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Artifactual detection of ADP-dependent sucrose synthase in crude plant extracts

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Results presented in a previous report from this laboratory indicated the presence, in crude extracts from sycamore (*Acer pseudoplatanus*) and spinach (*Spinacea oleracea*), of a sucrose synthase (EC 2.4.1.13) showing high affinity for ADP as the glucose acceptor in the sucrose-cleaving reaction. In the present paper we report that the modified enzymatic method previously used to measure sucrose synthase activities leads to the detection of artifactual ADP-dependent sucrose synthase, which in fact arises from the combined action of invertase (EC 3.2.1.26) and nucleoside diphosphate kinase (EC 2.7.4.6) activities. We also present data on the partial purification of nucleoside diphosphate kinase from sycamore cells.

Nucleoside diphosphate kinase; Sucrose synthase; Invertase

1. INTRODUCTION

Degradation of sucrose in plants is thought to involve sucrose synthase (SS) and/or invertase [1,2]. The reaction products of invertase are glucose and fructose, while SS cleaves sucrose producing fructose and glucose; the latter moiety transfers to a nucleoside diphosphate, usually UDP, thereby producing UDP-glucose.

In a recent paper, we reported the presence of an SS showing a high affinity for ADP as the glucose acceptor [3]. This was shown by a modification of the commonly used coupled enzymatic assay method [1], substituting ATP with GTP as the phosphate donor in the hexokinase reaction. Attempts to purify the ADP-dependent SS revealed that the previously reported activity [3] is an artifact produced by the combined action of invertase and nucleoside diphosphate kinase (NDPK) activities.

In the present paper we discuss data supporting this view and show the presence of NDPK activity in sycamore (*Acer pseudoplatanus*) extracts.

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Abbreviations: ADPG, ADP-glucose; NDPK, nucleoside diphosphate kinase; SS, sucrose synthase; G6PDH, glucose 6-P dehydrogenase; PGI, P-glucoisomerase.

2. EXPERIMENTAL

2.1. *Plant material and enzyme preparations*

For this investigation we used cultured cells of sycamore (*Acer pseudoplatanus*), protoplasts from which were obtained as previously described [4]. Protoplasts were disrupted, centrifuged, and the supernatant utilized.

2.2. *Enzyme assays*

The coupled invertase assay was performed as previously described [3] using 1 mM ATP and 150 mM sucrose. Invertase activity in partially purified preparations was also assayed by testing for glucose and fructose released from sucrose after incubation of the sample (50 μl) in 150 μl of sucrose (150 mM in 100 mM HEPES buffer, pH 7.0). After boiling the sample for 1 min, glucose was quantitated using the hexokinase-G6PDH (glucose 6-P dehydrogenase) assay method measuring NADH production (A_{340} nm). Fructose was then assayed after addition of P-glucoisomerase (PGI). Absorbances were compared with those obtained with known amounts of glucose and fructose.

ADP-dependent SS was assayed as previously described [3].

NDPK was assayed by two methods. Method 1 was as described [5], with minor modifications. The assay was performed spectrophotometrically at 20°C in a 0.5 ml reaction mixture containing ADP (100 μM), dGTP (170 μM), $MgCl_2$ (2 mM), KCl (15 mM), NAD (0.4 mM), glucose (200 μM), HEPES-KOH buffer (50 mM, pH 7.5) and 1 U each of hexokinase and G6PDH. The activity without ADP is negligible. Method 2 is the coupled assay method employing pyruvate kinase and lactate dehydrogenase described by Bergmeyer [6]. Method 1 was employed routinely except where otherwise indicated.

Adenylate kinase was assayed as described by Oliver [7].

2.3. *Partial purification of the enzymes*

Extracts were concentrated by ammonium sulphate precipitation (80% saturation) and the resulting pellet was resuspended in triethanolamine buffer (50 mM, pH 7.5) and applied to a Blue-Sepharose affinity column (HiTrap Blue, 5 ml column, Pharmacia) equilibrated with triethanolamine buffer. After sample loading the column was washed several times with buffer until no material absorbing at 280 nm was apparent in the effluent. Proteins bound to the column were eluted by loading ADP (10 mM, 2 column vols.) and collecting the

eluted proteins. Finally, the remaining proteins bound to the column were eluted with 2 M KCl. Fractions eluted with KCl were found to contain invertase activities (see Results). Although it had been reported that not only enzymes utilizing adenylates or adenyl containing cofactors but also several other proteins can bind to Blue-Sepharose [8], to our knowledge binding of invertase to this matrix has not been reported previously; the presence of invertase in KCl-eluted fractions was confirmed by testing the equimolar liberation of glucose and fructose from sucrose.

