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A Specific Sucrose Phosphatase from Plant Tissues

By J. S. HAWKER and M. D. HATCH
David North Plant Research Centre, Colonial Sugar Refining Co. Ltd., Indooroopilly, Queensland, Australia

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1. A phosphatase that hydrolyses sucrose phosphate (phosphorylated at the 6-position of fructose) was isolated from sugar-cane stem and carrot roots. With partially purified preparations fructose 6-phosphate, glucose 6-phosphate, fructose 1-phosphate, glucose 1-phosphate and fructose 1,6-diphosphate are hydrolysed at between 0 and 2% of the rate for sucrose phosphate. 2. The activity of the enzyme is increased fourfold by the addition of Mg^{2+} ions and inhibited by EDTA, fluoride, inorganic phosphate, pyrophosphate, Ca^{2+} and Mn^{2+} ions. Sucrose (50mM) reduces activity by 60%. 3. The enzyme exhibits maximum activity between pH 6·4 and 6·7. The Michaelis constant for sucrose phosphate is between 0·13 and 0·17mM. 4. At least some of the specific phosphatase is associated with particles having the sedimentation properties of mitochondria. 5. A similar phosphatase appears to be present in several other plant species.

A derivative of sucrose is probably involved in the process of sugar accumulation in sugar-cane (Glasziou, 1961; Sacher, Hatch & Glasziou, 1963; Hawker & Hatch, 1965). Hatch (1964) provided evidence consistent with the proposition that this derivative is sucrose phosphate (phosphorylated at the 6-position of fructose). He also described phosphatases which hydrolyse sucrose phosphate but at the pH values chosen for assay no evidence for a specific sucrose phosphatase was obtained. Other workers (Leloir & Cardini, 1955; Mendicino, 1960; Bird, Porter & Stocking, 1965; Haq & Hassid, 1965) have briefly described phosphatases which hydrolyse sucrose phosphate but no detailed evidence has been provided to show that a specific enzyme was involved. The present paper describes the partial purification and properties of a sucrose phosphate phosphohydrolase (referred to below as sucrose phosphatase) extracted from sugar-cane and carrot tissues.

MATERIALS

Stem tissue from expanding internodes of sugar-cane (var. Pindar) was classified as immature and tissue from fully expanded internodes as mature. Sugar-cane shoots and roots, and etiolated seedlings of wheat, barley and peas, were obtained by germinating sets or seeds in vermiculite in the dark at 26°. Carrot, potato and parsnip storage tissues were obtained from the local market.

Sucrose phosphate, phosphorylated at the 6-position of fructose and containing radioactivity only in the fructose moiety, was prepared enzymically (Hatch, 1964). Hypatite C (modified calcium phosphate) was obtained from Clarkson Chemical Co., Williamsport, Pa., U.S.A., and invertase from Difco Laboratories, Detroit, Mich., U.S.A.

METHODS

Preparation of sucrose phosphatase

From immature stem tissue of sugar-cane. The tissue (500g.) was cooled to 2°, sliced and homogenized with 500ml. of medium containing 100mM-tris–HCl buffer, pH 7·6, 300mM-mannitol, 10mM-EDTA, 10mM-$MgCl_2$, 20mM-sodium diethyldithiocarbamate, 3mM-cysteine hydrochloride and 500mg. of bovine serum albumin. All operations were carried out at 2°. During homogenization the pH was maintained between 7·0 and 7·5 by the addition of 3N-KOH. The filtrate obtained by squeezing the homogenate through muslin was centrifuged for 5min. at 2000*g*. The supernatant fluid was centrifuged at 10000*g* for 15min. and the mitochondrial pellet was washed twice with 300mM-mannitol containing 1mM-tris–HCl buffer, pH 7·2. The final pellet was suspended in 5ml. of the same medium, frozen and thawed twice, and then centrifuged at 100000*g* for 30min. The supernatant (5ml.) contained about 70mg. of protein. Half of this was poured on to a calcium phosphate gel column (Hypatite C, volume 5ml.) that had been previously washed with 5mM-potassium phosphate buffer, pH 6·5. The eluting solutions applied were 8ml. of the above buffer, 8ml. of 40mM-potassium phosphate buffer, pH 6·5, 8ml. of 80mM-potassium phosphate buffer, pH 6·5, and 8ml. of 250mM-potassium phosphate buffer, pH 6·5; 4ml. fractions were collected. After the addition of 20mg. of bovine serum albumin, each fraction was dialysed for 12hr. against 2l. of diluted (1:100) tris–maleate buffer, pH 6·5, described by Gomori (1955). Most of the sucrose phosphatase appeared in the first 4ml. of the 80mM-buffer that emerged from the column. This fraction is referred to below as 'partially purified enzyme of sugar-cane'.

