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Localization of the Enzymes of Fructan Metabolism in Vacuoles Isolated by a Mechanical Method from Tubers of Jerusalem Artichoke (*Helianthus tuberosus* L.)[1]

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ABSTRACT

Vacuoles isolated by a mechanical slicing method from developing tubers of Jerusalem artichoke (*Helianthus tuberosus* L.) contain activities of the two principal enzymes responsible for fructan synthesis: sucrose-sucrose fructosyl transferase and fructan-fructan fructosyl transferase. Both enzymes are associated with the vacuolar sap and not with the tonoplast. In vacuoles isolated from dormant tubers, the fructan-fructan fructosyl transferase activity remains in the vacuolar sap but the fructan exohydrolase activity is associated with the tonoplast. Fructan is hydrolysed by these vacuoles to fructose, which can be exported to the suspending medium. The localization of the enzymes of fructan metabolism in the vacuole has implications for the maintenance of fructan polymerisation.

Fructan is a polyfructosylsucrose which occurs widely as a storage carbohydrate in the vegetative parts of plants (13). In tubers of Jerusalem artichoke, as in other species, the synthesis of fructan from sucrose is catalysed by the sequential action of two enzymes: SST,[3] which takes two molecules of sucrose to form a trisaccharide, 1-kestose; and FFT, which catalyses the transfer of fructosyl residues from the trisaccharide to elongating fructan chains (6). When the tubers are dormant SST activity disappears and activity of a specific exohydrolase, FEH, appears. This enzyme removes fructosyl residues from the ends of the fructan chains, thus initiating the depolymerisation and remobilization of the stored fructan.

Fructan accumulates in the large central vacuole of the storage parenchyma (6, 13). To account for the biosynthesis of vacuolar fructan from cytosolic sucrose Edelman and Jefford (6) proposed that SST was cytosolic, and FFT was located in the tonoplast so that the fructosyl residues were transferred from the cytosolic trisaccharide across the tonoplast to the elongating chains of fructan within the vacuole. Similarly they suggested (6) that FEH could most effectively mobilize fructan if the enzyme were located at the tonoplast transferring fructosyl residues from vacuole to cytosol. This scheme (6) elegantly accounts for many features of fructan synthesis but it failed to find support in the recent work of Frehner *et al.* (8), who concluded that both SST and FFT were located in the vacuole.

Edelman and Jefford (6) had shown that while SST has a high K_m for sucrose, the FFT-catalyzed polymerisation of fructan is effectively inhibited by sucrose because FFT donates fructosyl residues preferentially to sucrose instead of to oligomeric fructans. It was because of the contrasting sucrose requirements of SST (high sucrose levels) and FFT (low sucrose levels) that Edelman and Jefford (6) allocated SST and FFT to the cytosol and vacuole, respectively. These contrasting requirements could be accommodated by a vacuolar localization of SST and FFT if the SST were organized at the tonoplast so as to receive sucrose from the cytosol and supply 1-kestose to FFT in the vacuolar sap. The principal aim of the present work therefore was to determine whether the enzymes of fructan synthesis (SST and FFT) and mobilisation (FFT and FEH) are located at the tonoplast or within the vacuolar sap.

Frehner *et al.* (8) had isolated vacuoles from lysed protoplasts. This procedure involved an extensive incubation (12 to 16 h at 27° C) for the preparation of the protoplasts. Moreover release of the vacuoles from the protoplasts appeared to be incomplete, since nearly 40% of the extravacuolar marker enzymes were reported to be present in vacuoles from developing tubers. In the present work we have used a mechanical procedure to isolate vacuoles from Jerusalem artichoke tubers. This procedure, originally developed for the isolation of beet vacuoles (11), avoids extensive tissue incubation, and offered the possibility of a more complete isolation of the vacuoles (1).

MATERIALS AND METHODS

Plant Material

Developing tubers of Jerusalem artichoke (*Helianthus tuberosus* L.) were obtained during autumn from plants growing in the experimental grounds of the Plant Science Laboratories, University of Reading. Dormant tubers were allowed to remain in the ground over winter or were stored at 5° C.

