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INHIBITION OF UDP GLUCOSE: D-FRUCTOSE 2-GLUCOSYLTRANSFERASE FROM SUGAR CANE STEM TISSUE BY PHENOL OXIDATION PRODUCTS

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Abstract—Sucrose synthetase (UDP glucose: D-fructose 2-glucosyltransferase E.C 2.4.1.13) is inhibited during extraction from elongating internodes of sugar cane by oxidation products of phenolic compounds. *p*-Benzoquinone and *p*-chloromercuribenzoate inhibit the enzyme; the inhibition is reversed by thiol compounds. It is suggested that the inhibition of sucrose synthetase which occurs during the oxidation of phenolic compounds results from a reaction between quinones and sulphydryl groups on the enzyme. In elongating internodes, sucrose synthetase is localized in the vascular strands.

INTRODUCTION

SUCROSE synthetase (UDP glucose: D-fructose 2-glucosyltransferase, E.C. 2.4.1.13)[1-5] and sucrose phosphate synthetase (UDP glucose: D-fructose-6-phosphate 2-glucosyltransferase, E.C. 2.4.1.14)[6,7,9] are both found in higher plants. In mature internodes of sugar cane, sucrose synthetase is localized in the vascular strands,[8] and indirect evidence suggests that sucrose phosphate synthetase is present in the parenchyma.[9] Rapidly elongating internodes were reported to contain much lower levels of sucrose synthetase than mature internodes.[4] However, the present paper shows that much of the activity of the enzyme may be lost during its isolation from elongating internodes owing to inhibition by oxidation products of phenolic compounds. Isolation procedures are described for avoiding inhibition of sucrose synthetase, and the nature of the inhibition is discussed.

RESULTS

Inhibition of Sucrose Synthetase by Products of Phenol Oxidation

Rapidly elongating internodes of sugar cane contain, besides phenolic substrates, a highly active phenol oxidizing system which is completely absent from mature internodes and when macerated in air elongating internodal tissue becomes dark brown within a few minutes. Extracts were found to contain much more sucrose synthetase activity when prepared under

[1] C. E. CARDINI, L. F. LELOIR and J. CHIRIBOGA, *J. Biol. Chem.* **214**, 149 (1955).
[2] E. S. REREM, H. G. WALKER and R. M. MCCREADY, *Plant Physiol* **35**, 269 (1960).
[3] J. V. DUTTON, A. CARRUTHERS and J. F. T. OLDFIELD, *Biochem. J.* **81**, 266 (1961).
[4] M. D. HATCH, J. A. SACHER and K. T. GLASZIOU, *Plant Physiol.* **38**, 338 (1963).
[5] A. G. AVIGAD, *J. Biol. Chem.* **239**, 3613 (1964).
[6] C. F. LELOIR and C. E. CARDINI, *J. Biol. Chem.* **214**, 157 (1955).
[7] J. MEDICINO, *J. Biol. Chem.* **235**, 334 (1960).
[8] J. S. HAWKER and M. D. HATCH, *Physiol. Plantarum.* In Press.
[9] M. D. HATCH, *Biochem. J.* **93**, 521 (1964).

conditions designed to prevent browning (Table 1); the specific activity of the enzyme was approximately a 1000-fold greater than previously reported.[4] In contrast, the activity of invertase was unaffected by the browning reaction. Both cysteine and sodium diethyldithiocarbamate (dieca), an inhibitor of phenol oxidase, were effective in preventing the inhibition of sucrose synthetase when present in the extraction medium at 0·01 M concentration. Neither increased the activity of sucrose synthetase when added to extracts after browning had occurred. It was necessary to keep the extracts under nitrogen prior to precipitation of the protein by $(NH_4)_2SO_4$ to prevent extracts from darkening at the surface.

TABLE 1. EFFECT OF PHENOL OXIDATION DURING TISSUE EXTRACTION ON THE ACTIVITY OF SUCROSE SYNTHETASE AND INVERTASE IN EXTRACTS

Extraction procedure	Sucrose synthetase activity (μmole sucrose synthesized/ mg protein/hr)	Invertase activity (μmole sucrose hydrolyzed/ mg protein/hr)
Phenol oxidation prevented	9·7*	7·0
	10·2	8·1
	0	6·5
Phenol oxidation occurred	0	7·0

* Extracts from duplicate batches of tissue.