Alternatively, soluble enzyme (4ml.), obtained from frozen-and-thawed mitochondria by the procedure already described, was applied to the top of a column of Sephadex G-100 (35cm. × 2·5cm.) which had been washed with 2mM-tris–HCl buffer, pH 7·0. The same buffer was passed through the column and 5ml. fractions were collected and immediately mixed with 25mg. of bovine serum albumin. Most of the sucrose phosphatase appeared in the fourth, fifth and sixth fractions after the emergence of one void volume of eluent.

From carrot-root tissue. Soluble enzyme was obtained from frozen-and-thawed mitochondria by the procedure described above. A sample (4ml.) of this preparation was treated on Sephadex G-100 as described for sugar-cane enzyme. The fourth, fifth and sixth fractions were pooled and are referred to below as 'partially purified enzyme of carrot'.

From other tissues. Mitochondria were isolated by the same procedure described for sugar-cane stem tissue except that 20g. of tissue was ground in a mortar with 2vol. of medium without diethyldithiocarbamate. After centrifuging the frozen-and-thawed mitochondria at 100000*g* the supernatant (0·5ml.) was stored. The fragmented mitochondria in the pellet were washed twice with 1ml. portions of tris–maleate buffer, pH 6·5, containing 5mg. of bovine serum albumin/ml. The washings were discarded and the pellet was suspended in 0·5ml. of the same solution.

Measurement of phosphatase activity

Hydrolysis of sucrose phosphate. Except where otherwise indicated reaction mixtures contained enzyme solution, [*fructosyl*-^{14}C]sucrose phosphate (4·9mμmoles, 38000 disintegrations/min.), 0·8μmole of $MgCl_2$, 3·5μmoles of tris–maleate buffer, pH 6·5, and 0·4mg. of bovine serum albumin in a final volume of 80μl. In studies on release of sucrose phosphatase from mitochondria, reaction mixtures contained, in addition, 24μmoles of mannitol. Mixtures were incubated at 22° and the reaction was stopped by heating at 100° for 2min. Activity was determined and the product of the reaction identified as described by Hatch (1964). Saturating concentrations of sucrose phosphate were not used in the standard assay because of the limited supply of sucrose phosphate. Generally, conditions were chosen which gave less than 15% hydrolysis of sucrose phosphate and initial rates were obtained directly. In other cases initial rates could be calculated since under the conditions employed the kinetics of the reaction were essentially first-order. The maximum velocity for hydrolysis of sucrose phosphate was calculated from the initial rates at various substrate concentrations (Lineweaver & Burk, 1934). In subsequent assays maximum velocities of sucrose phosphate hydrolysis could be calculated for comparison with rates of hydrolysis of other substrates.

Hydrolysis of other phosphorylated compounds. Reaction mixtures contained 0·4ml. of enzyme, 6μmoles of substrate, 5μmoles of tris–maleate buffer, pH 6·5, and 2·5mg. of bovine serum albumin in a final volume of 0·5ml. In studies on release of phosphatase from mitochondria, reaction mixtures contained, in addition, 150μmoles of mannitol. The reactions were stopped by adding 0·5ml. of 15% perchloric acid. After removal of precipitated protein the inorganic phosphate in the reactions and blanks was determined (Allen, 1940). At a concentration of 12mM, enzyme preparations were saturated with respect to fructose 6-phosphate.

Determination of protein. The protein content of mitochondrial suspensions was determined by digestion followed by distillation and nesslerization. The protein content of solutions was measured by the method of Warburg & Christian (1941).

RESULTS

Purification of sucrose phosphatase. Most of the phosphatase which hydrolyses fructose 6-phosphate in sugar-cane mitochondrial extracts was separated from sucrose phosphatase by treatment on calcium phosphate gel (Fig. 1). Partially purified enzyme of sugar-cane (fraction 5 in Fig. 1) hydrolysed fructose 6-phosphate, fructose 1-phosphate, glucose 6-phosphate and UDP at less than 5% of the rate for sucrose phosphate with saturating concentrations of all substrates. When fructose 6-phosphate and sucrose phosphate were supplied at the same concentration, 60μM, the rate of hydrolysis of sucrose phosphate was more than 200 times that of fructose 6-phosphate. However, fructose 1,6-diphosphate was hydrolysed at the same rate as sucrose phosphate. After this preparation had been heated at 40° for 10min. it hydrolysed fructose 1,6-diphosphate at the same rate as before heating but sucrose phosphate was hydrolysed at only 1% of its original rate. The preparation obtained by chromatography of mitochondrial extracts of sugar-cane on Sephadex G-100 hydrolysed fructose 1,6-diphosphate at only 5% of the rate for sucrose phosphate. However, this preparation hydrolysed UDP at 35% of the rate for sucrose phosphate. When a sequence of the above purification procedures was used a large part

