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CARBOHYDRATE AND ENZYME DISTRIBUTION IN PROTOPLASTS FROM VALENCIA ORANGE JUICE SACS*

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Key Word Index—*Citrus sinensis*; Rutaceae; enzyme compartmentation; metabolite compartmentation; sugar breakdown; protoplasts; vacuole.

Abstract—The subcellular distribution of neutral sugars, organic acids and their metabolic enzymes was investigated in protoplasts from 'Valencia' orange juice sacs. The vacuole was found to contain 70% of the malic acid, 75% of the fructose and glucose, 89% of the citric acid and 100% of the sucrose. Of the enzymes assayed, α-mannosidase, phosphohexoisomerase and phosphoglucomutase showed activity in the vacuole fraction at 100, 5 and 29%, respectively. The activities of acid and neutral invertases, UDPG pyrophosphorylase and both ATP and PPi phosphofructokinase were present only in the cytoplasmic fraction. The remaining enzymes (sucrose synthase, hexokinase, fructokinase and aconitase) showed no activity in any of the samples assayed (protoplast, vacuole and cytoplasm). From the data presented, it appears that vacuolar sugars are the major form of carbohydrate supplying energy to the mature juice sac cells. Possible mechanisms of sugar transport across the tonoplast prior to their oxidation are discussed.

INTRODUCTION

Cellular components must be carefully distributed into separate compartments if normal biochemical functions are to be maintained. Disruption of this compartmentation can cause deterioration of physiological activities and in many instances may result in the death of the cell. In plant cells, the major fluid compartments are the vacuolar sap and the cytosol. Other compartments include the soluble portions of plastids, mitochondria and endoplasmic reticulum.

Studies on the compartmentation of cellular components have been performed on a wide variety of tissues including beet roots [1], sugarcane [2], apple cotyledons [3], *Tulipa* and *Hippeastrum* petals [4], maize endosperm [5] and leaves of *Bryophyllum* [6], barley [7], and maize [8]. Most of the studies have investigated the intracellular distribution of sugars [3,4], organic acids [3,9], amino acids [4], organic and inorganic metabolites [10–12] and some enzymes [1,2,11]. Secondary metabolites such as nicotine [13], diurrhin [14] and pigments [4,12] have also been studied. In fruits, however, compartmentation studies have only been conducted on grape skin tissue [12] and apple flesh [3].

Mature citrus fruits store large amounts of sugars (glucose, fructose and sucrose), organic acids (citric and malic) and vitamin C [15–17], all of which are major parameters of fruit quality [18]. Prolonged periods of storage may cause significant changes in the levels of such metabolites despite the fruit's low respiratory rates [19–21].

Knowledge of the location of metabolites and their enzymes within cellular compartments is crucial for understanding the biochemical events of the cell. The present study was undertaken to determine the localisation of several sugars, organic acids and related metabolic enzymes in protoplasts from orange juice sacs. This information will help in determining the mechanisms involved in the utilisation of these metabolites during prolonged storage periods.

RESULTS AND DISCUSSION

Isolation and purity of vacuoles

A satisfactory number of vacuoles was obtained upon osmotic shock of the protoplasts. It was estimated that over 70% of the protoplasts lysed releasing their vacuoles to the medium. The unlysed protoplasts were largely separated from the vacuoles by centrifugation in a two-step Ficoll gradient. Reducing the osmotic strength of the solution caused a large number of the released vacuoles to attain sizes over 200 μm which made the vacuoles too fragile for further handling.

The presence of BSA in the vacuole breaking media was essential for the maintenance of tonoplast integrity; however, it proved ineffective in reducing adherence of cytoplasmic material to the tonoplast as reported in ref. [14]. Some particulate cytoplasm and/or plasma membrane remained attached to some vacuoles. These could not be removed by further purification steps including centrifugation at 100 000 *g*. This type of contaminated vacuole has been referred to as vacuoplast [22]. Due to the scope of this study and the nature of the compounds and enzymes being investigated, the level of cytoplasmic contamination in the vacuole preparations was assessed in every sample by measuring the presence and activity of alcohol dehydrogenase. The percent contamination varied between 15 and 35% with an average of 23%.

By staining the vacuoles with neutral red prior to

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protoplast lysis and measuring the amount of dye recovered in the vacuole layer after centrifugation, it was calculated that 24–30% of the vacuolar space was recovered. In experiments using α-mannosidase as a vacuolar marker, approximately 26% of the enzyme activity was recovered within the vacuole fraction. From the above results we estimate the vacuolar recovery to be 26%. This recovery is comparable to those obtained from tobacco [13], *Tulipa* [4] and barley leaves [11].