Isolation and Purification of Vacuoles

Three hundred g of peeled tubers, cut into cubes 1 to 2 cm square, were sliced at room temperature in the tissue-slicing

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[3] Abbreviations: SST, sucrose-sucrose fructosyl transferase; FFT, fructan-fructan fructosyl transferase; FEH, fructan exohydrolase.

apparatus described by Leigh and Branton (11) for the large-scale isolation of vacuoles from roots of red beet. The vacuoles were collected into 1 L of collection medium containing 0.8 M sorbitol, 50 mM Tris-Cl (pH 7.6), 5 mM disodium EDTA, 0.1 mM polyethyleneglycol mol wt 4000 (PEG-4000), 0.1% (w/v) dextran sulfate, and 2 mM DTT. The sliced material was filtered through a layer of Miracloth (Calbiochem) supported on a stainless steel sieve (11) and the retained tissue was sliced again into the filtrate. Remaining unsliced tissue was removed by filtration through Miracloth as above, and the vacuoles were sedimented from the filtrate by centrifugation at 2000*g* for 10 min at 4° C. The resulting supernatant was decanted away carefully, and the remaining supernatant removed from the pellets by aspiration. The pellets were resuspended in 20 mL of a medium containing 0.8 M betaine, 50 mM Mes (adjusted to pH 5.2 with Tris), 0.1 mM PEG-4000, and 20 mM EGTA. The suspension was filtered through Miracloth, and the vacuoles were allowed to sediment at room temperature for 1 h. The supernatant was discarded, and the vacuoles were resuspended in 20 mL of the resuspension medium. The vacuoles were centrifuged at 2000*g* for 5 min, and the pellets were resuspended in 2 mL of a medium containing 0.8 M betaine, 20 mM Tris-Mes (pH 5.2) and 1 mM DTT.

Vacuoles were lysed by resuspending the vacuole pellet in 2 ml of 5 mM Tris-Mes (pH 5.2) containing 2 mM DTT. The tonoplast membranes were sedimented by centrifugation at 350,000*g* for 1 h and resuspended in 20 mM Tris-Mes (pH 5.2) containing 1 mM DTT. The supernatant was retained as the sap fraction.

Assays of SST and FFT

Activities of SST and FFT were determined from the amounts of glucose, fructose and trisaccharide (kestose) formed when enzyme samples were incubated with sucrose and fructan (8). The trisaccharide formed was not identified chemically but is assumed to be 1-kestose (1^F-fructosylsucrose) as this is the only trisaccharide found in extracts of Jerusalem artichoke (6). However, as it may also have included 6-kestose and neokestose, it is here termed kestose. Enzyme sample (100 μL) was incubated at 25° C for 3 h with 100 μL of a solution containing sucrose (100 mg/mL) and chicory inulin (70 mg/mL, Sigma Chemical Co.) dissolved in 100 mM Tris-Mes (pH 5.2). The reaction was stopped by the addition of 150 μL of 0.2 M Na_2HPO_4, heated in a water bath at 100° C for 2 min, and cooled in an ice bath. Precipitated protein was removed by centrifugation. Glucose and fructose in the supernatant were assayed enzymatically using a commercial kit (Boehringer) and kestose was measured after separation by HPLC. A sample (100 μL) of the supernatant was mixed with 50 μl of rhamnose (4 mg/mL) as an internal standard and desalted by passage through a small column held in a 1 mL disposable pipette tip. The column consisted of Amberlite MB-3 resin overlain with Dowex $50H^+$ (200–400 mesh). The column was rinsed with 200 μL of water, and the eluates combined. An equal volume of acetonitrile was added, the samples were left overnight at room temperature and any precipitated inulin was sedimented by centrifugation. The samples were filtered through 0.45 μm HPLC disposable filters. Aliquots (100 μL) were injected onto a prepacked Spherisorb-5-NH_2 column (Phase Separations, Queensferry, Clwyd, UK) fitted with a Zorbax CN guard column (Dupont, UK). The mobile phase, acetonitrile:H_2O (70:30, v/v) was pumped at a flow rate of 2 mL/min. Peaks were monitored using a Waters R401 differential refractometer linked to a Spectra Physics 4270 computing integrator. Peaks were quantified using reference compounds, melizitose being used for kestose (8). Preliminary experiments with crude extracts revealed that the SST and FFT assays were linear with both time (up to 3 h) and extract concentration.