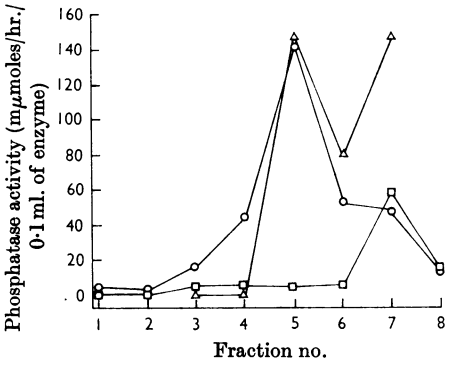

Fig. 1. Separation of phosphatase activities in mitochondrial extract from sugar-cane by chromatography on calcium phosphate gel. The rates of hydrolysis of sucrose phosphate (○), fructose 6-phosphate (□) and fructose 1,6-diphosphate (△) at saturating concentrations are shown. The concentration of inorganic phosphate in the eluate was 5mM in fractions 1 and 2, 40mM in fractions 3 and 4, 80mM in fractions 5 and 6, and 250mM in fractions 7 and 8. Other details are given in the Methods section.

of the sucrose-phosphatase activity was lost but the more stable contaminating enzymes remained active. Since the phosphatase or phosphatases which hydrolysed fructose 1,6-diphosphate in partially purified enzyme of sugar-cane did not hydrolyse sucrose phosphate at a significant rate this fraction was used for studies of the properties of sucrose phosphatase.

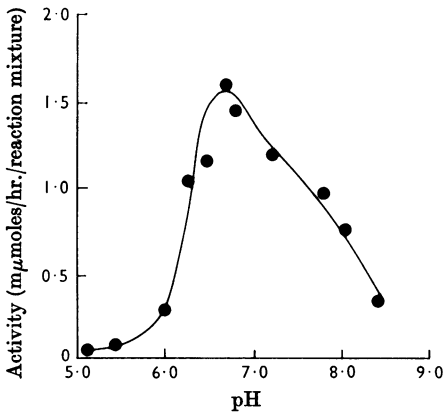

Fig. 2. Effect of pH on the activity of partially purified sucrose phosphatase from sugar-cane. Reaction mixtures were as described in the Methods section except that the tris–maleate buffer was adjusted to different pH values with NaOH. The incubation time was 1 hr. at 22°.

Michaelis constant. Reaction conditions were adjusted to give less than 15% hydrolysis of sucrose phosphate with a range of concentrations up to 0·3 mM. Several determinations of the Michaelis constant by the procedure of Lineweaver & Burk (1934) gave values between 0·13 and 0·17 mM.

Reaction product. The product of the reaction with sucrose phosphate co-chromatographed with sucrose. Hydrolysis of the product with analytical invertase yielded a radioactive compound which co-chromatographed with fructose.

Effect of pH. With sucrose phosphate as substrate the enzyme had optimum activity between pH 6·4 and 6·7 (Fig. 2). Results of isoelectric precipitation trials suggested that the sharp fall on the acid side of the pH optimum may have been due to inactivation of the enzyme rather than to low initial rates at these pH values.

Activators and inhibitors. The activity of the partially purified enzyme was increased about four-fold by the addition of 10 mM-magnesium chloride (Table 1). EDTA (10 mM) in the presence of 5 mM-magnesium chloride inhibited activity by 81% and by 100% in the absence of magnesium chloride. Calcium chloride (10 mM), manganese chloride (10 mM) and potassium fluoride (50 mM) inhibited activity by 89%, 37% and 66% respectively. Inorganic phosphate (50 mM) and pyrophosphate (30 mM) inhibited sucrose-phosphatase activity by 75% and 87% respectively. Sucrose (50 mM) reduced activity by 60% and toluene either inhibited or partially inactivated the enzyme.

Table 1. *Activators and inhibitors of sucrose phosphatase from sugar-cane*

The standard reaction mixture (described in the Methods section) contained 10 mM-$MgCl_2$. The incubation time was 1 hr. at 22°.

