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INVERTASES IN YOUNG AND MATURE LEAVES OF *CITRUS SINENSIS*

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Key Word Index—*Citrus sinensis*; Rutaceae; invertase; source–sink relationships.

Abstract—The sucrose catabolic enzymes acid invertase (EC 3.2.1.26) and alkaline invertase (EC 3.2.1.27) were studied in young and mature *Citrus sinensis* leaf tissue. In young, expanding leaves (60% final length) soluble acid invertase activity predominated, while soluble alkaline invertase activity predominated in mature leaves. The acid and alkaline invertase activities were separated on Sephadex G-200. The acid invertase had an M_r of approximately 60 000, pH maximum of 4.5 and apparent K_m of 3.3 mM sucrose. The alkaline invertase had an M_r of approximately 200 000, pH maxima of 6.8 and an apparent K_m of 20 mM sucrose. Alkaline invertase was strongly inhibited by 10 mM Tris while acid invertase was not. Possible physiological roles for the two invertases are discussed.

INTRODUCTION

Sucrose is the transportable form of photosynthate in most higher plants and its catabolism can occur either via invertase, which catalyses the hydrolytic reaction:

$$\text{sucrose} \rightarrow \text{glucose} + \text{fructose},$$

or via sucrose synthetase, which can catalyse the reversible reaction

$$\text{sucrose} + \text{UDP} \leftrightarrow \text{UDP-glucose} + \text{fructose}\ [1].$$

Invertases with acidic and neutral, or alkaline, pH maxima have been reported in many plant tissues [1].

A transition in the form of invertase has been reported to occur during the development of certain storage organs. Hatch and Glasziou [2] reported that acid invertase is present in immature sugar cane storage tissue and is replaced by an alkaline invertase in the mature storage tissue. Kato and Kaboto [3] showed that both acid and alkaline invertases were present in immature *Citrus* juice sacs while only the alkaline form was present in the mature sacs that store sucrose. Ricardo and ap Rees [4] showed that in developing carrot roots there was a decrease in acid invertase and an increase in the alkaline form. However, such a transition has not been reported in leaves.

The objective of this work was to characterize the sucrose catabolizing enzymes in *Citrus* leaves in order to shed light on their role in photosynthate partitioning. We report a change in invertase form from the young to the mature *Citrus* leaf, an organ not generally considered as adapted for storage.

RESULTS AND DISCUSSION

When invertase activity over a broad pH range was assayed in crude extracts of immature and mature *Citrus* leaves it was observed (Fig. 1) that the immature leaves displayed acid invertase activity with no noticeable second peak in the alkaline range. The mature leaves showed peak activity only in the neutral range. Coincidentally, the activity at pH 7 of both tissues was similar, despite the difference in enzyme activity at the respective pH optima.

Sephadex G-200 separation of the invertases of mature and immature leaf tissue showed that in young developing leaves there is one broad peak of activity at pH 5 and an indication of an additional larger molecular weight fraction with activity at pH 7 (Fig. 2). When mature tissue was similarly examined the larger molecular weight fraction with higher activity at pH 7 than at pH 5 predominated, while the smaller molecular weight fraction with higher activity at pH 5 was present in a smaller amount. The pH activity of the two partially purified enzymes is shown in Fig. 2 (inset). The lower molecular weight fraction showed peak activity from pH 4–4.5 while the higher molecular weight fraction showed peak activity from 6.8–7.2. The apparent M_r of the two enzymes was approximately 200 000 and 60 000, similar to values reported in *Citrus* fruit [3].

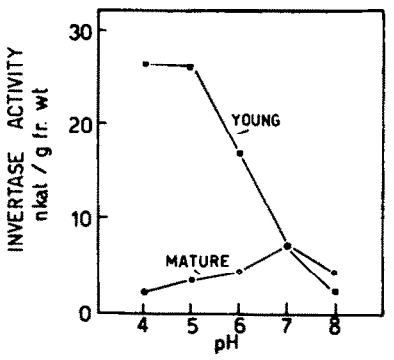

Fig. 1. The effect of pH on invertase activity in extracts of young (1 month old) and mature (4 months old) *Citrus* leaves. Reaction mixtures were standard as in Experimental. Each point is the average of two samples.

