Vacuolar Acid Hydrolysis as a Physiological Mechanism for Sucrose Breakdown

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ABSTRACT
Sucrose breakdown in mature acidic 'Persian' limes (Citrus aurantifolia [Christm.] Swing.) occurred at a rate of 30.6 pico-moles per milliliter per day during 9 weeks storage at 15°C. Neither enzyme of sucrose catabolism (sucrose synthase or acid/alkaline invertase) was present in extracts of mature storage tissue. The average vacuolar pH, estimated by direct measurement of sap from isolated vacuoles and by the methylamine method, was about 2.0 to 2.2. In vitro acid hydrolysis of sucrose at physiological concentrations in a buffered solution (pH 2.2) occurred at identical rates as in matured limes. The results indicate that sucrose breakdown in stored mature acidic limes occurs by acid hydrolysis.

Sucrose degradation in plant cells occurs by means of two distinct enzymic mechanisms, namely invertase (acid and alkaline or neutral) and sucrose synthase (22). Acid invertase is distinguished by its ability to hydrolyze sucrose optimally in the pH range of 4.0 to 5.3 (12) and considered to be of vacuolar origin (1). Alkaline invertase expresses maximum activity near neutral pH and is present in the cytosol (1). Both enzymes hydrolyze sucrose to form two hexose molecules. Sucrose synthase, which is also localized in the cytoplasm, produces a fructose and a nucleotide diphosphate glucose molecule. The products of both enzymic mechanisms can be used as substrates for the glycolytic pathway (4, 9, 22) as well as for other metabolic processes.

Mature citrus fruits contain large quantities of sugars (sucrose, glucose, and fructose) and organic acids (citric and malic) located mostly in the large central vacuole (2, 6). Generally, the sugar content of the fruit increases during maturation, and, in many instances, continues to increase for a time after harvest (5, 7, 14). Concurrent with the increase in sugar concentration, a decline in the acid content is observed (8, 20). In the more acidic cultivars, such as lemons, sucrose content declines after reaching maximum levels early in development, while the concentration of organic acids increases (19). The decline in sugars and the simultaneous increase in organic acids persists after harvest (19). The continuous increase in the levels of organic acids (mainly citric acid) during ontogeny results in a highly acidic fruit.

The mechanisms involved in the breakdown of sucrose in the highly acidic citrus cultivars (lemons and limes) have not been thoroughly investigated. In other citrus cultivars ('Satsuma' mandarins for example), both acid and neutral invertases are present in tissue extracts from immature juice sacs. At maturity, nonetheless, only small levels of alkaline invertase remained (10). In 'Valencia' orange protoplasts, low levels of cytoplasmic invertase activity were detected at later stages of maturity (6). In neither case was sucrose synthase activity found, a condition similar to the mature grapefruit (K. Koch, personal communication).

Considering the low levels of sucrose-degrading enzymes present in mature citrus fruits (6, 10), their cytoplasmic compartmentation (1, 6), pH optima (10), and the highly acidic cellular milieu, enzymic hydrolysis of sucrose would be an unlikely event. This investigation was aimed at elucidating the physiological factors involved in sucrose breakdown in mature limes. Our results show that in the absence of sucrose-degrading enzymes and at a vacuolar pH of approximately 2.1, sucrose breakdown in mature limes occurs via acid hydrolysis. This is the first report of acid hydrolysis as a physiological mechanism for sucrose breakdown.

MATERIALS AND METHODS

Plant Material

For protoplast preparation, 'Persian' limes (Citrus aurantifolia [Christm.] Swing.) and 'Palestine' sweet limes (Citrus limettiodes Tanka) were obtained from the Citrus Arboretum at the Division of Plant Industry (Florida Department of Agriculture), Winter Haven, FL. 'Valencia' oranges (Citrus sinensis [L.] Osbeck) were harvested from the orchards of the Citrus Research and Education Center, Lake Alfred, FL.

For sugar and acid determination, 'Persian' limes were obtained as a gift from Thomas Davenport at the Tropical Research and Education Center, Homestead, FL. The fruit were washed and treated with thiabendazole (1000 ppm) to minimize decay during storage at 15°C and 95% RH. Sampling was performed at the time of harvest and at 3, 6, and 9 weeks of storage. Unless otherwise indicated, all activities are described on per mL of expressed juice basis.

Protoplast Preparation

Protoplasts from juice sacs were prepared as previously described by Echeverria (3) with the following modifications. For 'Valencia' orange and 'Palestine' lime, 4 g of juice sac tissue were incubated overnight (25°C) in a solution containing 900 mM mannitol, 200 mM Mes Buffer (pH 5.6), 100 mM CaCl₂, 0.1% PVP, 5 mM spermidine, 1% Cellulysin (Calbiochem), and 30 units of pectinase (Sigma P-5146).