Expt. no.	Addition to or omission from the standard mixture	Sucrose phosphate hydrolysed (mμmoles/hr./reaction mixture)
1	None	1·76
	$MgCl_2$ omitted	0·40
	With 1 mM-$MgCl_2$	1·40
	With 5 mM-$MgCl_2$	1·75
	With 5 mM-$MgCl_2$ and 10 mM-EDTA	0·34
	With 10 mM-EDTA, $MgCl_2$ omitted	0
	With 10 mM-$CaCl_2$	0·19
	With 10 mM-$MnCl_2$	1·12
	With 50 mM-KF	0·63
	With 5 mM-inorganic phosphate	1·63
	With 50 mM-inorganic phosphate	0·44
	Enzyme boiled	0
	Enzyme omitted	0
2	None	1·1
	With 30 mM-inorganic pyrophosphate	0·14
3	None	2·8
	With 50 mM-sucrose	1·1
	With 10 μl. of toluene	1·64

Table 2. *Purification of sucrose phosphatase from carrot-root tissue*

The muslin filtrate and washed mitochondrial fractions were frozen and thawed twice before assay. Rates of hydrolysis of sucrose phosphate and of fructose 6-phosphate were determined as described in the Methods section.

Fraction	Sucrose phosphate hydrolysis (mμmoles/g. of tissue/hr.)	Sucrose phosphate hydrolysis (μmoles/mg. of protein/hr.)	Fructose 6-phosphate hydrolysis (mμmoles/g. of tissue/hr.)	Ratio of activity (sucrose phosphate/ fructose 6-phosphate)
Filtrate through muslin	25600	—	7150	3·6
Washed mitochondria	373	1·71	24·7	15
100000*g* supernatant	194	5·4	8·8	22
Sephadex fraction 4	38	15·7	1·72	22
Sephadex fraction 5	56	46·0	1·85	30
Sephadex fraction 6	41	42·5	1·25	33

Enzyme stability. Sucrose phosphatase in the soluble preparations obtained from sugar-cane mitochondria remained stable when stored for 10 days at −15°. Upon further purification losses of up to 50% were observed during storage for 7 days at −15°. Activity was completely destroyed by heating the partially purified enzyme at 40° for 10min. or at 100° for 2min. Exposure to pH 5·2 for 15min. at 20° destroyed 97% of the activity.

Substrate specificity. Preparations of sucrose phosphatase from sugar-cane showed relatively low activity towards most other substrates tested. Attempts to obtain a preparation virtually free of other phosphatase activity by a combination of procedures were hindered by low recoveries of the sucrose phosphatase. Compared with mitochondrial extracts from sugar-cane, carrot-root preparations contained more sucrose phosphatase (Table 4) and showed lower activity towards other substrates. The results of the further purification of this extract are shown in Table 2. The ratio of sucrose phosphate hydrolysis to fructose 6-phosphate hydrolysis increased from 3·6 in the first filtrate to 33 in fraction 6 from the Sephadex column. On a protein basis the enzyme from mitochondria was purified 27-fold. The rates of hydrolysis of several other phosphorylated compounds relative to the rate of sucrose phosphate hydrolysis by the partially purified enzyme of carrot are shown in Table 3. These rates would be maximum values for hydrolysis of these compounds by sucrose phosphatase since the preparation would still contain traces of other phosphatases.

Properties of carrot sucrose phosphatase. Partially purified enzyme of carrot requires Mg^{2+} ions for activity, is inhibited by sucrose and is unstable in dilute solution. The enzymes from carrot and sugar-cane have similar Michaelis constants for sucrose phosphate and have the same mobility on Sephadex G-100.

Release of sucrose phosphatase from mitochondria. When washed sugar-cane mitochondria were frozen and thawed sucrose-phosphatase activity increased 9·3-fold whereas activity towards fructose 6-phosphate increased only 2·3-fold. The same treatment released 90% of the sucrose phosphatase in a soluble form whereas only 60% of the phosphatase that hydrolysed fructose 6-phosphate was rendered soluble.

Table 3. *Substrate specificity of carrot sucrose phosphatase*

The partially purified enzyme was incubated at 22° for 3hr. with all substrates (12mM) except sucrose phosphate. A sample of the enzyme treated under the same conditions without substrate was then diluted and incubated for 10min. with 61μM-[*fructosyl*-^{14}C]sucrose phosphate. The maximum velocity for sucrose phosphate hydrolysis was calculated and compared with the rates of hydrolysis of other compounds. Other details of the assay procedures are given in the Methods section.

