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INSOLUBLE INVERTASE FROM GRAPES: AN ARTIFACT OF EXTRACTION

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Abstract—Both soluble invertase (supernatant from 10,000 *g* centrifugation) and insoluble invertase (precipitated with cell-wall fraction) were obtained from sultana grape berries homogenized under a variety of conditions. Treatment of the insoluble fraction with borate buffer, pH 8·5, polyethylene glycol (Carbowax 4000) or non-ionic detergents solubilized most of the invertase. Homogenates of grape berries prepared in the presence of Carbowax 4000, non-ionic detergents or protein (bovine serum albumin) contained up to 100 per cent of the total invertase in the soluble form. It is suggested that insoluble invertase from grapes is an artifact of extraction caused by the formation of a tannin–protein complex and/or a protein–tannin–cell-wall complex. Invertase in grapes is probably located in the cytoplasm or vacuoles of cells. In contrast to grapes, insoluble invertase from aged aseptic carrot disks or from corn coleoptiles could not be solubilized.

INTRODUCTION

INVERTASES (β-D-fructofuranoside fructohydrolases, E.C.3.2.1.26) in higher plants are often firmly bound to easily sedimented material in crude homogenates ([1–3] and references cited therein). In some plants the insoluble invertase has different properties to those of soluble invertase which also occurs in the same tissue[1,4] while in other plants the properties of the insoluble and soluble invertase are similar.[5,6] Bound invertases are generally resistant to solubilization by salts and buffers. However, Arnold[2] solubilized grape-insoluble invertase by treating cell-wall preparations with borate buffer and he suggested that the effect may have been due to a complex formation between borate ion and polysaccharide and a subsequent release of enzyme. Grapes contain phenols and tannins.[7] Tannins are capable of binding enzymes to cell-walls[8] and several compounds, including borate, break tannin–protein complexes.[9] It is therefore possible that during the extraction of invertase from grapes some of the soluble invertase is precipitated by the formation of cell-wall–tannin–invertase and/or insoluble tannin–invertase complexes. Since invertase may be involved in sugar accumulation in grapes[10] it is important to know whether it is bound to cell-walls *in vivo*. Experiments using compounds which break protein–tannin complexes have now been carried out to

[1] J. A. SACHER, *Plant Physiol.* **41**, 181 (1966).
[2] W. N. ARNOLD, *Biochim. Biophys. Acta* **128**, 124 (1966).
[3] D. VAUGHAN and I. R. MACDONALD, *J. Expt. Botany* **18**, 578 (1967).
[4] J. S. HAWKER and M. D. HATCH, *Physiol. Plantarum* **18**, 444 (1965).
[5] D. VAUGHAN and I. R. MACDONALD, *Plant Physiol.* **42**, 456 (1967).
[6] W. N. ARNOLD, *Biochim. Biophys. Acta* **128**, 196 (1966).
[7] V. L. SINGLETON, *Am. J. Enol. Viticult.* **17**, 126 (1966).
[8] R. E. YOUNG, *Arch. Biochem. Biophys.* **111**, 174 (1965).
[9] J. L. GOLDSTEIN and T. SWAIN, *Phytochem.* **4**, 185 (1965).
[10] J. S. HAWKER, *Phytochem.* **8**, 9 (1968).

answer this question. Insoluble invertases from carrot and corn coleoptiles have also been studied.

RESULTS

Activity of Invertase during the Development of Sultana Grape Berries

During the 1966–67 grape season, soluble and insoluble invertase was extracted from berries without the addition of carbowax. Early in the development of the berries, about 90 per cent of the invertase activity was in the insoluble fraction. This uneven distribution of invertase slowly changed until about half-way through the development of the berries the invertase activity was almost equally distributed between the soluble and insoluble fractions. At this stage sugar accumulation commenced and the berries began to increase in size rapidly by cell expansion. Little change in the relative amounts of soluble and insoluble invertase occurred during further development. The concentration of extractable phenols decreases during the development of many grape varieties[7] and estimations of phenols from sultana berries showed that a decrease also occurred in this variety.

