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ENZYMES OF STARCH METABOLISM IN LEAVES AND BERRIES OF *VITIS VINIFERA*

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Abstract—Leaves of *Vitis vinifera* L., cv. Cabernet Sauvignon contained 2·0 mg of starch per g fresh weight, whereas young green berries and maturing grape berries contained less than 0·03 mg of starch, despite the presence of abundant substrates (reducing sugars and sucrose) in berries for starch synthesis. The activities of several enzymes likely to be involved in starch synthesis were determined in extracts of berries and leaves. Fractionation procedures resulted in final recoverable ADPglucose-starch glucosyltransferase activity which was 2–3 times the activity measured in crude extracts of leaves. Compared to leaves, berries contained low activities of ADPglucose-starch glucosyltransferase and ADPglucose pyrophosphorylase. These enzymes increased only 2- to 3-fold from young to maturing berries. ADPglucose-starch glucosyltransferase activity in the absence of added primer was found in leaf extracts but not in berry extracts. The activities of UDP-glucose pyrophosphorylase, phosphorylase and amylase were comparable in both leaves and berries and increased 6- to 7-fold during berry development. The low activities of ADPglucose-starch glucosyltransferase and ADPglucose pyrophosphorylase probably account for the paucity of starch in grape berries.

INTRODUCTION

LEAVES of grapevine usually contain appreciable amounts of starch which is easily assayed chemically.[1] Although starch has not been detected chemically in extracts of grape berries, traces of starch have recently been found in berries examined by light microscopy.[2] This paper proposes an explanation for the low concentrations of starch in grape berries based on a comparative study of enzymes associated with starch metabolism in leaves and berries of grapevine.

RESULTS AND DISCUSSION

Fractionation of ADPglucose-starch Glucosyltransferase in Leaves

Centrifugation of the crude homogenates of grape leaves resulted in recovery of only about 20% of the total leaf complement of ADPglucose-starch glucosyltransferase in the supernatant fraction (Table 1—supernatant A). The pellet was treated with 20 mM buffer to lyse chloroplasts and other organelles. However, use of the hypotonic buffer and solubilization with Tween 20 released only an additional 20% activity (Table 1—supernatant B). The final pellet was subdivided into an upper green fraction and a lower starch pellet. Much of the total leaf activity (40%) remained associated with starch grains.

[1] BUTTROSE, M. S. and HALE, C. R. (1971) *Planta* **101**, 166.
[2] SWIFT, J. G., BUTTROSE, M. S. and POSSINGHAM, J. V. (1973) *Vitis* in press.

Since recent work indicated that soluble ADPglucose-starch glucosyltransferase predominates in spinach leaves,[3] we subjected spinach to our fractionation schedule. The enzyme was easily solubilized (Table 1—supernatants A and B) with about 10% remaining associated with starch grains. The crude extracts of grapevine and spinach leaves accounted for only 35 and 56% respectively of the total activities eventually recovered from each extract following solubilization of the enzyme with hypotonic buffer and detergent.

TABLE 1. DISTRIBUTION OF ADPGLUCOSE-STARCH GLUCOSYLTRANSFERASE IN FRACTIONS OF GRAPEVINE AND SPINACH LEAVES

Fraction	% of total activity in each fraction		Fraction	% of total activity in each fraction	
	Grapevine	Spinach		Grapevine	Spinach
Supernatant A (0·35 M buffer)	22	50	Starch pellet	40	11
Supernatant B (0·02 M buffer)	21	36	Total activity (nmol/min/g fr. wt)	58·6	57·2
Green pellet	17	3	Crude extract (0·35 M buffer), % of total activity	35	56

Tissues were extracted in 0·35 M Tris-acetate buffer pH 8·5 containing 10 mM EDTA, 2 mM sodium metabisulphite and 0·1% BSA. See Experimental for fractionation procedure.

A further attempt was made to solubilize the enzyme in grape leaves to facilitate the study of ADPglucose-starch glucosyltransferase. Plants were placed in the dark and the leaves extracted 2·5 days later. Table 2 shows that the proportion of easily solubilized enzyme increased from 43 to 65%, but that the total activity was only 29% of the original activity.