Sugar and acid distribution

The vacuole/extra vacuole content and the distribution of neutral sugars and acids in protoplasts of citrus juice sacs was investigated by comparing the contents of the protoplasts and isolated vacuoles (Table 1). Glucose and fructose were present in equal ratios in all three fractions investigated. A glucose: fructose ratio of 1:1 was present in mature 'Valencia' juice [23, 24]. After accounting for cytoplasmic contamination, it was estimated that approximately 75% of each of the hexoses was present in the vacuole, the remaining 25% being located in the cytosol. This distribution differs from those of apple fruit flesh and tulipa leaf where 100% of both hexoses is found within the vacuole [3, 4]. However, in *Hippeastrum* petals, the percent of glucose and fructose in the vacuoles was found to be 81 and 85%, respectively [4]. It is interesting to note that in tulipa petals 100% of the glucose was found in the vacuole but only 50% of the fructose was within the same compartment [4]. Sucrose, however, appears to be exclusively located in the vacuoles of citrus protoplasts as is the case for sugarcane suspension culture cells [2].

The above results indicate that in protoplasts of 'Valencia' juice sacs approximately 90% of the total sugars is found in the vacuoles, with the remaining 10% being located in the cytosol. Since vacuoles generally constitute 90% of the cell volume [25], the total sugar concentration would be approximately equal in both the cytoplasm and vacuole. Sucrose concentration, however, would be different in both compartments.

The content and distribution of the organic acids investigated are presented in Table 1. Approximately 70% of the malate and 90% of the citrate are vacuolar. Malate has been estimated to be entirely located in the vacuoles of *Bryophyllum* [6] and *Sedum* [9] leaf cells. However, in *Melilotus* [26] and apple flesh protoplasts [3], 8% of the malate was located in the cytosol. The presence of citrate in the cytoplasm has not been previously reported. In apple flesh protoplasts, citrate was completely stored within the vacuole [3]. Our results indicate that 10% of the citric acid is found in the cytosol of protoplasts from orange juice sacs. The presence of citrate in the cytosol may have a significant effect on the cell's metabolism. This will be discussed in a following section.

Table 1. Distribution of neutral sugars and acids between the Vacuole and the cytosol in protoplasts of 'Valencia' orange juice sacs

Compound	Protoplast content (mg, 2.48×10^5 protoplasts)	% in cytosol	% in vacuole
Glucose	62.3 ± 7.8	25.1	74.9
Fructose	63.1 ± 12.6	25.4	74.8
Sucrose	118.5 ± 8.3	0.6	99.4
Total sugars	243.9 ± 9.9	9.9	90.1
Citrate	25.5 ± 1.2	11.1	88.9
Malate	4.1 ± 0.4	30.4	69.6

Enzyme distribution

The distribution of enzymes of carbohydrate metabolism is presented in Table 2. Of the enzymes investigated, only phosphoglucomutase and phosphohexoisomerase were found to be present in the vacuole fraction. The presence of these two enzymes in the vacuolar compartment has been previously reported for tobacco suspension cultured cells, pineapple leaves and tulipa petal vacuoles [27]. Acid and neutral invertase, UDPG pyrophosphorylase and both ATP and PPi dependent PFK were exclusively located in the cytosol. A cytoplasmic location for invertase is not surprising since sucrose was found only in the vacuole. Acid invertase was reported to be 83% vacuolar in preparations from tobacco suspension culture [27]. However, in sucrose storing tissues such as beet roots and sugarcane, vacuolar invertase activity is either inversely proportional to the sucrose content or virtually absent [1, 2]. Cytoplasmic invertases (both acid and neutral) show low levels of activity (Table 2). Acid invertase would be more active under optimum conditions; however, since the pH of the cytoplasm is close to neutrality [28], it is probable that acid invertase would not be participating in the cellular metabolism. The presence of a neutral invertase in the cytoplasm may explain the absence of sucrose in this compartment.

Recoveries for all enzymes were satisfactory (over 80%, Table 2). The percent of enzyme recovered was estimated as the fraction of the protoplast activity present in the combined vacuole and cytoplasm samples. In separate experiments, known amounts of commercial enzymes were added to a protoplast sample and fractionated as described above. The activity of all enzymes was measured and compared to a set of standards. The isolation and purification steps had negligible effect on enzyme activities.