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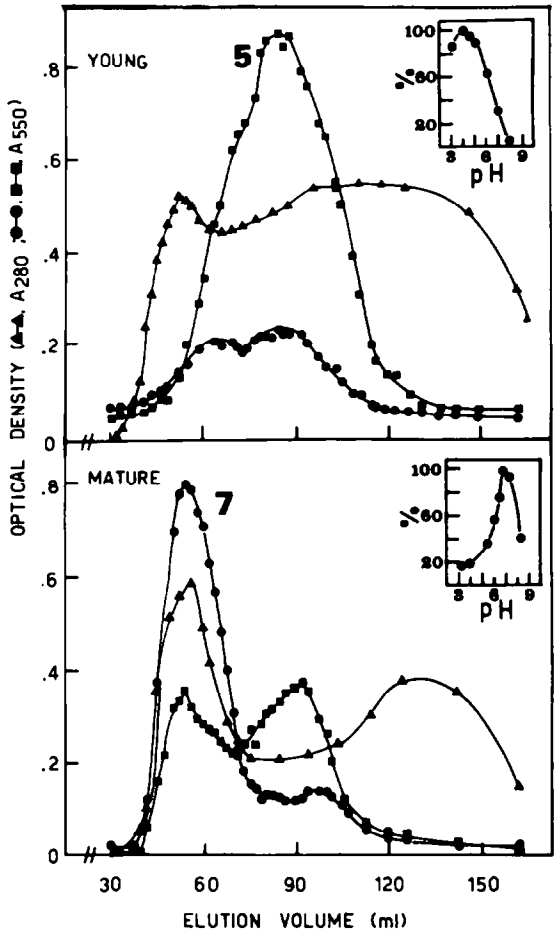

Fig. 2. Sephadex G-200 elution patterns of invertases from young (1 month old) and mature (4 months old) *Citrus* leaves. Two ml fractions were collected and assayed according to Experimental. (■) Activity at pH 5; (●) activity at pH 7. Activity is expressed as optical density at 550 nm after assay with dinitrosalycilic acid. (▲) Absorbance at 280 nm. Absorbance at 280 nm of the mature leaves is after a fifteen-fold dilution. Fractions from 40–70 ml elution volume for mature leaves and fractions from 75–105 ml elution volume for young leaves were pooled for further characterization. Inset: The effect of pH on the activities of partially purified invertase enzymes from *Citrus* leaves. (●) Enzyme from pooled 75–105 ml elution volume from young leaves. (●) Enzyme from pooled 40–70 ml elution volume from mature leaves. Maximum activity of partially purified enzymes from young and mature leaves was 11.7 and 0.3 nkat/mg protein, respectively.

The acid invertase fraction from immature leaves showed activity with raffinose (gal–glc–fru, 45% of activity with sucrose) but not with melezitose (glc–fru–glc), indicating that it is a β-fructofuranosidase. The alkaline invertase activity from mature leaves showed activity with both raffinose and melezitose, indicating a mixture of enzyme activities. pH activity with melezitose showed peak activity at pH 5 suggesting that the melezitose hydrolysing activity was not due to the alkaline invertase but rather to contamination, perhaps by an α-glucosidase which has peak activity in the acid range [5].

K_m values for sucrose, calculated from Lineweaver–Burk plots of the two enzyme fractions, were 3.3 mM at pH 5 and 20.0 mM at pH 7 for the acid and alkaline enzymes respectively. In *Citrus* fruit juice sacs K_m values of 7.3 and 35.7 mM sucrose were reported for the two enzymes [3]. In general, the K_m value of the alkaline enzyme is higher than that of the acid enzyme [1, 4, 6, 7]. The alkaline invertase was completely inhibited by 10 mM Tris at pH 7 while the acid invertase showed only a 6% inhibition at pH 5, a distinction previously reported [3].

The physiological significance of the transition of invertase forms during development has been proposed [1, 8–10]. Acid invertase, presumably compartmentalized in the vacuole, primarily functions in tissues undergoing rapid growth and development, where hexoses are rapidly utilized and sucrose rapidly hydrolysed. Therefore, the acidic pH optima and low K_m for sucrose that we report make the enzyme efficient for the task. In mature storage tissues, the presence of the vacuolar, highly efficient acid invertase is unnecessary and even undesirable. The less efficient, cytoplasmic alkaline invertase, or sucrose synthetase [11], can satisfy the requirements of the storage cell for hexoses.

The mature *Citrus* leaf is not generally considered a storage tissue for carbohydrates, but in fact it functions as such. The starch content in leaves on a dry wt basis is high and may approach that found in roots [12]. During periods of high sink demand (i.e. excessive fruit set) the stored non-structural carbohydrate content of both leaves and roots decreases dramatically [12]. During the diurnal cycle [unpublished results] the non-structural carbohydrate content of the leaves remains high throughout the period and fluctuates by only 20%, in contrast to the situation reported in annuals [13].

The mature *Citrus* leaf, although perhaps a storage organ, presumably does not function as a sucrose importer. The presence of an invertase is, therefore, surprising. However, the alkaline form may function efficiently in source leaves where sucrose export is inhibited and excess sucrose in the cytoplasm must be hydrolysed and utilized, or in source leaves that have been manipulated and converted into physiological sinks that import sucrose [14].

EXPERIMENTAL

Plant material. Leaves were harvested from field grown Shamouti orange trees [*Citrus sinensis* (L.) Osbeck cv Shamouti] during March and June. In March young, developing leaves (60% final length), *ca* 1 month old, were sampled. In June, new flush leaves, also *ca* 1 month old, and mature new leaves *ca* 4 months old, were sampled. Leaves were quick frozen and ground in liquid N_2, lyophilized and stored at $-20°$.