TABLE 2. EFFECT OF DARKNESS ON ADPGLUCOSE-STARCH GLUCOSYLTRANSFERASE ACTIVITY IN LEAVES

Tissue	Regular light/dark cycle	Darkness for 2–2·5 days	Tissue	Regular light/dark cycle	Darkness for 2–2·5 days
	% of activity solubilized			Total activity (nmol/min/g fr. wt)	
Grapevine	43	65			
Spinach	86	98	Grapevine	58·6	17·2
			Spinach	57·2	50·9

Extraction and fractionation procedure as for Table 1.

Spinach leaves stored in darkness for 2 days, on the other hand, still retained 89% of activity. Virtually all of the spinach enzyme was soluble. The starch pellet from centrifugation was much smaller following dark treatment of the spinach leaves. It seems likely that spinach leaves 1 or 2 days after harvest (as obtained from supermarkets) would have all of their ADPglucose-starch glucosyltransferase in soluble form.[3] Further work is necessary to find suitable procedures for liberating the rather large component of ADP-

[3] OZBUN, J. L., HAWKER, J. S. and PREISS, J. (1972) *Biochem. J.* **126**, 953.

glucose-starch glucosyltransferase associated with starch grains in leaves of grapevine. In this paper, activities quoted in Tables 4 and 5 are the sums of activities present in supernatants and precipitates resuspended in 20 mM buffer, and represent maximum activities obtained by our procedures for leaves of grapevine.

The high percentage of enzyme associated with the pellet after the initial centrifugation and the amount bound to starch grains suggests that the enzyme is located in organelles. Starch grains in leaves are found only in chloroplasts and it therefore seems probable that the enzyme is located in chloroplasts. Attempts to isolate whole washed chloroplasts were made difficult by release of starch grains. Non-aqueous fractionation of leaves may help to confirm the location of ADPglucose-starch glucosyltransferase.

TABLE 3. EXTRACTION MEDIA FOR ADPGLUCOSE-STARCH GLUCOSYLTRANSFERASE FROM LEAVES AND YOUNG BERRIES OF GRAPEVINE

Extraction medium	Activity as % of maximum measured: Leaves	Young berries
0·35 M Tris-acetate, 10 mM EDTA and 5 mM DTT	95	—
0·35 M Tris-acetate, 10 mM EDTA, 0·1% BSA and 2 mM $Na_2S_2O_5$	86	5
0·35 M Tris-acetate, 10 mM EDTA, 0·1% BSA, 2 mM $Na_2S_2O_5$ and 5% Carbowax 4000	—	32
0·35 M Tris-acetate, 20 mM EDTA, 11 mM DIECA, 15 mM cysteine-HCl and 6% Carbowax 4000	100	100

Extraction Media for Isolation of Enzymes from Grapevine Tissue

Grapevine leaves and berries contain phenols and tannins.[4] Various substances known to decrease the rate of phenol oxidation[5] were added to extracting media. For leaves (Table 3), buffer, EDTA and a reducing agent such as dithiothreitol (DTT) or sodium metabisulphite gave adequate protection. However, with green berries, other protective agents were necessary. Higher activities of ADPglucose-starch glucosyltransferase were obtained with Carbowax 4000 (polyethylene glycol, *ca.* MW 4000). Carbowax has an affinity for tannins and can split tannin-protein complexes.[6] Highest activities in berries were recorded when sodium diethyldithiocarbamate (DIECA) was included with Carbowax. DIECA inhibits polyphenol oxidase by chelating the copper required for its activity.[5,7] This final mixture, similar to that prevously used by Hawker[8] for Sultana berries, also gave the highest activity of ADPglucose-starch glucosyltransferase in grapevine leaves.

[4] HAWKER, J. S., BUTTROSE, M. S., SOEFFKY, A. and POSSINGHAM, J. V. (1973) *Vitis* **11**, 189.
[5] ANDERSON, J. W. (1968) *Phytochemistry* **7**, 1973.
[6] GOLDSTEIN, J. L. and SWAIN, T. (1965) *Phytochemistry* **4**, 185.
[7] GRNCAREVIC, M. and HAWKER, J. S. (1971) *J. Sci. Food Agric.* **22**, 270.
[8] HAWKER, J. S. (1969) *Phytochemistry* **8**, 9.

Comparative Enzymology of Starch Metabolism in Leaves and Berries of Grapevine

Coloured berries contained high concentrations of reducing sugars (7·9%—Table 4) and were, therefore, certainly not deficient in potential substrate for starch synthesis. However, the starch content of grape berries was at, or lower than, the limits detectable by our sensitive assay.