Ripening of citrus fruits is characterized by an accumulation of ethanol in the juice vesicles [29]. Concurrently, aerobic respiration declines during ripening [30] and through storage. These metabolic changes indicate that the NADH oxidase pathway is inadequate to maintain the redox equilibrium and alcoholic fermentation occurs [31]. The anaerobic pathway is dependent on the oxidation of hexoses in the cytoplasm through glycolysis, the pentose phosphate pathway or the conversion of organic acids to pyruvate or PEP. Both pathways for hexose oxidation have been reported to be operative in citrus fruits at different stages of maturity [32]. The presence of citrate in the cytoplasm (Table 1) could be the controlling factor in switching from a glycolytic oxidation of hexoses to the pentose phosphate pathway. Citrate is an inhibitor of ATP-PFK and utilization of hexoses through glycolysis could not be possible in its presence.

Citrus juice sac cells can metabolize sugars; however, phosphorylation of the saccharides must occur prior to reaching the glycolytic or pentose phosphate pathway compartment (cytosol). The absence of hexokinase or fructokinase from the cytosol makes the utilization of hexoses improbable. Furthermore, even in the presence of invertase, the absence of such enzymes prevents sucrose

Table 2. Activities of enzymes of carbohydrate metabolism in desalted orange juice sac protoplasts (values are the mean of a minimum of three experiments)

Enzyme	Activity in protoplasts (nKat/mg protein)	% in cytosol	% in vacuole	% recovery
UDPG pyrophosphorylase	33.6 ± 0.18	100.0	0	117.3
Phosphoglucomutase	1.93 ± 0.09	71.0	29.0	93.2
Phosphohexoisomerase	0.32 ± 0.04	95.0	5.0	81.7
Acid invertase	0.16 ± 0.05	100.0	0	82.8
Neutral invertase	0.06 ± 0.00	100.0	0	93.3
ATP phosphofructokinase	6.60 ± 0.12	100.0	0	90.7
PPi phosphofructokinase	Na	—	—	—
+ 2 μM F-2, 6-p	0.08 ± 0.02	100.0	0	—
Sucrose synthase*	NA	—	—	—
Hexokinase	NA	—	—	—
Fructokinase	NA	—	—	—
Aconitase	NA	—	—	—
α-Mannosidase	0.36 ± 0.01	—	100	—
Alcohol dehydrogenase	0.96 ± 0.01	100	0	91.4

NA; no activity.
*Both activities.

utilization after its hydrolysis. It is possible that the presence of equal amounts of fructose and glucose in the cytosol is the result of sucrose leakage into the cytoplasm and its subsequent hydrolysis by the neutral invertase. Eventually, these hexoses accumulate in the cytosol since they cannot be metabolized. Additional evidence for the inability of mature 'Valencia' orange juice sac cells to metabolize hexoses is presented in Table 3. When desalted, saturated ammonium sulphate protein extracts were supplied with different substrates, production of triose-P was only measured in the presence of hexose-P or UDPG.

If sugars are to reach the cytosol in a phosphorylated state, phosphorylation must occur during transport across the tonoplast. A mechanism for sugar transport from the vacuoles that releases phosphorylated hexoses to the cytoplasm has not been reported. However, Thom and Maretzki [33] have described a multienzyme complex sucrose translocator in the tonoplast of sugarcane where UDPG is converted to sucrose-phosphate during transport to the vacuole. Phosphorylation of sugars during transport from the vacuole could be possible in the presence of a tonoplast bound hexokinase or fructokinase, or a system where sucrose is broken down into UDPG and F-6-P. This mechanism would require the involvement of at least two enzymes (sucrose synthase and fructokinase). Sucrose carbon would reach the cytoplasm in the form of UDPG and F-6-P. The F-6-P can enter the glycolytic or pentose phosphate pathway without further conversion. The UDPG can be converted into UDP and F-6-P in the cytoplasm by the combined action of UDPG pyrophosphorylase, phosphoglucomutase and phosphohexoisomerase all of which are present in the cytoplasm.

Citric acid has been suggested as the other possible energy source in harvested citrus fruits [21]. Our data indicates that, at this stage of maturity, this is not possible due to the absence of aconitase (Table 2) and citrate lyase [15]. Furthermore, it has been demonstrated that the decline in citric acid content in stored citrus fruits is mostly the result of translocation of the acid to the peel tissue where it is metabolized [34]. The translocation of citric acid to the peel may explain its presence in the cytoplasm.