Assay of FEH

The enzyme sample (100 μL) was incubated for 4 h at 25° with 100 μL of 50 mM Mes-Tris (pH 5.2) containing 50 mg/ml of oligomeric fructan. The reaction was stopped by heating the samples in a boiling water bath for 2 min. When the glucose and fructose were assayed enzymatically as described above the glucose formed was insignificant compared with the fructose, and therefore fructose formed by FEH activity was routinely assayed by the Nelson-Somogyi method (14).

The substrate, oligomeric fructan, was prepared according to Edelman and Jefford (5) except that sodium diethyldithio-

Table I. *Activities of Enzyme Markers in Different Fractions during Isolation of Vacuoles from Developing Tubers*

Fraction	Vacuoles	Total Fructose	α-Mannosidase	Alcohol Dehydrogenase	Glucose-6-P Dehydrogenase	NADH Cyt *c* Oxidoreductase	NADPH Cyt *c* Oxidoreductase	Glutamate Oxaloacetate Transaminase	SST	FFT
	×10^{-7}	μmol			μmol/min				μmol/h	
Sliced tissue filtrate		43,650	2.460	669	42,288	125.1	5.108	2.250	532.8	1,091.0
2,000*g* supernatant		44,500	2.092	643	42,673	121.0	5.173	2.121	534.0	979.0
2,000*g* Pellet	16.7	700	0.0726	9.51	2,040	5.676	0.227	0.457	8.4	14.0
1*g* Supernatant	13.6	515	0.0597	7.60	1,466	5.670	0.229	0.449	8.24	13.9
1*g* Pellet (vacuoles)	1.6	70	0.0051	0.35	21	0.005	<0.005	0.001	1.05	2.52
Percent recovery[a]	91	84	89	84	73	100	100	97	110	117

[a] Percent recovery = $\frac{1g \text{ supernatant} + 1g \text{ pellet}}{2{,}000g \text{ pellet}} \times 100$.

Table II. *Ratio of Total Fructose to Enzyme Markers when Vacuoles Are Isolated from Developing Tubers*

Fraction	Total Fructose/ α-Mannosidase	Total Fructose/ Alcohol Dehydrogenase	Total Fructose/ Glucose-6-Phosphate Dehydrogenase	Total Fructose/ NADH Cyt *c* Oxidoreductase	Total Fructose/ NADPH Cyt *c* Oxidoreductase	Total Fructose/ Glutamate Oxaloacetate Transaminase
Sliced tissue filtrate	17,744[a]	65.2	1.03	348.9	8,545	19,400
1*g* Pellet	13,725	200.0	3.3	14,000	>14,000	70,000
Purification factor[b]	0.77	3.0	3.2	40	>1.6	3.6

[a] Data from Table I. [b] Purification factor = $\frac{\text{1g pellet ratio}}{\text{sliced tissue filtrate ratio}}$.

Table III. *Activities of SST and FFT Compared with Total Fructose and Marker Enzyme Activities during Isolation of Vacuoles from Developing Tubers*

Fraction	SST/Total Fructose	FFT/Total Fructose	SST/ α-Mannosidase	FFT/ α-Mannosidase	SST/Alcohol Dehydrogenase	FFT/Alcohol Dehydrogenase	SST/Glucose-6-P Dehydrogenase	FFT/Glucose-6-P Dehydrogenase
Sliced tissue filtrate	0.012[a]	0.025	217	443	0.796	1.630	0.013	0.026
2000*g* Supernatant	0.012	0.022	255	468	0.830	1.522	0.012	0.023
2000*g* Pellet	0.012	0.020	117	193	0.883	1.472	0.004	0.007
1*g* Supernatant	0.016	0.027	137	232	1.084	1.829	0.006	0.009
1*g* Pellet	0.015	0.036	206	494	3.000	7.20	0.05	0.12
Purification factor[b]	1.25	1.44	0.95	1.12	3.76	4.4	3.85	4.61

[a] Data from Table I. [b] Purification factor = $\frac{\text{1g pellet ratio}}{\text{sliced tissue filtrate ratio}}$.