Fractions showing NDPK activity were pooled, dialyzed, and applied to a Mono S FPLC column pre-equilibrated with 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES, pH 5.5). The column was washed and eluted with a 0–1 M NaCl gradient in the same buffer.

2.4. *Electrophoresis*

SDS-polyacrylamide gel electrophoresis was performed on slab gels (15% polyacrylamide) as described by Laemmli [9].

Proteins were stained using a silver stain kit. Molecular weight standards used were: phosphorylase *b* (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100) and α-lactalbumin (14,400).

2.5. *Thin-layer chromatography of adenylates*

The separation of radiolabeled adenylates was carried out by using ascending cellulose TLC (isobutyric acid/water/ammonia/EDTA (37 g/l) = 500:280:21:8, v/v) followed by autoradiography.

2.6 Chemicals

All the chemicals used were purchased from Sigma (USA).

3. RESULTS AND DISCUSSION

Preliminary results indicated that the strong ADP-dependent SS activity detected in the extracts was easily lost after a single chromatographic step. This was true also for the Blue-Sepharose affinity column, but the activity was restored if fractions eluted with ADP were mixed with fractions eluted with KCl (not shown). This suggested that the apparent SS detected in the crude extracts was due to the combined action of distinct enzymes. Fractions eluted with KCl were found to contain invertase activity (Fig. 1), indicating the possibility that the enzyme eluted with ADP had, as a substrate, the glucose and/or fructose produced by invertase. Subsequent experiments excluded the possibility that this activity was due to hexokinase or G6PDH activated by ADP.

The replacement of ATP with GTP in the proposed assay method for the ADP-dependent SS [3] was the main modification of the classical method [1]. The affinity of yeast hexokinase (present in the coupling mixture) for GTP is several times lower than that for ATP [10], and the relative maximal velocities for the phosphate donor are 100 for ATP and 0.1 for GTP [10]. Since the assay mixture contains both GTP and ADP, we tested whether or not the fractions eluted with ADP contained an enzymatic activity capable of transferring the phosphate group from GTP to ADP. If so, the resulting production of ATP would strongly increase the rate of phosphorylation of glucose and fructose (resulting from the action of invertase) by hexokinase.

By incubating [^{14}C]ADP and [^{14}C]ADP+GTP together with the dialyzed pooled fractions eluted from the Blue-Sepharose column with ADP, we realized that ADP was effectively phosphorylated to ATP when incubated with GTP (Fig. 2, lanes B and C). It was therefore postulated that this activity was a NDPK catalyzing the following reaction: GTP+ADP <=>; GDP+ATP. A low adenylate kinase activity (ADP+ADP <=>; ATP+AMP) was also evident from the small amounts

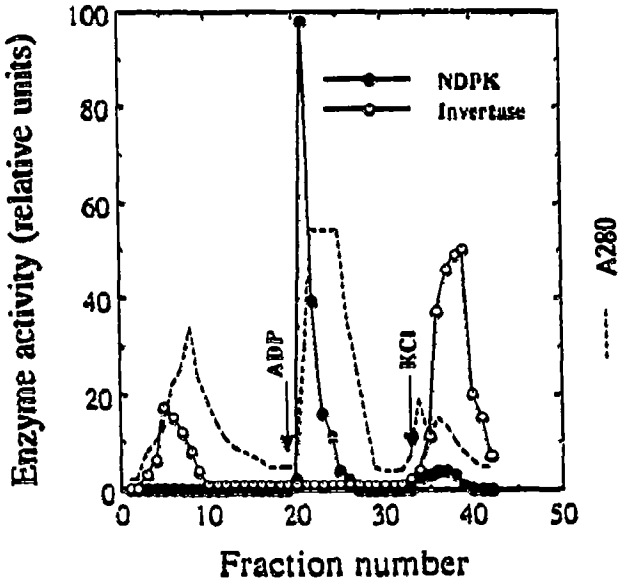


Fig. 1. Elution of invertase and NDPK from a Blue-Sepharose affinity column. ADP (20 mM) and KCl (2 M) were used to elute bound proteins as indicated by the arrows.