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Omitting vacuum infiltration of the incubation media resulted in a significant increase in protoplast yield. At the end of the incubation period, the juice vesicle segments were transferred to 10 mL of a cold solution similar to the above described (with the exception of the hydrolytic enzymes), and the protoplasts were extracted from the tissue as previously described (3).

For the highly acidic 'Persian' lime, the incubation media contained 500 mM mannitol, 800 mM Mes (pH 5.6), 100 mM CaCl₂, 0.1% PVP, 5 mM spermidine, 1% Cellulysin, and 30 units of pectinase. Protoplasts were extracted in a similar solution without the hydrolytic enzymes.

Vacuole Isolation

Vacuoles were obtained by the application of shearing force during centrifugation of the protoplasts in unbuffered Ficoll gradients at 100,000g for 90 min. For 'Valencia orange' and 'Persian' lime, protoplasts were layered on a discontinuous Ficoll gradient of 15, 10, and 5% Ficoll. The Ficoll (Sigma F-9378) solutions contained 1 M mannitol, 100 mM CaCl₂, 0.1% PVP, 0.1% BSA, and 1mM DTT. Vacuoles from both tissues were recovered at the 10 to 5% Ficoll interface.

‘Palestine’ lime vacuoles were obtained in similar manner except that the discontinuous density gradient consisted of three layers of 5, 3, and 1% Ficoll solutions. The Ficoll solutions were similar to those described above. Vacuoles were recovered at the 3 to 1% Ficoll interface.

Direct pH Measurement of Vacuolar Extract

Vacuoles (1 mL) were collected from their respective interfaces and transferred to a graduated test tube. To the vacuole-containing sample, 9 mL of 95% ethanol were added. The resulting solution was shaken vigorously, placed in ice for 20 min, and centrifuged at 7500g for 10 min. The pellet containing the precipitated Ficoll was discarded. The supernatant was transferred to a 30 mL beaker and placed on a warm plate to evaporate the ethanol. After the ethanol evaporated, water was added to a final volume of 500 µL and the pH measured using a finely calibrated pH meter (Fisher Accumet 915).

Measurement of pH by the "Methylamine Method"

Determination of vacuolar pH by the use of [14C]methylamine was performed as described by Strack et al. (21). The vacuoles (prepared as earlier described) were incubated at 30°C for 1 h in the presence of [14C]methylamine (0.03 mM; 0.26 µCi mL⁻¹) and H₂O (8 µCi mL⁻¹). A second set of vacuoles was incubated with [14C]inulin (2.1 µCi mL⁻¹) to estimate adherent medium. After 1 h, the vacuole sample was diluted with an equal volume of a corresponding solution (without substrates) and centrifuged for 15 min at 7500g in a discontinuous gradient of 10 and 5% Ficoll. After centrifugation, the vacuoles were collected from the 5 to 10% interface and radioactivity (14C and 3H₂O) was measured in a scintillation counter (Beckman LS 1800). Correction for the adherent medium was based on the determination of pelleted [14C] inulin. Vacuolar pH (pHv) was calculated by determining the concentration of methylamine distributed between intra (Ci) and extravacuolar (Cv) spaces according to Rottenberg et al. (18):

\[
\frac{Cv}{Ci} = 1 + 10^{pK_{\text{pH}}}/1 + 10^{pK_{\text{pH}}},
\]

Enzyme Preparation

For enzyme preparation, 100 mL of 'Persian' lime was expressed directly into 100 mL of 1 M Hepes buffer (pH 7.0) containing 0.5 mM cysteine and 0.1% PVP-40. The pH was continually adjusted by adding NaOH. The resulting solution was filtered through two layers of cheesecloth and centrifuged at 10,000g for 15 min. To the supernatant, ammonium sulfate was added to saturation. After overnight incubation at 4°C, the saturated solution was centrifuged for 20 min at 25,000g and resuspended in a 2.5 mL of 10 mM Hepes buffer (pH 7.0). The resuspended solution was desalted through a Sephadex G-25M prepacked column (Pharmacia) and used as enzyme source immediately after.

Enzyme Assays

Invertase (EC 3.2.1.26) was assayed in a reaction mixture containing 100 mM buffer (sodium acetate [pH 2.0 to 5.0]; Mes [pH 6.0]; potassium phosphate [pH 7.0]) and 100 mM sucrose. The glucose produced was analyzed by the glucose oxidase method (11). Sucrose synthase (EC 2.4.1.13) was assayed in the synthesis direction in a reaction mixture containing 50 mM Hepes buffer (pH 8.5), 1 mM uridine-5'-diphosphoglucose, 25 mM fructose and 2 mM EDTA. The sucrose produced was analyzed as described below.