During the 1967–68 season, soluble and insoluble invertases were extracted from berries in the presence of carbowax. More than 85 per cent of the total invertase was soluble at all stages of development under these conditions of extraction. The pattern of activity of soluble invertase was similar to that measured in concentrated enzyme extracts of developing sultana grapes.[10] A maximum activity of about 8·5 units per g fresh weight was observed in the sixth week after flowering compared with a maximum of about 6 units on the same date measured in the concentrated extracts.[10]

Solubilization of Invertase with Borate Buffer, Carbowax 4000 *and Detergents*

When frozen sultana berries were ground in tris buffer, pH 8·5, most of the invertase appeared in the insoluble fraction (Table 1). Treatment of the insoluble fraction with 6% carbowax or borate buffer resulted in solubilization of a large part of the invertase (Table 1). When fresh berries were ground in phosphate–citrate buffer, pH 5·0, it was also found that the majority of the invertase activity appeared in the insoluble fraction. Most of this invertase was solubilized by treatment with carbowax or the non-ionic detergents, Triton X100 and Tween 20 (Table 2). The sum of the activities of the remaining bound invertase and solubilized invertase was greater than the activity of the original bound invertase (Table 2).

TABLE 1. SOLUBILIZATION OF BOUND INVERTASE EXTRACTED FROM FROZEN GRAPE BERRIES

	Units of invertase*/g fresh weight of berry			
	Soluble	Insoluble	Solubilized	Insoluble + solubilized
Whole berries	0·34	6·36	—	6·36
Insoluble fraction + 6% Carbowax 4000	—	1·74	7·0	8·74
Insoluble fraction + 0·1 M borate buffer, pH 8·5	—	1·82	8·7	10·52

* Assayed at pH 4·0.

TABLE 2. SOLUBILIZATION OF BOUND INVERTASE FROM FRESH GRAPE BERRIES

	Units of invertase*/g fresh weight of berry			
	Soluble	Insoluble	Solubilized	Insoluble + solubilized
Experiment 1				
Whole berries	0·35	2·28	—	2·28
Insoluble fraction + 6% Carbowax 4000	—	0·58	2·26	2·84
Experiment 2				
Whole berries	—	2·68	—	2·68
Insoluble fraction + 6% Carbowax 4000	—	0·26	3·90	4·16
Insoluble fraction + 1% Triton X100	—	0·38	3·42	3·80
Insoluble fraction + 0·2% Tween 20	—	0·74	2·78	3·52

* Assayed at pH 5·0.

Soluble and Insoluble Invertase in Grape Extracts Prepared with or without Carbowax, Detergents or Protein

After treatment of the insoluble grape fraction with carbowax, borate buffer or detergents a small but significant amount of invertase activity remained insoluble (Tables 1 and 2). However, when fresh sultana berries were ground in phosphate–citrate buffer containing 1% or 5% Tween virtually all of the invertase was soluble (Table 3). In the presence of carbowax 96 per cent of the invertase was soluble and with bovine serum albumin 69 per cent of the invertase was soluble.

TABLE 3. SOLUBLE AND INSOLUBLE INVERTASE IN GRAPE EXTRACTS PREPARED WITH OR WITHOUT CARBOWAX, DETERGENTS OR PROTEIN

	Units of invertase*/g fresh weight of berry		
Compound added	Soluble	Insoluble	Soluble as percentage of total
None	0·06	1·00	5·7
6% Carbowax 4000	2·33	0·11	95
10% Carbowax 4000	2·01	0·09	96
1% Tween 20	2·61	0·01	100
5% Tween 20	2·50	0	100
100 mg bovine serum albumin	1·15	0·52	69
None, boiled	0	0	—

* Assayed at pH 5·0.

Intracellular Location of Invertase in Grape Berries

In an attempt to determine whether invertase is bound to grape cell-walls *in vivo* or whether insoluble invertase is an artifact of extraction, two experiments were carried out.

In the first experiment unwashed slices of grapes were incubated in 6% Carbowax 4000. If the invertase was bound to cell-walls *in vivo* it would be expected that the carbowax would release a considerable part of the invertase into the incubation medium. However if the enzyme was located within a membrane (i.e. in the cytoplasm or vacuole) only invertase released from cut cells would appear in the medium. While 68 per cent of the reducing sugar was lost from the slices only 29 per cent of the invertase appeared in the medium (Table 4).

TABLE 4. LOSS OF INVERTASE AND REDUCING SUGAR FROM SLICES OF GRAPE BERRIES*

	Units of invertase*/g fresh weight of original whole berry	Reducing sugar (μmoles/g fresh weight of original berry)
Incubation medium	1·16	282
Slice homogenate		
Supernatant	2·18	130
Precipitate	0·65	—

* Slices of grape berry were incubated in a medium containing 6% Carbowax 4000 for 3 hr. Invertase was assayed at pH 5·0.