Table IV. *Distribution of Total Fructose, Marker Enzymes, SST, and FFT between Vacuole Sap and Tonoplast after Lysis of Vacuoles Isolated from Developing Tubers*

Fraction	Total Fructose	α-Mannosidase	Nitrate-Sensitive ATPase	SST	FFT
	μmol		*nmol/h*		
Unfractionated lysed vacuoles	30.13	184.8	213	186	1214
Vacuole sap	29.83	117	10	176	1053
Tonoplast	2.09	54	207	20	0
Percent recovery	106	93	102	105	87

Table V. *Activities of Enzyme Markers in Different Fractions during Isolation of Vacuoles from Dormant Tubers*

Fraction	Total Fructose	α-Mannosidase	Alcohol Dehydrogenase	Glucose-6-P Dehydrogenase	NADH Cyt *c* Oxidoreductase	NADPH Cyt *c* Oxidoreductase	Glutamate Oxaloacetate Transaminase	FFT	FEH
	μmol			*μmol/min*				*μmol/h*	
Sliced tissue filtrate	46,810	2.562	1,287	40.1	120.4	6.201	1.560	23.166	59.2
2,000*g* Supernatant	48,460	2.452	1,231	43.2	116.2	6.233	1.572	27.082	56.62
2,000*g* Pellet	707	0.038	25.80	<0.02	4.631	0.232	0.346	0.387	0.903
1*g* Supernatant	642	0.031	23.40	<0.02	4.020	0.232	0.340	0.328	0.842
1*g* Pellet (vacuoles)	60	0.003	0.35	<0.002	0.004	<0.002	0.0001	0.031	0.083
Percent recovery[a]	99	89	92		87	100	98	93	102

[a] Percent recovery = $\frac{\text{1g supernatant + 1g pellet}}{\text{2,000g pellet}} \times 100$.

Table VI. *Ratio of Total Fructose to Enzyme Markers when Vacuoles Are Isolated from Dormant Tubers*

Fraction	Total Fructose/ α-Mannosidase	Total Fructose/ Alcohol Dehydrogenase	Total Fructose/ Glucose-6-P Dehydrogenase	Total Fructose/ NADH Cyt *c* Oxidoreductase	Total Fructose/ NADPH Cyt *c* Oxidoreductase	Total Fructose/ Glutamate Oxaloacetate Transaminase
Sliced tissue filtrate	18,271[a]	36.37	1,167	388.8	7,549	30,006
1*g* Pellet	20,000	171.4	>30,000	15,000	>30,000	6×10^5
Purification factor[b]	1.09	4.7	>25.7	38.7	>3.97	20.0

[a] Data from Table V. [b] Purification factor = $\frac{\text{1g pellet ratio}}{\text{sliced tissue filtrate ratio}}$.

Table VII. *Activities of SST and FFT Compared with Total Fructose and Marker Enzyme Activities during Isolation of Vacuoles from Dormant Tubers*

Fraction	FFT/Total Fructose $\times 10^3$	FEH/Total Fructose $\times 10^3$	FFT/ α-Mannosidase	FEH/ α-Mannosidase	FFT/Alcohol Dehydrogenase	FEH/Alcohol Dehydrogenase
Sliced tissue filtrate	0.495[a]	1.26	9.04	23.11	0.018	0.046
2000*g* Supernatant	0.558	1.17	11.04	23.09	0.022	0.046
2000*g* Pellet	0.547	1.28	10.18	23.76	0.015	0.035
1*g* Supernatant	0.510	1.31	10.58	27.16	0.014	0.036
1*g* Pellet	0.516	1.38	10.33	27.66	0.089	0.237
Purification factor[b]	1.04	1.09	1.14	1.20	4.9	5.15

[a] Data from Table V. [b] Purification factor = $\frac{\text{1g pellet ratio}}{\text{sliced tissue filtrate ratio}}$.