TABLE 4. ACTIVITIES OF ENZYMES ASSOCIATED WITH STARCH METABOLISM IN LEAVES AND BERRIES OF GRAPEVINE

Enzyme	Activity (nmol/min/mg protein) Leaf	Young berry	Coloured berry		Activity (nmol/min/mg protein) Leaf	Young berry	Coloured berry
ADPglucose-starch glucosyltransferase:				Phosphorylase	4·9	2·1	4·0
added primer	2·92	0·34	0·34	Amylase	4·0	1·6	6·5
no added primer	0·54	0·0	0·0	Reducing sugar (g/100 g fr. wt)	0·27	0·78	7·9
UDPglucose-starch glucosyltransferase:				Sucrose (g/100 g fr. wt)	1·28	0·3	0·7
added primer	0·05	0·005	—	Starch (g/100 g fr. wt)	0·2	<0·003	<0·003
ADPglucose pyrophosphorylase	40	2·0	1·0	Chlorophyll (mg/g fr. wt)	1·9	0·09	—
UDPglucose pyrophosphorylase	450	267	530	Protein (mg/g fr. wt)	18·2	20·5	7·4

All tissues were extracted in 0·35 M Tris-acetate buffer pH 8·5 containing 20 mM EDTA, 11 mM DIECA, 15 mM cysteine–HCl and 6% Carbowax 4000. Supernatants and precipitates (resuspended in 20 mM buffer) were assayed and the values summed (see Experimental).

The activities of various enzymes associated with starch metabolism in leaves, and young and maturing berries of grapevine are shown in Table 4. For ADPglucose-starch glucosyltransferase assayed in the presence of primer (starch), activity in both young and coloured berries was very low compared to that in leaves. UDPglucose gave only about 2% of the rate measured with ADPglucose as substrate. Unprimed activity has recently been demonstrated in extracts from a variety of plant tissues[3,9,10] in the presence of high concentrations of certain anions such as citrate ions. When extracts from leaves of grapevine were assayed in the absence of added primer, but in the presence of citrate ions and bovine serum albumin (BSA), they exhibited, after correction for slight activity due to endogenous primer, about 20% of the ADPglucose-starch glucosyltransferase activity measured in the presence of primer. In supernatants of grape leaf extracts no activity was observed in the absence of primer, citrate ions and BSA; however, in the presence of citrate ions and BSA, activity was measured. No activity of ADPglucose-starch glucosyltransferase could be demonstrated in extracts of grapeberries in the absence of added primer. A more detailed examination of grapevine leaf and berry extracts by column chromatography is needed to confirm the present results and to ascertain whether multiple forms of ADPglucose-starch glucosyltransferase, such as are present in spinach leaf[3] and maize kernels,[10] also occur in grapevine tissues.

ADPglucose pyrophosphorylase is responsible for the synthesis of ADPglucose, required

[9] HAWKER, J. S., OZBUN, J. L. and PREISS, J. (1972) *Phytochemistry* **11**, 1287.
[10] OZBUN, J. L., HAWKER, J. S. and PREISS, J. (1971) *Plant Physiol.* **48**, 765.

by ADPglucose-starch glucosyltransferase as substrate. As with transferase activity, ADP-glucose pyrophosphorylase activity was very low in comparison to that in grapevine leaves.

The activities of phosphorylase, amylase and UDP-glucose pyrophosphorylase, on the other hand, were roughly comparable between leaves and berries, especially coloured berries. The product of UDPglucose pyrophosphorylase, UDPglucose, is a substrate for sucrose synthesis by sucrose phosphate synthetase and sucrose synthetase.[11] Table 4 shows that the sucrose content of berries remains low and that berries beginning to colour contain only 0·7% sucrose despite the reducing sugar level having risen to 7·9%. Kliewer[12] reports sucrose concentrations of less than 0·1% in ripe grape berries. The high activities of UDP-glucose pyrophosphorylase present in berries supports the suggestion that accumulation of reducing sugars in grape berries occurs with sucrose phosphate as an intermediate.[8] Although amylase is ubiquitous in higher plants,[13] its role in plant tissues, with the exception of germinating seeds, is uncertain.[14,15]