In the second experiment berries were squeezed gently in an attempt to extract juice only from the large thin-walled pericarp cells which did not contain tannin. Juice extracted in this way contained more soluble invertase than insoluble invertase whereas the extracts from the remainder of the berries (after removing some juice) or from whole berries contained more insoluble than soluble invertase (Table 5).

TABLE 5. THE EFFECT OF MILD EXTRACTION OF JUICE FROM GRAPE BERRIES ON THE DISTRIBUTION OF INVERTASE

	Units of invertase*/g fresh weight or /g juice	
	Soluble	Insoluble
Whole berry	0·18	1·05
Juice	0·85	0·60
Remainder of berry	0·34	0·74

* Assayed at pH 5·0. Carbowax was not used in this experiment.

Location of Phenols or Tannins in Sultana Grapes

Vanillin in HCl gives a red colour with leucoanthocyanidins, some catechins and polymers of each.[11] When transverse slices of sultana berries at all stages of development were placed in vanillin in HCl an intense red colour quickly appeared around the periphery of the slice and in the central portion. The embryos of sultana berries abort at a very early stage but the

[11] T. SWAIN and H. E. HILLIS, *J. Sci. Food Agr.* **10**, 63 (1959).

remaining septum and very small imperfect seeds stained red. Treatment of other slices with concentrated HCl slowly produced a red–brown reaction in the same regions of the berries. Aqueous ferric chloride slowly stained the same regions blue–black. With all stains used the pericarp did not stain appreciably.

Tests with vanillin in HCl on the supernatants and precipitates resulting from centrifuging sultana grape homogenates prepared either with or without carbowax showed that in each case virtually all of the red staining material remained in the precipitate.

Effect of Carbowax, Borate Buffer and Detergent on Insoluble Invertase from Aged Carrot Disks and Corn Coleoptiles

The activity of invertase in carrot disks which were cut and aged (washed) aseptically increased, as previously described by Vaughan and MacDonald,[5] with the majority of the activity appearing in the insoluble fraction. Treatment with carbowax or borate buffer of the insoluble invertase fraction from disks which had been aged for 48 hr solubilized only a small fraction of the invertase (Table 6).

TABLE 6. EFFECT OF CARBOWAX, BORATE BUFFER AND DETERGENT ON INSOLUBLE INVERTASE FROM AGED CARROT DISKS AND CORN COLEOPTILES

Aged carrot disks	Units of invertase*/g fresh weight of tissue		
	Soluble	Insoluble	Solubilized
Whole disks	0·014	0·084	—
Insoluble fraction + 6% Carbowax 4000	—	0·091	0·012
Insoluble fraction + 0·1 M borate buffer, pH 8·5	—	0·077	0·004
Corn coleoptiles	0·75	0·17	—
Coleoptiles + 6% Carbowax 4000	0·75	0·26	—
Coleoptiles + 1% Tween 20	0·62	0·19	—

* Assayed at pH 5·0.

Carbowax 4000 or Tween 20 made little difference to the proportion of soluble and insoluble invertase extracted from corn coleoptiles (Table 6).

DISCUSSION

The results presented in this paper suggest that all of the invertase in grapes is soluble and is located in either the cytoplasm or vacuoles of cells. The previously reported insoluble invertase[2, 6, 12] is probably an artifact produced during the extraction of invertase from the grapes. In the absence of compounds which break tannin–protein complexes a high proportion of the invertase of sultana grapes appears in the insoluble fraction after homogenization under a variety of conditions (Tables 1–3 and 5). Treatment of this fraction from frozen or fresh grapes with Carbowax 4000, borate buffer or non-ionic detergents solubilized most, although not all, of the invertase (Tables 1 and 2). However little, or no, insoluble invertase was obtained when fresh grapes were homogenized in the presence of carbowax or detergents

[12] W. N. ARNOLD, *Biochim. Biophys. Acta* **110**, 134 (1965).

(Table 3). Since grapes contain phenols and tannins[7] and tannin–protein complexes are broken by borate, carbowax and non-ionic detergents[9] it is probable that the insoluble invertase of grapes is a protein–tannin complex. The fact that added protein increases the proportion of soluble invertase obtained supports this hypothesis (Table 3) because added protein would be expected to compete with invertase in complex formation. Decreases in the ratio of insoluble to soluble invertase in grapes extracted without carbowax during the 1966–67 season which were accompanied by decreases in the concentration of phenols and the very low amounts of insoluble invertase extracted from grapes in the presence of carbowax in the 1967–68 season further support this hypothesis.