Table VIII. *Distribution of Total Fructose, Marker Enzymes, FFT, and FEH between Vacuole Sap and Tonoplast after Lysis of Vacuoles Isolated from Dormant Tubers*

Fraction	Total Fructose	Nitrate-Sensitive ATPase	FFT	FEH
	μmol		*nmol/h*	
Unfractionated lysed vacuoles	56.8	37	530	373
Vacuole sap	51.6	5	442	107
Tonoplast	4.0	40	0	351
Percent recovery	98	122	83	128

carbamate was omitted and the peeled tubers (200*g*) were heated in a microwave oven for 10 min to inactivate polyphenoloxidase. The oligomeric fructan was freed of inorganic ions with Amberlite MB-3 ion exchange resin and finally freeze-dried. The content of free fructose was <1 $\mu g \cdot mg^{-1}$.

Marker Enzymes

α-Mannosidase (vacuole sap) was determined from the release of *p*-nitrophenol from the appropriate *p*-nitrophenyl derivatives (2); glutamate oxaloacetate transaminase (plastid) was determined using a commercial assay system (Sigma Chemical Co.); alcohol dehydrogenase (cytosol) in a reaction mixture containing 44 mM NaPPi (pH 8.8) 1% (v/v) ethanol, 0.1 mM NAD at 25° C; glucose-6-P dehydrogenase (cytosol) from the formation of NADPH (4); both NADH-Cyt *c* oxidoreductase (mitochondrial inner membrane) and NADPH-

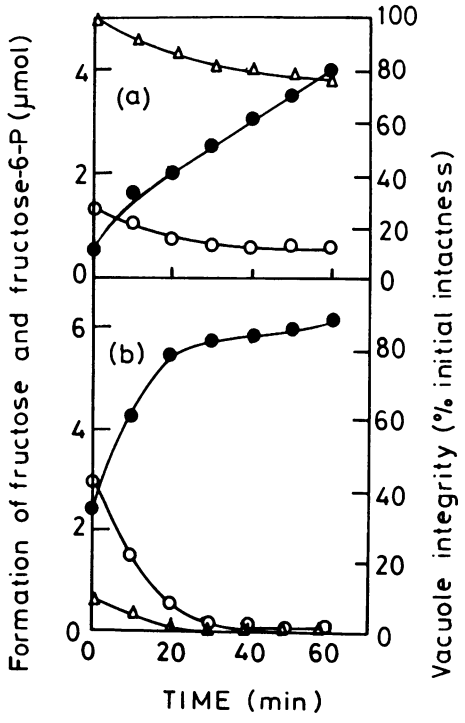

Figure 1. Hydrolysis of fructan by vacuoles isolated from dormant Jerusalem artichoke tubes. Fructose (○), fructose-6-P (●), and vacuole integrity (△) were estimated as described in "Materials and Methods." a, Untreated; b, sonicated for 30 s before the experiment.

Cyt *c* oxidoreductase (endoplasmic reticulum) from the reduction of Cyt *c* (9). Nitrate-sensitive ATPase (tonoplast) was assayed by a modification of the method of Walker and Leigh (16). Enzyme sample (100 μL) was incubated for 1 h at 30° C in 1 mL of a reaction mixture which contained 50 mM Tris-Mes (pH 8.0), 5 mM ATP, 5 mM $MgSO_4$, 100 μM ammonium molybdate, and 50 mM KCl. The reaction was stopped by the addition of 0.2 mL of 35% TCA, precipitated protein was removed by centrifugation and Pi determined by the method of Fiske and SubbaRow (7). The nitrate-sensitive fraction of the ATPase activity was given as the decrease in activity when 50 mM KNO_3 was included in the reaction mixture.

Other Determinations

Vacuoles were counted using a hemocytometer. Total fructose (monomeric and polymeric) was determined by the method of Percheron (12). In this method fructose is released by acid hydrolysis from the polymeric form, which forms the bulk of vacuolar fructose (8).

Hydrolysis of Endogenous Fructan

Vacuoles were resuspended and incubated at 25° C with gentle shaking in a medium containing 0.8 M betaine, 50 mM Mes-Tris (pH 6.0), 1 mM DTT, 10 mM disodium ATP, 10 mM $MgSO_4$, and 16 units/ml hexokinase. At intervals 100 μl aliquots were taken, heated in a boiling water bath for 2 min, the precipitated protein was removed by centrifugation, and the fructose-6-P and fructose were assayed using the Boehringer glucose/fructose UV test kit. Vacuole integrity was estimated by determining the sedimentability of total fructose (predominantly in the form of oligomeric and polymeric fructan), since fructan can be sedimented only when contained within an intact vacuole, and release of fructan from vacuoles would be likely only on lysis. Aliquots of the reaction mixture were centrifuged for 3 s in a Beckman microfuge and the supernatant assayed for total fructose as described above. Less than 5% of the total fructose was converted to fructose-6-P, and the total fructose content did not change significantly during the course of the experiment.