The basal portion of elongating internodes were quartered longitudinally and the tissue divided into 4 batches. Phenol oxidation was prevented by use of sodium diethyldithiocarbamate and nitrogen as described in the text. These phenol oxidase inhibitors were omitted in the preparation of extracts in which phenol oxidation occurred.

The two most likely explanations for the inhibition of sucrose synthetase were either (*a*) the enzyme was inhibited by quinones produced as intermediates in the oxidation of phenolic compounds or (*b*) the enzyme formed an inactive complex with the brown, polymerized products of phenol oxidation.

TABLE 2. INHIBITION OF SUCROSE SYNTHETASE ACTIVITY IN CRUDE EXTRACTS BY PREINCUBATION WITH PHENOLS AND *p*-BENZOQUINONE

Preincubation solutions	Inhibition (%)
Phenol extract from 25 mg fresh weight tissue	98
Phenol extract from 25 mg fresh weight tissue containing 5 mM dieca	10
Phenol extract from 0·1 mg fresh weight tissue	72
Oxidized phenol extract from 50 mg fresh weight tissue	70
Oxidized phenol extract from 50 mg fresh weight tissue containing 5 mM dieca	3
Catechol 100 mM	100
Catechol 1 mM	94
Catechol 1 mM containing 0·005 M dieca	10
p-Benzoquinone 1 mM	96
p-Benzoquinone 0·1 mM	72

Aliquots (0·1 ml) of crude enzyme extract were preincubated in air with 0·1 ml of the solutions described below in 0·025 M potassium phosphate buffer (pH 6·8) for 1 hr at 3°C. In controls enzyme was incubated with buffer. Aliquots (10 μl) were assayed for sucrose synthetase activity in presence of 0·005 M dieca.

In an attempt to define the nature of the inhibition, aliquots of a crude enzyme extract were incubated with phenols, oxidized phenols or *p*-benzoquinone. The extract contained an active phenol oxidase and became brown when incubated with phenols in the absence of dieca. The oxidation of both catechol and a preparation from elongating internodes containing phenolic compounds caused a very marked inhibition of sucrose synthetase (Table 2). The effectiveness of the phenol preparation as an "inhibitor" was reduced if the phenolic compounds were oxidized prior to mixing with the enzyme extract, and in the presence of dieca it caused very little inhibition. These observations suggested that the enzyme was inhibited by quinones formed as intermediates in the oxidation of phenols rather than by the final polymerized products. This conclusion was strengthened by the fact that inhibition was caused by *p*-benzoquinone, which did not produce any browning of the extract.

Quinones inhibit certain enzymes which catalyse oxidoreduction reactions and others which contain sulphydryl groups.[10] Inhibition of the latter is due to the oxidation of sulphydryl groups, which may be reversed by thiol reagents, and probably also to the formation of addition compounds between the sulphydryl groups of the enzyme and the quinone.[10] The partial reversal of the *p*-benzoquinone inhibition of sucrose synthetase by 2-mercaptoethanol (Table 3) was consistent with the view that the enzyme contained one or more sulphydryl groups which could be oxidized by quinones. Ascorbic acid was added to the reaction mixtures in order to reduce the quinone after the preincubation period. The activity of controls containing ascorbic acid or ascorbic acid plus 2-mercaptoethanol did not differ significantly.

TABLE 3. REVERSAL OF *p*-BENZOQUINONE INHIBITION BY 2-MERCAPTOETHANOL

Preincubation time (min)	% Inhibition in Ascorbic acid	% Inhibition in Ascorbic acid + 2-mercaptoethanol
2	47	5
6	62	14
30	80	46

Crude enzyme extract (0·2 ml) was preincubated with 1 mM *p*-benzoquinone (0·2 ml) at 3° C. At intervals aliquotes (10 μl) were transferred to reaction mixtures containing either 2 mM ascorbic acid or 2 mM ascorbic acid and 2 mM 2-mercaptoethanol. In controls, *p*-benzoquinone (5 μl) was added to reaction mixtures containing either ascorbic acid or ascorbic acid and 2-mercaptoethanol prior to the addition of crude extract (5 μl). Assays were otherwise as described in the text.