Enzyme preparation. Leaf tissue (8 g fr. wt for 1 month old leaves and 40 g fr. wt for 4 month old leaves) was homogenized in 100 ml of 25 mM HEPES–NaOH (pH 7.2) containing 0.5 mM EDTA, 5 mM $MgCl_2$, 3 mM DTT, 2 mM diethyldithiocarbamate and 2% Polyclar AT in a Turrex homogenizer. The extract was centrifuged at 20000 *g* for 15 min and the supernatant was brought to 80% satn with $(NH_4)_2SO_4$. The ppt was collected by centrifugation at 20000 *g* for 15 min and was resuspended in a small vol. of 50 mM KH_2PO_4–NaOH buffer (pH 7.5) and dialysed overnight against 15 mM KH_2PO_4–NaOH (pH 7.5). After dialysis the dialysate vol. was reduced to *ca* 3 ml with Aquacide II (Calbiochem). The resultant

dialysate was used for gel permeation chromatographic seperation. All operations were carried out at 5°.

For assay of enzyme activity in crude extracts 2 g fr. wt leaf tissue was extracted in 10 ml of the same extraction medium as above but including 1% BSA. After centrifugation the supernatant was dialysed against the same buffer but without BSA and Polyclar AT. The resulting dialysate was used as the crude enzyme extract. Invertase activity associated with the insoluble fraction, as measured by the method of Silvius and Snyder [15], was insignificant in extracts of both young and mature leaves.

Gel permeation chromatography. A Sephadex G-200 column (2.4 × 60 cm), preequilibrated with 5 mM KH_2PO_4–NaOH buffer (pH 7.2) with a flow rate of 1 ml/cm²/hr was used. Two ml fractions were collected, assayed for protein at 280 nm and for invertase activity at pH 5 and 7. Fractions showing activity were bulked for further study. The column was calibrated using M_r markers blue dextran (void vol.), cytochrome *c* (13 000), bovine serum albumin (55 000), ovalbumin (63 000) and catalase (225 000).

Enzyme assay. Invertase activity was assayed at various pHs (modified with 0.1 M phosphate–citrate buffer) and sucrose concn (final concns 1, 2, 5, 10, 15, 20, 50, 100 and 200 mM). Standard assay conditions were pH 5 and 7 and 100 mM sucrose. For comparison of activity with raffinose and melezitose 50 mM sugars were used. Reaction mixtures, in a total vol. of 1 ml, consisted of 0.6 ml buffer, 0.2 ml sucrose or other sugar, and 0.2 ml enzyme, according to a modification of Silvius and Snyder [15]. Reactions were incubated 30 min at 37° and reducing sugars formed during hydrolysis were measured with dinitrosalycilic acid at 550 nm according to ref. [16]. Enzyme blanks contained boiled enzyme or reaction mixtures incubated with dinitrosalycilic acid. Enzyme activities were linear over a period of at least 2 hr and linear with enzyme aliquot. Protein was measured according to the method of Bradford [17].

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REFERENCES

1. Avigad, G. (1982) in *Encyclopaedia of Plant Physiology* (Pirson, A. and Zimmerman, M. H., eds) New Series, Vol. 13a, pp. 217–347. Springer, Berlin.
2. Hatch, M. D. and Glasziou, K. T. (1963) *Plant Physiol.* **38**, 344.
3. Kato, T. and Kubota, S. (1978) *Physiol. Plant.* **42**, 67.
4. Cooper, R. A. and Greenshields, R. N. (1964) *Biochem. J.* **92**, 357.
5. Hutson, D. H. and Manners, D. J. (1965) *Biochem. J.* **94**, 783.
6. Hatch, M. D., Sacher, J. A. and Glasziou, K. T. (1963) *Plant Physiol.* **38**, 338.
7. Lyne, R. L. and ap Rees, T. (1971) *Phytochemistry* **10**, 2593.
8. Ricardo, C. P. P. and ap Rees, T. (1970) *Phytochemistry* **9**, 239.
9. Copping, L. G. and Street, H. E. (1972) *Physiol. Plant.* **26**, 350.
10. ap Rees, T. (1974) in *International Review of Biochemistry* (Northcote, D. H., ed.) Vol. 11, pp. 89–127. Butterworth, London.
11. Giaquinta, R. T. (1979) *Plant Physiol.* **63**, 828.
12. Goldschmidt, E. E. and Golomb, A. (1982) *J. Am. Soc. Hort. Sci.* **107**, 206.
13. Caspar, T., Huber, S. C. and Somerville, C. (1985) *Plant Physiol.* **79**, 11.
14. Fisher, D. G. and Eschrich, W. (1985) *Can. J. Botany* **63**, 1700.
15. Silvius, J. E. and Snyder, F. W. (1979) *Physiol. Plant.* **46**, 169.
16. Miller, G. L. (1959) *Analyt. Chem.* **31**, 426.
17. Bradford, M. M. (1976) *Analyt. Biochem.* **72**, 248.