Substrate	Relative activity
Sucrose phosphate	100
Fructose 6-phosphate	1·9
Fructose 1-phosphate	0
Fructose 1,6-diphosphate	1·5
Glucose 6-phosphate	1·0
Glucose 1-phosphate	0·4
UDP	1·7
AMP	0·9
IMP	0
Phosphoenolpyruvate	5·0
α-Glycerophosphoric acid	2·5
p-Nitrophenyl phosphate	3·9

Occurrence of sucrose phosphatase in other tissues from sugar-cane and other plants. Table 4 shows that the rate of hydrolysis of sucrose phosphate by soluble extracts of mitochrondia from several tissues greatly exceeded the rate of fructose 6-phosphate hydrolysis. As already observed with sugar-cane stem mitochondria the ratio of the two activities was much greater in the soluble extracts

Table 4. *Occurrence of sucrose phosphatase in different tissues of sugar-cane and other plants*

Mitochondria were isolated and enzyme preparations made and assayed as described in the Methods section.

Source of mitochondria	Activity in mitochondrial supernatant (mμmoles of sucrose phosphate hydrolysed/g. of fresh tissue/min.)	Ratio of hydrolysis (sucrose phosphate/fructose 6-phosphate)	
		Mitochondrial supernatant	Mitochondrial fragments
Sugar-cane			
Immature stem tissue	0·9	12·5	—
Mature stem tissue	0·06	6·2	—
Roots	0·3	4·4	—
Leaf laminae	0·7	11·0	1·1
Shoots	0·9	22·0	5·3
Etiolated barley seedlings	1·7	16·0	1·7
Etiolated oat seedlings	2·0	12·6	1·4
Etiolated pea seedlings	2·2	8·8	1·6
Carrot root	2·7	31·9	7·8
Parsnip root	0·8	4·9	3·3
Potato tuber	1·2	14·0	5·2

from mitochondria than in the fragments remaining after freezing and thawing.

DISCUSSION

A preparation from wheat germ which hydrolysed sucrose phosphate 2·5 times as fast as fructose 6-phosphate has been reported (Mendicino, 1960). Bird *et al.* (1965) observed a much higher percentage hydrolysis of 0·1 mM-sucrose phosphate than 5 mM-fructose 6-phosphate or glucose 6-phosphate by a chloroplast preparation. A similar result was obtained by Haq & Hassid (1965) with 4·5 μM-sucrose phosphate and 71·5 μM-fructose 6-phosphate as substrates for a chloroplast preparation from sugar-cane. Since different concentrations of substrates were used and maximum velocities were not determined conclusions about the specificity of these enzymes are not possible.

The phosphatase now isolated from carrot tissue is highly specific for sucrose phosphate. An enzyme with similar specificity occurs in sugar-cane. These enzymes have a number of other properties in common. Sucrose phosphatase requires a bivalent metal ion for activity. Although the partially purified enzyme from sugar-cane does not show an absolute requirement for added Mg^{2+} ions, addition of EDTA completely inhibits activity. The behaviour of the enzyme in this regard is similar to that of uridine diphosphatase (Hatch, 1963).

Stetten & Taft (1964) reported the synthesis of glucose 6-phosphate from glucose and inorganic pyrophosphate by a specific glucose 6-phosphatase. When sucrose phosphatase from sugar-cane was incubated with [U-^{14}C]sucrose and 30 mM-inorganic pyrophosphate no sucrose phosphate was detected.

At least some of the sucrose phosphatase present in plant tissues is clearly associated with particles which behave like mitochondria during differential centrifugation. The ninefold increase in sucrose-phosphatase activity resulting from freezing and thawing mitochondria strongly suggests that the enzyme is located inside the mitochondrial membrane and is released when the membrane is ruptured. Apparently the enzyme is not tightly bound to a membrane since it is not precipitated at 100 000*g* and, from studies on Sephadex G-100, it has a molecular weight of less than 100 000. In contrast, the rate of fructose 6-phosphate hydrolysis is only doubled by freezing and thawing mitochondria and much of the phosphatase activity towards fructose 6-phosphate is precipitated at 100 000*g*. Of the phosphatases active towards sucrose phosphate in the original tissue extracts only a small fraction was recovered in the mitochondria. Much of the sucrose phosphate hydrolysis in unfractionated extracts was probably due to non-specific enzymes and considerable enrichment of the specific sucrose phosphatase was obtained by isolating mitochondria. The loss of phosphatase activity towards sucrose phosphate during isolation would be due in part to removal of non-specific phosphatases and in part to incomplete recovery of mitochondria.

The identification of a sucrose phosphatase in sugar-cane tissue provides support for the concept that sucrose phosphate is involved in sucrose accumulation in the manner proposed by Hatch (1964). Further studies on inter- and intra-cellular

distribution and the nature of the inhibition by sucrose may provide additional information on the role of sucrose phosphatase in plant tissues.

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