TABLE 5. ACTIVITIES OF ENZYMES IN GRAPE BERRIES OF TWO MATURITIES

Enzyme	Activity (nmol/min/berry) Young berry	Coloured berry	Fold increase	Enzyme	Activity (nmol/min/berry) Young berry	Coloured berry	Fold increase
ADPglucose-starch glucosyltrans-ferase-primed	0·35	1·1	3·1	UDPglucose pyrophosphorylase	280·8	1804	6·4
ADPglucose pyrophosphorylase	1·8	3·3	1·8	Amylase	2·2	14·8	6·7
Phosphorylase	2·2	13·4	6·1	Berry fr. wt (g)	0·052	0·41	7·9
				Reducing sugars (g/100 g fr. wt)	0·78	7·9	10·1

Extraction and assay was as described in Table 4.

Table 5 compares enzyme activities per berry in young and maturing berries. Between the two ages, berry weight increased 8-fold, reducing sugar content, 10-fold and the activities of the enzymes, phosphorylase, amylase and UDPglucose pyrophosphorylase, 6- to 7-fold. Sucrose phosphate synthetase and sucrose synthetase in developing Sultana berries show similar increases over a comparable period.[8] ADPglucose pyrophosphorylase and ADPglucose-starch glucosyltransferase failed to show this trend and increased only 2- to 3-fold despite a 10-fold increase in potential substrate for starch synthesis. Evidence suggesting that starch synthesis in developing maize endosperm occurs via ADPglucose has been discussed.[16] It is still possible that phosphorylase is involved in starch synthesis in plants, but it is more generally accepted that ADPglucose-starch glucosyltransferase is the enzyme responsible.[17]

[11] HASSID, W. Z. (1967) *Annu. Rev. Plant Physiol.* **18**, 253.
[12] KLIEWER, W. M. (1966) *Plant Physiol.* **41**, 923.
[13] GATES, J. W. and SIMPSON, G. M. (1968) *Can. J. Botany* **46**, 1459.
[14] AKAZAWA, T. (1965) in *Plant Biochemistry* (BONNER, J. and VARNER, J. E., eds.), p. 258, Academic Press, New York.
[15] BUTTROSE, M. S. (1969) *Australian J. Biol. Sci.* **22**, 1297.
[16] OZBUN, J. L., HAWKER, J. S., GREENBERG, E., LAMMEL, C., PREISS, J. and LEE, E. Y. C. (1973) *Plant Physiol.* **51**, 1.
[17] PREISS, J. and KOSUGE, T. (1970) *Annu. Rev. Plant Physiol.* **21**, 433.

The lack of starch accumulation in berries probably results from the low activity of both ADPglucose-pyrophosphorylase and ADPglucose-starch glucosyltransferase. Little or no starch accumulates in grape berries despite the presence of high activities of phosphorylase indicating that phosphorylase is not involved in starch synthesis in grapevine, at least in the berries.

EXPERIMENTAL

Materials. Plants of grapevine (*Vitis vinifera* L., cv. Cabernet Sauvignon) were established from cuttings and maintained in a glasshouse according to Mullins.[18] Spinach was grown in solution culture under controlled conditions of 14 hr, 24° day/10 hr, 22° night. Potato starch and *E. coli* alkaline phosphatase were purchased from Sigma, St. Louis, MO., U.S.A. Invertase and Carbowax 4000 were obtained from British Drug Houses Ltd., Poole, England.

Preparation of enzyme extracts. Washed leaves (3 g) were ground in a mortar with 7 ml of 0·35 M Tris-acetate buffer pH 8·5 and other additives as indicated in the text. 100 berries (4–5 mm dia.) weighing *ca.* 5 g were ground in 15 ml of the above buffer with additives. Berries (minus seeds) that had begun to turn purple (*ca.* 1 cm dia.) were similarly ground using the same ratio of tissue to buffer as for green berries. These vols. were sufficient to maintain the pH of the extracts above 7·0. Homogenates were filtered through cheesecloth and centrifuged at 30 000 *g* for 15 min (supernatant A). The pellet was suspended in a hypotonic buffer (20 mM Tris-acetate pH 8·5, 10 mM EDTA, 2 mM DTT), Tween 20 added to a final concentration of 0·5%, and the preparation was allowed to stand for 15 min with occasional stirring. Centrifugation at 30 000 g for 15 min gave supernatant B and a pellet. This pellet was subdivided into an upper green fraction and a lower starch fraction and resuspended in hypotonic buffer. Supernatants A and B were designated 'soluble enzyme' for ADPglucose-starch glucosyltransferase assays. In some cases total ADPglucose-starch glucosyltrans ferase activity was determined by assaying the first pellet suspended in hypotonic buffer as well as the supernatant. For amylase measurements, crude homogenates were dialysed overnight against two changes of 20 mM Tris-acetate pH 8·5 containing 1 mM EDTA. All operations were performed at 0–4°.