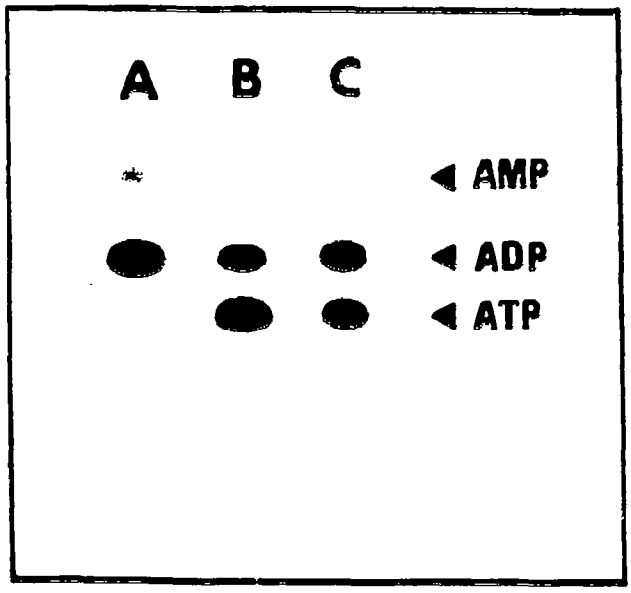
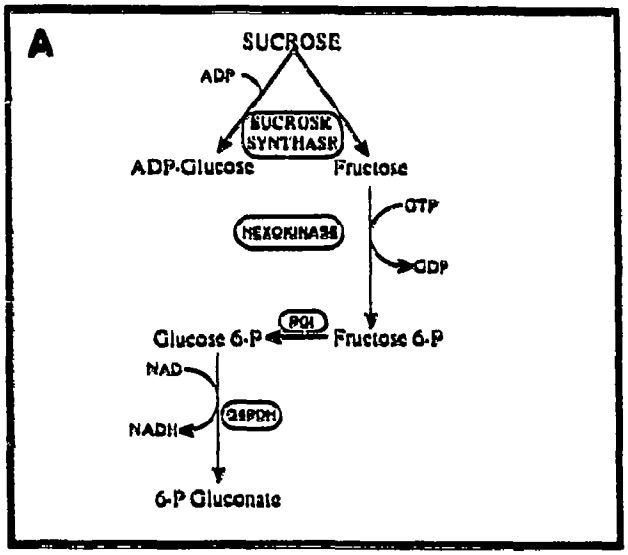


Fig. 2. TLC of adenylates after incubation (30 min, 37°C) with partially purified NDPK (pooled fractions from a Blue-Sepharose affinity column). Lane A, incubation with [^{14}C]ADP (200 μM); lane B, incubation with [^{14}C]ADP (1 mM) + GTP (1 mM); lane C, incubation with [^{14}C]ADP (200 μM) + GTP (1 mM). AMP, ADP, and ATP were identified using the corresponding labeled adenylates.



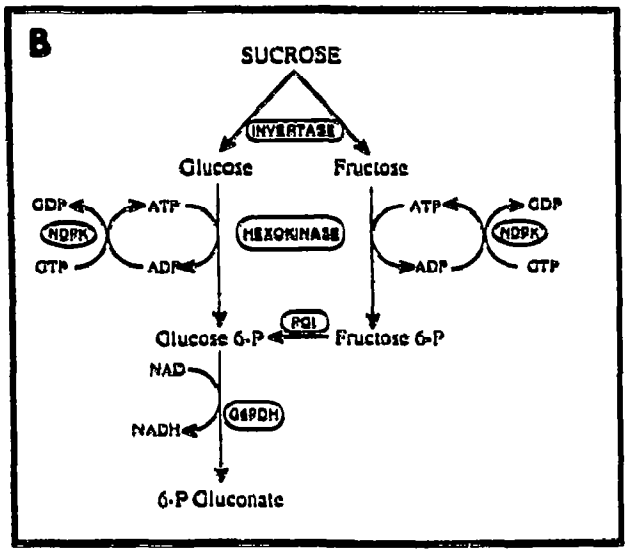


Fig. 3. Schematic diagram of coupling assay method. (A) SS activity detection as proposed previously [3]: addition of ADP activates SS producing ADP-glucose and fructose. (B) Artifactual SS from the combined action of invertase and NDPK: addition of ADP leads to the formation of ATP by the action of NDPK leading to a faster phophorylation of glucose and fructose resulting from invertase activity.

of AMP and ATP produced by the incubation of the partially purified enzyme preparations with radioactive ADP (Fig. 2, lane A).