Young[8] concluded that an insoluble aldolase–tannin–cell-wall complex was formed during thorough homogenization of immature banana fruit. The positive reaction to vanillin obtained with cell-wall preparations from sultana berries prepared in the presence or absence of carbowax suggests that in grapes insoluble invertase is due to a complex of invertase, tannin and cell-wall. However it must be pointed out that there is no evidence in either the present work or in the work of Young[8] that the vanillin positive tannins are involved in complex formation. Many other types of tannins could play a role in complex formation.[13]

That insoluble invertase probably does not exist in grapes *in vivo* but is due to an artifact of extraction is suggested by the effect of carbowax on whole berries and cell-wall fractions and by slice and juicing experiments. Fresh berries, ground in the presence of carbowax, contained only a small proportion of the invertase in the insoluble form. More insoluble invertase occurred when cell-wall fractions, prepared in the absence of carbowax, were subsequently treated with carbowax (Tables 2 and 3). Still more insoluble invertase occurred in the case of frozen berries (Table 1). An explanation for these results could be that carbowax is more effective in preventing enzyme–tannin complex formation than in breaking such a complex. Phenolic compounds combine with proteins reversibly by hydrogen bonding and irreversibly by oxidation followed by covalent condensations.[13] Secondly the small release of invertase from slices incubated in carbowax compared to the release of reducing sugar suggested that much of the invertase was located within cell membranes (Table 4). The invertase which did appear in the medium could have come from large pericarp cells which had been cut or broken. Thirdly the higher proportion of soluble invertase in juice from gently squeezed berries compared to homogenized berries could have resulted from the bursting of pericarp cells while only a few of the smaller stronger cells containing tannin were ruptured (Table 5). Staining techniques showed that little, if any, polyphenols or tannins were present in the pericarp cells. The small amount of tannin present in juice extracted by squeezing berries would result in some precipitation of invertase but not as much as observed when all the tannin and invertase was extracted together. The results obtained support the hypothesis that grape invertase is not attached to cell walls *in vivo* (Table 5).

Several other plant tissues including carrot roots[5] and corn coleoptiles[14] contain soluble and insoluble invertases which have similar properties. Compounds which break tannin–protein complexes had practically no effect on insoluble invertase from carrot roots or corn coleoptiles (Table 6). There is good evidence that part of the invertase which appears in ageing disks of storage tissues is bound to cell-walls *in vivo*.[3,5] The fact that this invertase is not solubilized by Carbowax 4000 or borate buffer shows that it is bound to the insoluble fraction in a different way to that of invertase in grape homogenates. These findings are consistent with the hypothesis that insoluble invertase from grapes is an artifact. It is possible

[13] W. D. LOOMIS and J. BATTAILE, *Phytochem.* **5**, 423 (1966).
[14] A. KIVILAAN, T. CABRERA BEAMAN and R. S. BANDURSKI, *Plant Physiol.* **36**, 605 (1961).

that other tissues, particularly fruit which contain tannins, may form invertase–tannin complexes and care should be taken to avoid the formation of such complexes by the addition of carbowax or other protective agents.

The possible role of invertase in sugar accumulation in grapes has been briefly discussed.[10] The almost equal concentrations of glucose and fructose (about 10 per cent fresh weight of each) and very low concentrations of sucrose which occur in ripe grapes suggest that invertase may be involved in sugar accumulation. The possibility arising from the present work that the enzyme occurs in the cytoplasm, vacuoles or both of grape cells would allow the operation of several alternative pathways for sugar accumulation. However, because of the changes in concentrations of enzymes known to occur in grapes, it is conceivable that sucrose is hydrolysed in the cytoplasm, transported into the vacuole as sucrose phosphate and finally converted to glucose and fructose in the vacuole.[10]

EXPERIMENTAL

Materials

Grape berries from young vines of *Vitis vinifera* L. cv. Sultanina (syn. Sultana, Thompson Seedless) were obtained from a vineyard near Adelaide, South Australia or from small plants grown in a glasshouse.

Unit of Invertase

In this paper a unit of invertase is defined as the amount which hydrolyses 1 μmole of sucrose per min at 30° and under other conditions as described for each experiment.