Inhibition of Sucrose Synthetase by Thiol Inhibitors

The inhibition of sucrose synthetase by thiol inhibitors and reversal of the inhibition by thiol compounds is shown in Table 4. In experiment 2 the enzyme was assayed spectrophotometrically using a large excess of yeast hexokinase, phosphohexoisomerase and glucose-6-phosphate dehydrogenase. Yeast hexokinase is itself inhibited by *p*-chloromercuribenzoate (PCMB),[11] however, the inhibition described in Experiment 2 (Table 4) was not the result of

[10] O. HOFFMANN-OSTENHOF, *Metabolic Inhibitors* (Edited by R. M. HOCHSTER and J. H. QUASTEL) Vol. 2, p. 145. Academic Press, New York (1963).
[11] K. BAILEY and E. C. WEBB, *Biochem. J.* **42**, 60 (1948).

TABLE 4. EFFECT OF THIOL INHIBITORS ON SUCROSE SYNTHETASE ACTIVITY

Experiment	Reagent added		Inhibition (%)
1	*p*-chloromercuribenzoate (PCMB)	100 μM	93
		25 μM	63
	$HgCl_2$	50 μM	94
		10 μM	21
	$CuSO_4$	200 μM	65
		20 μM	5
2	PCMB 10 μM		100
	1 μM		89
	1 μM + 1 mM cysteine		10
	1 μM + 0·1 mM 2-mercaptoethanol		12
	1 μM + 0·1 mM 2,3-dimercaptopropanol		0
	0·1 μM		0

In Experiment 1 aliquots (10 μl) of crude enzyme extract were incubated in tris-HCl buffer and inhibitor for 10 min at 3° C prior to the addition of substrates. In Experiment 2 the inhibitor was added to reaction mixtures containing the partially purified enzyme preparation 5 min prior to addition of substrates. Reaction rate was established over a 5 min period, then a thiol compound added where indicated. Assay conditions were otherwise as described in the text. The reaction mixtures in Experiments 1 and 2 contained 2·0 and 0·025 mg protein/ml respectively.

TABLE 5. ACTIVITY OF SUCROSE SYNTHETASE AND ACID INVERTASE IN EXTRACTS OBTAINED FROM INTERNODAL TISSUE BY DIFFERENT DEGREES OF MACERATION

Experiment	Region of internode used	Extraction method	Sucrose synthetase activity (μmole sucrose synthesized/hr) /extract	/mg protein	Invertase activity (μmole sucrose hydrolyzed/hr) /extract	/mg protein
1	Fully elongated cells	Light squash	N.D.*	1·1	N.D.	1·7
		Isolated vascular strands	N.D.*	15·1	N.D.	0·2
2	Fully elongated cells	Light squash	4·1	1·6	4·7	1·9
			3·0	1·4	4·2	2·0
		Complete maceration	50·0	15·6	2·8	0·9
			43·0	21·4	2·0	1·0
3	Elongating cells	Light squash	14·0	8·7	13·9	8·7
			15·6	8·2	15·9	8·4
		Complete maceration	52·0	8·4	32·0	5·4
			61·5	9·0	31·8	4·7

* N.D. not determined.

Longitudinal slices taken from a specified region of elongating internodes were lightly squashed to rupture some parenchyma cells, then thoroughly macerated with sand to extract vascular strands and unbroken parenchyma cells. Details are given in the text. In Experiment 1 vascular strands were dissected from the slices after the initial light squashing.

UDP glucose-pyrophosphorylase was also assayed in Experiment 1; the specific activity was 0·68 and 0·81 μmole glucose liberated/mg protein/hr for extracts of parenchyma and vascular strands respectively.

hexokinase inhibition since 1×10^{-5} M PCMB did not affect the rate of NADP reduction when fructose was added to the assay system in either saturating or subsaturating concentrations.

Location of Sucrose Synthetase in Elongating Internodes

In studies on the location of sucrose synthetase in mature internodes Hawker and Hatch[8] separated parenchyma and vascular strands by dissection. This approach was not applicable to tissues of elongating internodes since these contained fragile, thin-walled parenchyma. The method adopted utilized the fact that parenchyma cells rupture more readily by squashing in a pestle and mortar than cells of the vascular strands. The method was first used to sample cells from the region of fully elongated cells above the cell-elongation zone (Experiments 1 and 2, Table 5). The results strongly suggested that sucrose synthetase was localized in the vascular strands and invertase in the parenchyma. The light squash released 7 per cent of the sucrose synthetase and 65 per cent of the acid invertase from the tissue. The results for the region of cell elongation were less conclusive; the proportions of sucrose synthetase and acid invertase liberated by the light squash were 21 and 32 per cent respectively (Experiment 3). In this region the cell walls of both parenchyma and vascular strands are fragile and it is considered that the cells of both tissues were ruptured by the squashing procedure.