NDPK activity was then easily detected in the fractions from the Blue-Sepharose column (Fig. 1).

A faint adenylate kinase activity was detectable spectrophotometrically in crude extracts but was below the detection limit of the method in partially purified preparations.

Table I shows data indicating the lower efficiency of GTP in the coupled assay for invertase. Addition of

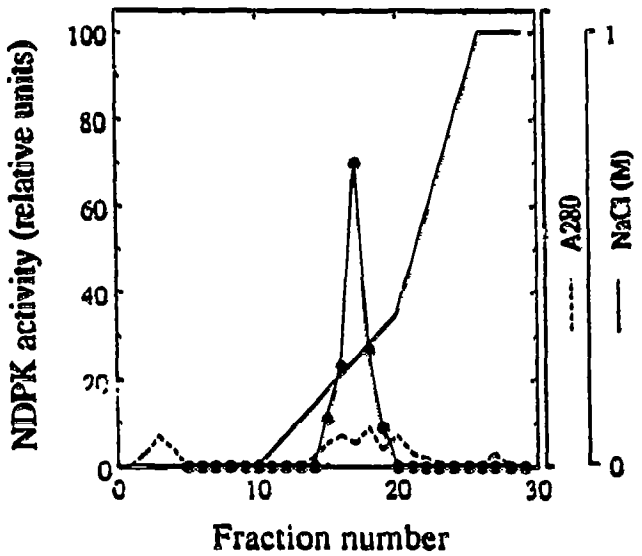


Fig. 4. Elution of NDPK from a Mono S column. Dialyzed active fractions pooled from 10 separate Blue-Sepharose affinity columns (total volume 11 ml) were loaded onto a Mono S HR5/5 column (0.5 × 5 cm) and eluted with a NaCl gradient as shown in the figure.

both GTP and ADP results in the detection of a higher activity, previously attributed to an ADP-dependent SS. This activity is comparable to that of invertase when assayed using ATP. We tend to think that the increased activity after addition of ADP is due to the GTP-mediated conversion of ADP to ATP, resulting in a higher efficiency of the coupled assay system. If addition of ADP is delayed with respect to the addition of the other components present in the assay mixture, a higher activity is observed. This is probably due to the accumulation of glucose and fructose, resulting from the invertase activity, which are not efficiently phosphorylated by hexokinase in the presence of GTP; addition of ADP results in its conversion to ATP which, together with the higher initial hexose concentration leads to a higher NADH production. NDPK activity was high enough to

Table I

Invertase (1,2), apparent ADP-dependent SS (3,4) and NDPK (5-7) activities in crude extracts from Sycamore protoplast

Enzyme assay	Nucleotide added after		Activity (nmol/min/mg prot.)
	0 min	1 min	
1 Invertase	ATP	-	209
2 Invertase	GTP	-	72
3 Invertase (apparent ADP-SS)	GTP, ADP	-	217
4 Invertase (apparent ADP-SS)	GTP	ADP	330
5 NDPK*	dGTP, ADP	-	466
6 NDPK*	dGTP	ADP	453
7 NDPK**	dTDP, ATP	-	418

Activity is expressed in nmol of NADH produced or NADH utilized**. *Assay 1 for NDPK; **Assay 2 for NDPK. Nucleotides were added at the times indicated.