Extraction and Assay of Invertase

Each value given is the mean of determinations on at least three samples of tissue. All enzyme extractions were carried out at less than 4°. It was established that different speeds and times of centrifugation between 1000 *g* for 5 min and 20,000 *g* for 15 min had no effect on the proportion of soluble and insoluble invertase present in homogenates. The higher speeds and longer times were used in some experiments to obtain more compact pellets.

From sultana berries during their development. In the 1966–67 season frozen berries (10 g) were ground in a mortar with 50 mg sodium diethyldithiocarbamate, 0·2 ml 0·5 M EDTA, 0·1 ml toluene and sufficient 0·1 M Na_2CO_3 to obtain a final pH between 7 and 8. The homogenate was centrifuged at 20,000 *g* for 10 min. The supernatant containing soluble invertase was dialysed against distilled water overnight and the pellet containing insoluble invertase was washed with 5 mM potassium phosphate buffer, pH 7·0. During the 1967–68 season frozen berries (10 g) were ground in 20 ml 0·5 M tris buffer, pH 8·5, containing 30 mg sodium diethyldithiocarbamate, 20 mg cysteine–HCl, 0·1 ml 0·5 M EDTA and 1.6 g Carbowax 4000. The homogenate was treated as for the 1966–67 season homogenates. Invertase was assayed at pH 4·0 as described by Arnold[12] except that the final reaction mixtures containing the insoluble fraction were filtered prior to heating.

From frozen berries and insoluble fraction. Soluble and insoluble invertase was prepared from frozen berries as described for the 1967–68 season. The pellet (insoluble invertase fraction) was suspended in 100 ml 5 mM potassium phosphate buffer, pH 7·0. Two 40-ml samples were centrifuged at 20,000 *g* for 10 min and the pellets were suspended in either 30 ml 0·2 M borate buffer, pH 8·5, or 30 ml of 6% (w/v) Carbowax 4000 in 5 mM potassium phosphate buffer, pH 7·0. After 30 min the suspensions were centrifuged at 20,000 *g* for 10 min. The supernatants were dialysed against distilled water overnight and the pellets suspended in 40 ml 5 mM potassium phosphate buffer, pH 7·0. Invertase was assayed at pH 4·0 as described above.

From fresh berries and insoluble fraction. Single washed sultana berries (approximate weight 1 g) were ground with glass beads in a mortar in 15 ml 0·1 M citrate–phosphate buffer, pH 5·0, and centrifuged and assayed at 30° as described by Vaughan and MacDonald[5] with the following modifications. Supernatants were sometimes dialysed, reducing sugar was determined as described by Arnold[12] and samples of the insoluble fraction were filtered between the addition of the 3,5-dinitrosalicylic acid reagent and heating. Carbowax 4000, detergents or protein were added to the homogenizing medium in some experiments. In other experiments the pellet from the first centrifugation was washed with 10 ml of 0·1 M citrate–phosphate buffer, pH 5·0, and then incubated with 5 ml of solutions of Carbowax 4000 or detergents in 5 mM potassium phosphate buffer, pH 7·0, for 30 min. After centrifugation at 10,000 *g* for 10 min the supernatant was assayed at pH 5·0 by adding citrate–phosphate buffer and sucrose. The pellet was washed with 10 ml of carbowax or detergent solution and assayed for invertase as described in this paragraph.

From aged carrot slices and corn coleoptiles. Carrot disks were cut and aged aseptically and soluble and insoluble invertase was extracted as described by Vaughan and MacDonald.[5] The insoluble fraction was treated with borate buffer or Carbowax 4000 and invertase was assayed as described for fresh grape berries.

Invertase was extracted from corn (*Zea Mays* L.) coleoptiles and assayed as described for fresh grape berries.

Experiments on Intracellular Location of Invertase

A single sultana berry was cut into transverse slices 1–2 mm thick and incubated in 15 ml 6% Carbowax 4000 in 0·1 M citrate–phosphate buffer, pH 5·0, for 3 hr at 4°. The slices were separated from the medium and soluble and insoluble invertase extracted from the slices and soluble invertase in the medium was assayed as described for fresh berries. Zero time determinations gave values for the amounts of reducing sugar which had leaked into the medium and which remained in the slices.

A single sultana berry was squeezed gently to release about one-third of its weight as juice. Soluble and insoluble invertase was extracted without carbowax and assayed at pH 5·0 as described for fresh berries.

Staining Techniques

Slices of grape berries were incubated in solutions of 1% vanillin in concentrated HCl and in 5% ferric chloride in water.

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