DISCUSSION

The above results are consistent with the view that the sucrose synthetase from stem tissue of sugar cane is a sulphydryl enzyme which can be inhibited by quinones produced during the oxidation of phenols. Its susceptibility to the products of phenol oxidation may be gauged from the fact that the phenolic compounds from 0·1 mg fresh wt. of tissue inhibited the enzyme in 0·1 ml (equivalent to 500 mg fresh wt. tissue) of crude extract, which contains the phenol oxidases, by 72 per cent (Table 2). Inhibition of sucrose synthetase during its extraction from other plant sources has not been reported. However, Cardini *et al.*[1] were unable to obtain consistently sucrose synthetase activity in extracts from sugar cane leaves; this they attributed to interference from invertase in the assay system. The explanation seems unlikely since the sugar cane leaf invertase is completely inactive under their conditions of assay (0·13 M tris-HCl, pH 7·2) (unpublished observation). A possible alternative explanation is that sucrose synthetase was inhibited by products of phenol oxidation.

The prevention of phenol oxidation to give quinones may be important in the successful extraction of other sulphydryl enzymes. In this regard Pojnar and Cocking[12] showed that cysteine was necessary for the successful extraction of nitrate reductase from tomato leaves and attributed the effect to inhibition of phenol oxidation.

Although sucrose synthetase is associated with the vascular tissue of sugar cane stems its precise location and function within the strands is unknown. Reported values for the equilibrium constant of the reaction catalysed by the enzyme range from 1·2[5] to 8[1]. Since UDP-phosphatase, which hydrolyses UDP to UMP, is absent from the vascular strands[13] the enzyme could provide a supply of UDP-glucose if located in a compartment containing UDP and sucrose. It is interesting to note that in sugar beet leaves the vascular strands contain higher levels of both UDP and UDP-glucose than other tissues.[14] Alternatively the

[12] E. POJNAR and E. C. COCKING, *Biochem. J.* **91**, 29P (1964).
[13] M. D. HATCH, *Biochem. J.* **88**, 423 (1963).
[14] A. L. KURSAROV, *Advan. Botan. Res.* **1**, 259 (1963).

enzyme may act in association with UDP glucose pyrophosphorylase to maintain, in some way, the high level of sucrose in the sieve tubes.

EXPERIMENTAL

Materials

p-Benzoquinone was prepared from hydroquinone by chromate oxidation and purified by steam sublimation. Other chemicals and enzymes were obtained from commercial sources.

Preparation of Enzyme Extracts

Elongating internodes were obtained from sugar cane plants (cv Pindar) grown in an irrigated field plot. The soft basal portion of the internodes was selected and the rind removed. The tissue was thoroughly macerated with an equal volume (w/v) of nitrogen saturated 0·2 M potassium phosphate buffer (pH 6·8) containing 0·01 M dieca, and centrifuged to remove cell debris. Saturated $(NH_4)_2SO_4$ (4 vol.), previously adjusted to pH 7·0 with NH_4OH, was added to the supernatant and after 30 min the precipitated protein was recovered by centrifugation. The pellet was dissolved in a minimum volume of 0·01 M phosphate buffer (pH 6·8) containing 0·01 M dieca and dialysed against 0·005 M phosphate buffer for 24 hr. All operations were carried out at 3° and where possible under nitrogen. This preparation is referred to as the crude extract.