RESULTS

Localization of SST and FFT during Fructan Biosynthesis

The slicing machine proved to be as effective in releasing vacuoles from tubers of Jerusalem artichoke as from roots of red beet. Thus when 300 g of dormant tuber had been sliced the initial pellet typically contained from 10 to 16 × 10^7 vacuoles. This yield compares with a yield of 10 × 10^7 vacuoles reported to be released from 450 g of red beet root (11). However the accelerated flotation method used by Leigh and Branton (11) to purify red beet vacuoles failed to separate the Jerusalem artichoke vacuoles from the cytosolic marker enzymes, although the vacuoles readily separated from marker enzymes of microsome, mitochondria, and plastids. Modifications of the gradient in terms of density and constitution (using silica sols, Nycodenz and Ficoll instead of Metrizamide) all failed to elicit a significant purification. Thus the vacuoles were separated by sedimentation at 1*g*, a technique used by Frehner *et al.* (8) to purify vacuoles from protoplasts of Jerusalem artichoke.

Table I shows that about 10% of the countable vacuoles and of the vacuole marker, total fructose, present in the 2000*g* pellet sedimented at 1*g*. Activities of NADH-Cyt *c* oxidoreductase, NADPH-Cyt *c* oxidoreductase, and glutamate oxaloacetate transaminase remained in the supernatant after 1 h at 1*g*, and thus the vacuoles were virtually free from contamination by, respectively, mitochondria, microsomes and plastids. However significant activities of the cytosolic marker enzymes, alcohol dehydrogenase and glucose-6-P dehydrogenase, also sedimented. In addition to α-mannosidase we have used as vacuolar marker total fructose (predominantly present as fructan), since the vacuole is widely recognised to be the sole site of fructan accumulation (8, 15).

In Table II the total fructose per unit marker enzyme activity is calculated for both the initial sliced tissue filtrate and the 1*g* pellet. It can be seen that the total fructose per unit α-mannosidase activity does not change greatly in the two fractions, whereas for all other markers there is at least a 3-fold purification factor apparent in comparing the 1*g* pellet with initial homogenate.

When SST and FFT activities were assayed during vacuole purification the ratio of their activities to the total fructose and to the α-mannosidase activity remained relatively constant, while the ratio of their activities to the cytosolic markers alcohol dehydrogenase and glucose-6-P dehydrogenase increased 4- to 5-fold (Table III). Thus it is concluded that in Jerusalem artichoke tubers SST and FFT are both vacuolar enzymes, in agreement with Frehner *et al.* (8).

In order to determine whether SST and FFT are located at the tonoplast or within the vacuole sap, the isolated vacuoles were lysed, separated into soluble and membrane fractions, and the distribution of SST and FFT activities compared with that of appropriate markers. An ATPase activity that is stimulated by Cl^- and inhibited by NO_3^- was used as tonoplast marker (16); and total fructose (8) and α-mannosidase activity (3) as markers for the vacuole sap. Table IV shows that of the recovered activities 97% of the nitrate-sensitive ATPase was sedimented, while 63% of the α-mannosidase remained in the supernatant along with 90% of the SST and 100% of the FFT activity. The partial sedimentation of the α-mannosidase indicates that some of the activity may have been retained by the tonoplast. However further washes of the membranes led to unacceptably high losses of enzyme activity.

Nevertheless it is apparent that both SST and FFT activities are almost entirely associated with the vacuole sap.