Chlorophyll. Chlorophyll was extracted in 80% acetone and measured at 652 nm according to Bruinsma.[19]

Protein assay. The method of Lowry *et al.*[20] was used, with bovine serum albumin as standard.

Carbohydrate determinations. Reducing sugars and starch in the tissues were determined as described previously.[16] Sucrose was determined by the difference between reducing sugar values obtained before and aftet treatment of extracts with invertase. Chromatography of extracts on Whatman No. 1 paper in EtOAc–pyridine–H_2O (8:2:1) confirmed the presence of sucrose.

Measurement of enzyme activities. ADPglucose-starch glucosyltransferase. Transfer of [^{14}C]-labelled glucose to a primer, or formation of a [^{14}C]-labelled glucan in the absence of primer was determined at 30° as previously described[9] except that GSH and amylopectin were replaced with DTT and potato starch. In some cases ADP[^{14}C]glucose was replaced with 1 μmol of UDP[^{14}C]glucose (250 cpm/nmol).

Phosphorylase. Incorporation of [^{14}C]glucose from [U-^{14}C]glucose-1-phosphate into potato starch at 30° was determined. Reaction mixtures contained 700 nmol of [U-^{14}C]glucose-1-phosphate (150–200 cpm/nmol), 10 nmol of Hepes buffer, pH 6·3, 0·8 mg potato starch and enzyme in a final vol. of 80 μl. The reaction was stopped by heating at 100° for 1 min and carrier potato starch (0·1 ml, 1 mg) was added prior to precipitation by 2 ml of 1% KCl in 75% aq. MeOH.[21] The washed ppt. was dissolved in 0·2 ml of H_2O and reprecipitated with methanolic KCl to further reduce the counts in controls containing boiled enzyme.

ADPglucose and UDPglucose pyrophosphorylases. α-[^{14}C]Glucose-l-phosphate was used to measure synthesis of ADP[^{14}C]glucose or UDP[^{14}C]glucose at 30°.[22] The reaction mixture contained 40 μmol of Hepes buffer, pH 7·7, 5 μmol of $MgCl_2$, 100 μg of bovine serum albumin, 0·2 μmol of α-[^{14}C]glucose-l-phosphate (1000 cpm/nmol), 0·6 μmol of ATP or UTP, and enzyme in a final vol. of 0·2 ml. 3-Phosphoglycerate (2 μmol) was added as an activator of ADPglucose pyrophosphorylase. The reaction was stopped by heating at 100° for 1 min. The remaining [^{14}C]glucose-l-phosphate was hydrolysed to [^{14}C]glucose by incubating the reaction mixture with 50 μg of *E. coli* alkaline phosphatase at 40° for 3 hr. After the addition of 30 μl of 0·25 M Na_4EDTA, 30 μl of the mixture was chromatographed on Whatman No. 1 paper for 20 hr in 95% EtOH–1 M NH_3OAc, pH 7·5 (5:2). Radioactivity was detected with a strip chart detector and the radioactive spots were cut out and counted on a planchet counter.

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[21] GHOSH, H. P. and PREISS, J. (1966) *Biochemistry* **4**, 1354.
[22] DICKINSON, D. B. and PREISS, J. (1969) *Arch. Biochem. Biophys.* **130**, 119.

Amylase. The release of reducing sugars from potato starch was determined at 30°. Reaction mixtures contained 60 μmol Na acetate buffer, pH 5·0, 12 mg of potato starch, 6 μmol of $CaCl_2$, 0·6 μmol of DTT and enzyme in a final vol. of 1 ml. At intervals, 0·1 or 0·2 ml samples were withdrawn, heated at 100° for 1 min and the reducing sugar content measured.[23]

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[23] NELSON, N. (1944) *J. Biol. Chem.* **153,** 375.