The enzyme were purified 20-fold from a crude extract prepared as described above except that 0·01 M cysteine was used in place of dieca. The crude extract was fractionated with saturated $(NH_4)_2SO_4$ and the protein which precipitated between 25 and 50 per cent saturation was retained. This was dissolved in a minimum volume of 0·01 M-tris-HCl buffer (pH 7·5) and 4 ml applied to a Sephadex G-200 column (36 × 2·5 cm, void volume 45 ml) which was eluted with tris-HCl buffer. The eluate fraction (45–60 ml) was collected and passed down a DEAE-cellulose column (14 × 0·6 cm) pre-equilibrated with 0·01 M-tris-HCl buffer (pH 7·5). The column was eluted with 9 ml of tris-HCl buffer containing 0·1 M NaCl, then with 8 ml of buffer containing 0·3 M NaCl. The enzyme was collected in the second 4·0 ml fraction of this eluant. This procedure completely removed UDP-phosphatase and other phosphatases present in the crude extracts which hydrolyse UDP at pH 7·5.

Extraction of Parenchyma and Vascular Strands

Longitudinal slices (1 mm thick) were cut from a defined region of elongating internodes using a hand microtome and washed in ice-cold 0·05 M-tris–HCl buffer (pH 7·5) containing 0·01 M cysteine. In turn, each slice was placed flat down in a large mortar containing 4 ml of the same tris-HCl-cysteine mixture and squashed under a pestle with a slight downward pressure. The slices were rinsed and macerated in pestle and mortar with sand and an equal volume of the above buffer mixture. The extracts were centrifuged to remove cell debris and saturated $(NH_4)_2SO_4$ (4 vol.) added to the supernatants. After 30 min the precipitated protein was recovered by centrifugation, dissolved in a minimum volume of 0·01 M-tris–HCl (pH 7·5) containing 0·01 M cysteine and dialysed over-night against tris buffer without cysteine. The above operations were carried out under nitrogen at 3°.

Preparation of the Phenol Extract

Internodal tissue was macerated in an equal volume of nitrogen saturated 0·05 M phosphate buffer (pH 6·8), the extract was centrifuged to remove cell debris and boiled to inactivate

phenol oxidase and other enzymes and to coagulate protein which was removed by centrifugation. The operations were conducted at 3° under nitrogen. This extract was a pale yellow colour. To obtain a preparation of oxidized polyphenols 5 ml of the above preparation was vigorously shaken in air at room temperature for 6 hr with 0·5 ml of crude enzyme extract.

Enzyme Assays

Sucrose synthetase was assayed in crude extracts by measuring the incorporation of labelled fructose into sucrose. The reaction mixture contained tris–HCl buffer pH 7·5 (5 μmole); UDP-glucose (0·5 μmole); [U-^{14}C]fructose (0·8 μmole, 5×10^4 disintegrations/min) and crude enzyme extract (approximately 0·06 mg protein) in 0·05 ml. Assays were conducted at 30° and aliquots (10 μl) were removed at intervals and spotted onto Whatman No. 1 paper together with unlabelled sucrose and fructose. When the activity of the extract was known, the reaction mixtures were incubated until 30 per cent of the fructose had been utilized and the reaction stopped by heating to 100° for 2 min. The chromatograms were developed in pyridine–ethyl acetate–water (8:2:1) solvent and sprayed with *p*-anisidine phosphate. The activity in fructose and sucrose was counted directly on paper with a Geiger–Muller tube (efficiency 4·5%). The amount of sucrose formed was calculated from the proportion of total counts present in sucrose. Interference from invertase was prevented by the presence of 0·1 M tris–HCl in the reaction mixtures.[4] The rate of sucrose formation was linear until 60 per cent of the fructose had been utilized. Sucrose was identified as the labelled product since it co-chromatographed with authentic sucrose and was completely hydrolysed by analytical invertase to yield glucose and labelled fructose. In the absence of UDP-glucose no sucrose was produced.

Sucrose synthetase was assayed in the partially purified preparations by measuring the production of fructose from sucrose in the presence of UDP as described by Avigad,[5] (Method 1). Controls contained all reagents except UDP.

UDP-glucosepyrophosphorylase was assayed as described by Munch-Petersen and Kalckar,[15] and invertase as described by Hatch *et al.*[4]

Protein Determination

Protein was determined as described by Lowry *et al.*[16] using bovine serum albumin as a standard.

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[15] A. MUNCH-PETERSEN and H. M. KALCKAR, In *Methods in Enzymology* (Edited by S. P. COLOWICK and N. O. KAPLAN), Vol. 2, p. 675. Academic Press, New York (1955).
[16] O. H. LOWRY, M. J. ROSEBOROUGH, A. L. FARR and R. I. RANDALL, *J. Biol. Chem.* **193**, 156 (1951).