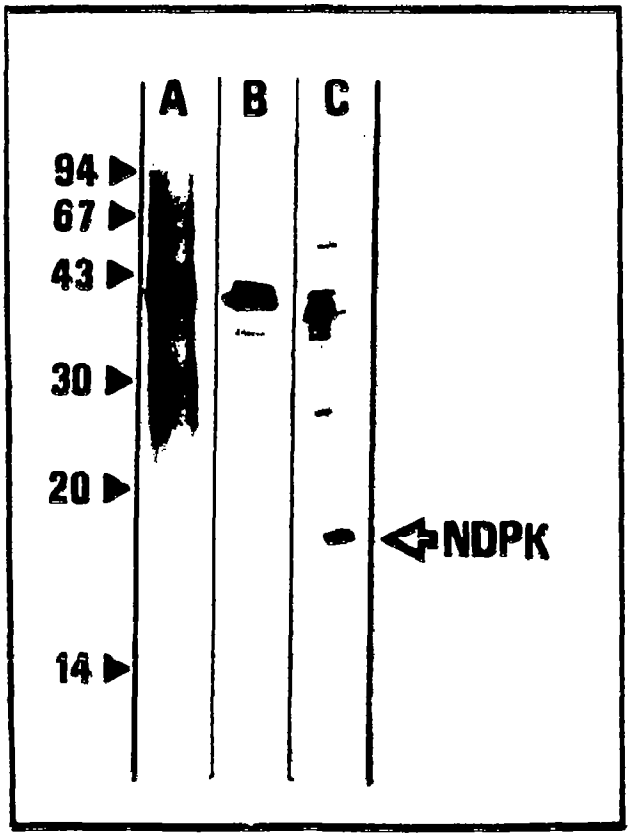


Fig. 5. Purification of NDPK, monitored by SDS-PAGE. Lane A, crude extract (5 μl); lane B, pooled active fractions after Blue-Sepharose affinity column (35 μl); lane C, pooled active fractions after Mono S column (16 μl). Lane C was run in a separate gel.

account for the apparent ADP-dependent SS activities (Table I).

Adding a small amount of ATP to the assay mixture containing GTP partially restores invertase activity, which is underestimated when using GTP alone (Table I). Since SS activity is estimated after subtracting the invertase activity measured without addition of ADP [3], the presence of even small amounts of ATP added to the reaction mixture thus reduces the activation due to the subsequent ADP addition. This explains the previously reported inhibitory effect of ATP in the ADP-dependent SS assay [3]. The observed inhibitory effect of Tris buffer [3] is probably due to the inhibition of invertase by this buffer [1].

Fig. 3 summarizes the reactions taking place during the assay. If an ADP-dependent SS is present, we can predict that the addition of ADP may increase the sucrose breakdown ([3]; Fig. 3A). However, our present results indicate that substitution of ATP with GTP as the phosphate donor for hexokinase generates artifactual SS activity. This is due to the NDPK-mediated conversion of ADP to ATP, resulting in an increased ability of hexokinase to phosphorylate glucose and fructose derived from the invertase activity (Fig. 3B). Adenylate kinase activity is unlikely to affect the SS assay since its activity is barely detectable in the crude extracts.

The presence of relatively high NDPK activity in the crude extracts of sycamore suggests that a caution should be given when using a coupled enzymatic assay method for the detection of SS activities. This is also true for the classical assay method [1] with ATP as the phosphate donor in the hexokinase reaction and UDP as the glucose acceptor in the SS reaction: under these conditions the interconversion, ATP+UDP <=>; ADP+UTP, may take place, thus leading to erroneous kinetics (a lower UDP concentration may be present) and nucleoside diphosphate specificity (ADP is also present together with UDP). Therefore, partial purification of crude extracts [1] is a prerequisite for the accurate estimations of SS. Alternatively, a non-coupled assay method may be used [11].

NDPK was further purified by Mono S FPLC. The results, shown in Fig. 4 are consistent with those reported by Nomura et al. [12].

Fig. 5 shows the polyacrylamide gel electrophoresis pattern of the partially purified NDPK. A more intense band, corresponding to a molecular mass of 18 kDa, was evident after Mono S chromatography. Since the intensity of this band was only increased after the Mono S purification step we tentatively assigned this band as the NDPK from sycamore. The data on NDPK from plant tissues is relatively small. Only a few reports concerning this enzyme have been published to date [5,12–14] and NDPK has only recently been purified to homogeneity from spinach leaves [12]. Our results may indicate that NDPK from sycamore cells is a polypeptide with a molecular mass of 18 kDa. Nomura et al. [12] have established that two NDPK subunit proteins exist in spinach, with molecular masses of 16 and 18 kDa. The native enzyme is composed of six identical subunits [12]. It has been shown that NDPK from pig brain is composed of at least three proteins having the same molecular mass (17 kDa) but different electrophoretic mobilities [15]. Our preliminary results appear to indicate that a single NDPK is present in sycamore. Further studies are needed to clarify this issue.

Our present report, while demonstrating that the previously described method for detecting ADP-dependent SS leads to artifactual results, cannot exclude the possibility that SS can use ADP as the glucose acceptor. Indeed, it has been reported that an SS using ADP with high efficiency is present in sugar beet root [16]. Further work is obviously needed to verify whether or not, apart from the plastid ADPG pyrophosphorylase, an extraplastid enzyme capable of producing ADPG is present in plant tissues.

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