Table 3. Production of triose phosphates by desalted, saturated ammonium sulphate protein extracts from 'Valencia' oranges

Substrate	Concentration (mM)	(nmol Triose-P produced/ min/mg protein)
Glucose	25	NA
Fructose	25	NA
Sucrose	50	NA
Fructose-6-P	2	8.72
Glucose-1-P	2	
− G-1,6-P		NA
+ G-1,6-P	0.1	4.05
UDPG	1.7	
− PPi		NA
+ PPi	0.5	1.13

The reaction mixture contained 100 mM HEPES (pH 7.5), 1 mM DTT, 3 mM $MgSO_4$, 15 mM KCl, 1 mM ATP, 1 mg/ml NADH, 1.1 unit of glycerol-P dehydrogenase and substrates as indicated in the table.
NA; no activity.

In conclusion, the present study suggests that sugars stored in the vacuole are the major carbon supply for mature 'Valencia' oranges. The data also implies the existance of a sugar translocator at the tonoplast where sugars are phosphorylated during transport to the cytosol. This is presently under investigation.

EXPERIMENTAL

Protoplast preparation and vacuole isolation. Protoplasts were prepared from juice sacs of late-season 'Valencia' oranges [*Citrus sinensis* (L.) Osbeck] as previously described [28]. Juice sac tissue

was incubated overnight in a 1% cellulysin containing medium. The protoplasts were filtered through a 200 μm nitex mesh and separated from the incubation medium in a discontinuous Percoll gradient at 1 g. Half of the protoplast sample was set aside as the protoplast fraction and the remaining was used for vacuole isolation. To the latter, two vols of a soln containing 20 mM mannitol, 200 mM Hepes (pH 8.0), 10 mM spermidine, 5 mg/ml BSA and 5 mM $CaCl_2$ were added. The soln containing the protoplasts was gently agitated for 60–90 sec and the vol. increased to 2 ml by the addition of 0.8 ml of 2 M sorbitol. Such treatment induced the release of vacuoles from over 70% of the protoplasts.

Mature vacuoles (*ca* 90 μm average diameter) were collected after centrifugation in a two step Ficoll gradient of 20 and 7% at 200 g for 10 min. The Ficoll solns contained 0.5 M mannitol, 10 mM Hepes (pH 7.0), 5 mM spermadine, 1 Mg/ml BSA, 5 mM $CaCl_2$ and Ficoll. Vacuoles were collected from the 20–7% interphase. Most unruptured cells remained on top of the 7% Ficoll layer; however, a small number were seen in the vacuole layer. The supernatant was recovered and considered to be the cytoplasmic fraction.

Sugars and acid analysis. The three samples of protoplast, vacuole and supernatant were vigorously agitated in a Vortex mixer and immediately centrifuged (13 000 *g*) for 5 min. The supernatant fraction was collected and frozen. Reducing sugars were measured by the method of ref. [35] and glucose was analysed by the glucose oxidase procedure (Sigma product G-6500 bulletin). Fructose was calculated as the difference between reducing sugars and glucose. Samples for sucrose determination were incubated for 2 hr with and without invertase (1 mg/ml Sigma grade X) prior to glucose analysis. Citrate and malate were measured using the enzymatic procedures described in ref. [36].

Enzyme assays. Samples of the supernatant (cytosol) and the solns containing the protoplasts and vacuoles were diluted with three vols of a soln containing 250 mM Hepes (pH 8.0), 1 mM $MgCl_2$, 1 mM EDTA and 10 mM KCl. For enzyme preparations, the resulting soln were sonicated and prepared as described in ref. [37].

Hexokinase (E.C. 2.7.1.1), phosphoglucomutase (E.C. 2.7.5.1), phosphohexoisomerase (E.C. 5.3.1.9) and aconitase (E.C. 4.2.1.3) were assayed as described by ref. [36]. The method of ref. [38] was used to assay for ATP (E.C. 2.7.1.11) and PP$_i$ (E.C. 2.7.1.90) dependent phosphofructokinases (PFK). For the breakdown activity of sucrose synthase (E.C. 2.4.1.13), the method of ref. [39] was employed except that non-radioactive sucrose was used and the UDPG formation was monitored spectrophotometrically [36]. The synthetic activity was estimated following the method of ref. [40].

Invertase (E.C. 3.2.1.26) was assayed in a reaction mixture containing 100 mM buffer (sodium acetate, pH 4.5 or Hepes, pH 7.0), 50 mM sucrose and enzyme. The glucose produced was analysed as previously described. The method of refs [27, 37] were used to measure α-mannosidase (E.C. 3.2.1.24) and UDPG pyrophosphorylase (E.C. 2.7.7.9) respectively.

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