For recovery experiments, known amounts of commercial enzyme were added to buffered lime extract and treated as described above. To test for possible pH enzymic inactivation, commercial enzyme was added directly to expressed juice and allowed to stand for 10 s. Immediately afterward, the extract was buffered and treated as described earlier. After treatment, the activity of the enzymes were compared to similar untreated standards. Invertase (grade VII) and sucrose synthase were both purchased from Sigma Chemical Co. (St. Louis, MO).

Sucrose Determination

Samples for sucrose determination were incubated for 2 h with and without invertase (1 mg/mL Sigma grade X) prior to glucose analysis. Glucose was analyzed by the glucose oxidase method (11).

RESULTS

In Vivo Sucrose Utilization

The concentration of sucrose in stored 'Persian' limes decreased from 1.75 mg/mL at the time of harvest to 1.01 mg/mL after 9 weeks in storage (Fig. 1). The rate of sucrose utilization was estimated to be 30.6 pmol/mL/d. The concentration of reducing sugars, however, remained unchanged as did citric and malic acids (data not shown). In contrast, lemons continue to accumulate citric acid as the levels of sugars decline (19).
contamination (<5%), eliminating the effect of its buffering capacity.

**Enzyme Activities**

Both enzymes of sucrose catabolism (sucrose synthase and invertase [pH range 2.0–8.0]) were tested in desalted extracts from storage tissue of mature 'Persian' limes. There was no detectable activity for either enzyme although recovery for commercial enzyme was over 80% (invertase 93% and sucrose synthase 86%, average of two experiments).

**Vacuolar pH**

For *in vivo* vacuolar pH measurement, 'Valencia' orange and 'Palestine' lime were also included for they possess distinctive characteristics. 'Palestine' lime is a high sugar-low acid cultivar, the crude extracts of which have a pH of about 5.5. 'Valencia' oranges is a highly popular citrus cultivar with a tissue extract of an intermediate pH value of approximately 3.6 (Table II). 'Valencia' oranges accumulate high levels of sugars and organic acids as well. 'Persian' lime vacuolar pH was determined to be 2.02 by the methylamine method (Table II). The accuracy of this procedure has been previously verified when compared to direct pH microelectrode measurements (21). Assessment of vacuolar pH using extracts of isolated vacuoles (as indicated in "Materials and Methods") gave similar results. The value obtained by the latter procedure of 2.26 (Table II) is within the statistical error range of the methylamine method estimate. It is possible that the slight difference in pH is due to dilution of the vacuolar contents during extract preparation. The method here described for direct pH measurement of the vacuolar contents offers the advantage of retaining possible leaking solutes from the vacuoles during isolation. The solutes would remain in the Ficoll gradient avoiding the loss of critical metabolites.

The pH of vacuoles isolated from 'Valencia' oranges and 'Palestine' limes were estimated to be 2.83 and 4.84, respectively (Table II). The citric acid content of 'Valencia' oranges (8.5 mg/mL) is intermediate between that of 'Persian' and 'Palestine' limes which contain 60 mg/mL and 1 mg/mL citric acid, respectively (2, 6).

**In Vitro Acid Hydrolysis of Sucrose**

Hydrolysis of sucrose in a solution containing 1.75 mg/mL of sucrose, 60 mg/mL citric acid and 6 mg/mL malic acid adjusted to pH 2.2, occurred at a rate of 32.1 pmol/mL/d (Fig. I). The concentration of sucrose and organic acids were those present in mature 'Persian' limes at the time of harvest. Figure 1 shows that the rates of acid hydrolysis *in vitro* are virtually identical to the rates of sucrose breakdown measured in the live fruit.

**Vacuole Purity and Isolation**

Attempts to isolate vacuoles from protoplasts by the use of DEAE dextran were unsuccessful, and the use of osmotic shock was avoided since it would have introduced inaccuracies in the measurement of vacuolar volumes and the corresponding pH determinations (13). The use of shearing force proved to be adequate by producing a satisfactory number of highly purified vacuoles (Table I). The purity of the vacuoles was determined by measuring the contaminating levels of [14C]inulin present in the vacuole preparations as compared to the total activity in the protoplast sample. Table I shows the average vacuole yield for each cultivar and the degree of cytoplasmic contamination present in the respective vacuoles. The vacuoles contain very low levels of cytoplasmic contamination (<5%), eliminating the effect of its buffering capacity.

