelon fruit by 62% and for sweetpotato roots by 57% (data not shown) but did not exert inhibitory effects on the Sigma purified SS from wheat germ and the partially purified muskmelon preparation (Figure 4).

Utilization of the PK/LDH system for SS assay involves four reactions, that is, an SS-catalyzed reaction and three coupling reactions (Figure 1). The SS-catalyzed reaction is completely reversible. In the coupling reactions, UDP derived from the SS-catalyzed reaction is a substrate for NDPK, ADP generated in the NDPK-coupling reaction is a substrate for PK, and pyruvate formed in the PK-coupling reaction is a substrate for LDH. The rates of the coupling reactions were 22- to 25-fold greater than that of the SS-catalyzed reaction (Figure 5). This characteristic directs the reaction toward sucrose synthesis and ensures the SS-catalyzed reaction is always rate-limiting. The coupling

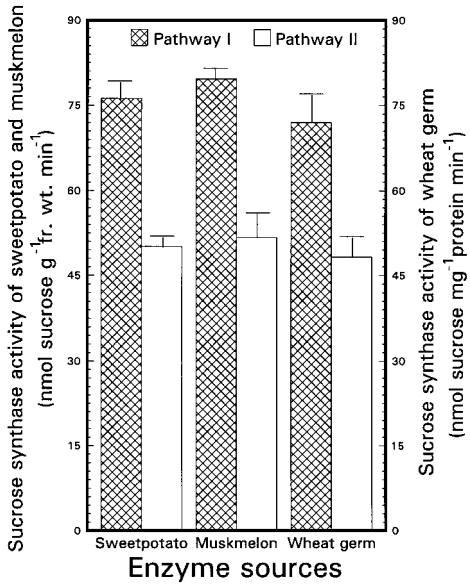

Figure 8. Specific activities of SS in wheat germ, muskmelon fruit, and sweetpotato roots determined by pathways I and II under identical assay conditions (see Materials and Methods) except for the inclusion of NDPK and ATP in pathway I. SS activities of muskmelon fruit and sweetpotato roots were expressed on a fresh weight basis, and SS activity of wheat germ was expressed on a protein basis. Assays were replicated four times. Vertical bars represent the standard error.

reactions occur so rapidly that the UDP yielded from sucrose synthesis is immediately utilized.

Utilization of the modified PK/LDH coupling system (pathway I) for SS assay gives accurate and repeatable results. The true rate of the SS-catalyzed reaction was obtained by simply subtracting the control rate derived from an incomplete mixture from that obtained for the full assay (Figure 6). The true rate was used to calculate SS-specific activities. The SS activities were 100.6 and 50.9 nmol of sucrose (g of fr wt)$^{-1}$ min^{-1} for muskmelon fruit and sweetpotato roots, respectively (Figure 6A). In sweetpotato roots treated with γ-irradiation, SS activity determined by the thiobarbituric method ranged from 20 to 80 nmol of sucrose (g of fr wt)$^{-1}$ min^{-1} (Ajlouni and Hamdy, 1988). In inner mesocarp tissue of ripened muskmelon fruit, SS activity measured with the H_2SO_4/anthrone procedure ranged from 42 to 93 nmol of

sucrose (g of fr wt)$^{-1}$ min^{-1} (Hubbard et al., 1989). The SS activity measured from pathway I was directly proportional to the amount of SS-containing protein from wheat germ (Figure 7A) and to the volumes of partially purified SS preparations from muskmelon fruit (Figure 7B) and sweetpotato roots (Figure 7C). This indicates it is suitable to use the partially purified preparations for SS assay in the PK/LDH coupling system.

SS-specific activities from pathways I and II were compared using three different plant materials. Pathway I showed 49.3−54.1% higher specific activity than pathway II (Figure 8). The difference in activity was due to the inclusion of NDPK and ATP in the assay procedure, which took advantage of the higher affinity of PK for ADP (K_m = 0.227 mM) compared with UDP (K_m = 2.68 mM) (Plowman and Krall, 1965) and the regeneration of ATP in the PK-coupled reaction. The advantage of pathway I is to significantly increase the sensitivity of the SS assay.

In conclusion, we have developed an accurate and reliable method for continuous assay of SS in the synthetic direction using a modified PK/LDH coupling system including ATP and NDPK.

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