Localization of FFT and FEH during Fructan Hydrolysis

Table V shows the distribution of total fructan and marker enzyme activities during the purification of vacuoles from dormant tubers. As with the preparations from developing tubers, NADH-Cyt *c* oxidoreductase, NADPH-Cyt *c* oxidoreductase and glutamate oxaloacetate transaminase remained in the supernatant after 1 h at 1*g*, and thus the vacuoles that sedimented were virtually free from contamination by respectively, mitochondria, microsomes and plastids. One of the cytosolic markers, glucose-6-P dehydrogenase, had a much

lower activity in these preparations than in the preparations from developing tubers (Table I), and the activity remained in the supernatant after the initial centrifugation. The other cytosolic marker, alcohol dehydrogenase again showed a significant activity in the 1*g* pellet. However, purification of the vacuoles with respect to this cytosolic marker enzyme is apparent when as previously (Table II), the total fructose is expressed per unit marker enzyme in the initial homogenate and in the 1*g* pellet (Table VI).

Table VII shows that the FFT and FEH activities copurified during vacuole isolation, following both the total fructose content and the mannosidase activity. Both FFT and FEH were purified some 5-fold in relation to the alcohol dehydrogenase activity (Table VII). Thus it is concluded that both FFT and FEH reside in the vacuole in agreement with an earlier proposal (7) and previous evidence (8).

When the isolated vacuoles were separated into fractions containing tonoplast and sap, the FFT activity was found in the sap together with the fructose, while the FEH activity was largely associated with the tonoplast together with the nitrate-sensitive ATPase (Table VIII).

In order to determine whether the isolated vacuoles were capable of exporting fructose released from the hydrolysis of internal fructan, vacuoles were incubated in the presence of MgATP and hexokinase so that exported fructose would be phosphorylated and internal fructose remain unphosphorylated. The results (Fig. 1a) show that vacuoles retain a steady level of fructose as well as exporting fructose to the suspension medium. When, in a control experiment, all the vacuoles are lysed, fructose disappears from the incubation medium, and there is a corresponding increase in the level of fructose-6-P (Fig. 1b). Activity of FEH shows a sharp optimum at about pH 5.2 (5). However at this pH we found that hexokinase would not operate. Consequently, the suspending medium was buffered to pH 6.0. Hydrolysis of MgATP by the tonoplast ATPase may have helped to maintain a lower pH in the vacuole, as it does with other vacuoles (3). When the vacuoles were lysed (Fig. 1b) the rate of fructose-6-P formation was greatly reduced, presumably both because the FEH was now exposed to the less acid suspending medium, and because the fructan was diluted by the suspension medium. Thus we interpret the formation of fructose-6-P shown in Fig. 1a to be the result of fructose export from intact vacuoles, and not the result of the limited vacuole lysis observed.

DISCUSSION

The simplest interpretation of the present findings is that the principal enzymes of fructan biosynthesis and mobilisation are all located in the storage vacuole, in agreement with previous findings (8). This conclusion may, however, need to be qualified when more is known of the ultrastructural changes accompanying fructan accumulation and vacuole development (10). Thus the vacuole isolation procedure used both in the present work and previously (8) is likely to select for the larger central storage vacuoles while smaller, developing vacuoles may escape collection. Moreover the cytosolic 'contamination' of the vacuoles observed both in the present work and previously (8) may arise from a dynamic and structurally complex relationship (3) between vacuolar and extravacuolar compartments that renders these terms oversimplifications. We also note that a vacuolar localization of the fructosyl transferases SST and FFT contrasts with the extravacuolar localization of the glycosyl transferase found in a variety of other biosynthesis pathways (3).

A tonoplast localization of the FEH allows this enzyme to export fructose from the vacuole directly on hydrolysis of the vacuolar fructan by a process of transmembrane ligand conduction. If this were the case then inside-out tonoplast vesicles would accumulate fructose from fructan supplied in the suspending medium. In preliminary experiments we have failed to observe this accumulation, and the maintenance of free fructose within intact vacuoles during fructan hydrolysis (Fig. 1) suggests that the fructose formed by FEH-catalysed hydrolysis is released to the vacuolar sap and then exported via a sugar carrier to the cytosol.

In preliminary experiments we have found that when vacuoles isolated from developing tubers are provided with [^{14}C] sucrose the fructans formed are restricted to chains containing 2 to 9 fructosyl residues. Thus the problem posed some 20 years by Edelman and Jefford (6) regarding the contrasting sucrose requirements of SST and FFT (see introduction) remains with the localization of both enzymes in the vacuolar sap.

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