**Table I. Vacuole Yield and Percentage of Cytoplasmic Contamination in Vacoles Obtained from Protoplasts of Fresh Storage Tissue of Three Citrus Cultivars**

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Vacuole Yield</th>
<th>Cytoplasmic Contamination %</th>
</tr>
</thead>
<tbody>
<tr>
<td>'Persian' lime</td>
<td>3.4 ± 0.8 × 10⁶</td>
<td>1.7</td>
</tr>
<tr>
<td>'Palestine' lime</td>
<td>1.8 ± 0.1 × 10⁵</td>
<td>1.4</td>
</tr>
<tr>
<td>'Valencia' orange</td>
<td>3.2 ± 1.4 × 10⁶</td>
<td>2.3</td>
</tr>
</tbody>
</table>

**Table II. pH of Vacoles from Storage Tissue of Three Citrus Cultivars**

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Direct measurement</th>
<th>Methylamine method</th>
<th>Extracta</th>
</tr>
</thead>
<tbody>
<tr>
<td>'Persian' lime</td>
<td>2.26 ± 0.11</td>
<td>2.02 ± 0.13</td>
<td>2.26 ± 0.21</td>
</tr>
<tr>
<td>'Palestine' lime</td>
<td>4.84 ± 0.10</td>
<td>NDb</td>
<td>5.46 ± 0.35</td>
</tr>
<tr>
<td>'Valencia' orange</td>
<td>2.83 ± 0.28</td>
<td>NDb</td>
<td>3.58 ± 0.43</td>
</tr>
<tr>
<td>Ficoll solution</td>
<td></td>
<td></td>
<td>5.43</td>
</tr>
</tbody>
</table>

a Extract from storage tissue.   b Not determined.
DISCUSSION

There are two known enzymic pathways for the breakdown of sucrose in higher plants (1, 9). One comprises the classical model involving invertase, whereas the other involves the action of sucrose synthase (1, 4, 9). During development of citrus fruits, sucrose synthase as well as both acid and neutral invertase are present in the storage tissue (10; K Koch, personal communication). As the fruit matures, the activities of both enzymes decline with only low levels of invertase remaining at maturity (6, 10). In the highly acidic ‘Persian’ lime, however, neither enzyme of sucrose catabolism is present at maturity, although sucrose is being degraded at a rate of 30.6 pmol/ml/d (Fig. 1). Sucrose breakdown during development and maturation also occurs in other highly acid citrus cultivars such as lemons. In lemons, the decline in sucrose is paralleled by an increase in the concentration of organic acids and a concurrent decline in pH (19). In limes, nonetheless, the pH of expressed juice remained unchanged during the 9 week storage period and, furthermore, neither organic acids nor reducing sugars accumulated suggesting the metabolic utilization of the sucrose breakdown products.

No respiratory data are available for limes; however, lemons (a closely related species) respire at a rate of approximately 2.4 mg CO₂/kg fresh weight/h (23). These rates of respiration would be sufficient to account for the respiratory utilization of the products of sucrose breakdown observed in limes. This indicates that the products of sucrose breakdown in limes are likely being respired.

As a result of the high levels of organic acids, extracts of lemon and lime storage tissue have a pH of 2.1 to 2.3. These crude pH estimates are from the products of the entire cell sap and not necessarily those of the vacuole where most of the organic acids and sucrose are stored (2, 6). Our studies indicate that the vacuolar pH of ‘Persian’ lime storage tissue is of 2.02 to 2.26 (Table II). The measurements were confirmed by the use of two separate techniques, including the methylamine method. At this pH, acid hydrolysis of sucrose occurs at identical rates to those observed in vivo (Fig. 1). It is apparent that in the absence of sucrose-degrading enzymes, breakdown of sucrose in ‘Persian’ limes occurs via acid hydrolysis. This is the first report of acid hydrolysis being used by a plant tissue as a physiological mechanism for the breakdown of sucrose.

How the rate of sucrose degradation could be controlled is a matter of speculation; however, by altering the organic acid concentration in the vacuole (and consequently the pH) the rates of acid hydrolysis could be metabolically manipulated. All the necessary enzymic machinery for the oxidation of hexoses and the production and utilization of organic acids have been reported to be present in the close time relative, Citrus acida (15–17). In our extracts, similar necessary enzymic machinery (such as hexokinase and phosphohexoisomerase) were recovered and measured and were not inactivated by the highly acidic pH during extraction. Further studies are necessary to resolve remaining questions concerning the transport of hexoses from the vacuole into the cytoplasm and its regulatory properties.