Phytochemistry, 1970, Vol. 9, pp. 139 to 144. Pergamon Press. Printed in England.

L-PHENYLALANINE AMMONIA-LYASE ACTIVITY AND NARINGENIN GLYCOSIDE ACCUMULATION IN DEVELOPING GRAPEFRUIT

V. P. MAIER and S. HASEGAWA

Fruit and Vegetable Chemistry Laboratory,* Pasadena, California 91106, U.S.A.

(*Received* 19 *June* 1969)

Abstract—A direct relationship was observed between L-phenylalanine ammonia-lyase (PAL) activity and the rate of naringenin glycoside accumulation in developing grapefruit (*Citrus paradisi* Macf.). The fruit tissues had potential PAL activity sufficient to account for the accumulated naringenin glycosides and still allow for turnover and synthesis of them and other cinnamate derived phenols. These results suggest that PAL is an element of an enzyme-regulator system that controls the synthesis of naringenin glycosides (and other cinnamate derived phenols) in grapefruit. Chalcone-flavanone isomerase activity was also present but L-tyrosine ammonia-lyase activity was not detected.

INTRODUCTION

CITRUS fruits are unusual in that they accumulate large amounts of flavanone glycosides. A number of workers have shown that flavanone glycoside content per fruit increases very rapidly during early growth.[1–7] As the fruit grows further and eventually matures, accumulation slows appreciably. Because flavanones are early products in flavonoid biosynthesis,[8] citrus fruit with their high ratio of flavanones to total flavonoids are a very useful and convenient plant system for studies of the enzyme chemistry and regulatory mechanisms involved in the early steps of the flavonoid pathway. The grapefruit is particularly useful in this regard because it primarily accumulates glycosides of the flavanone naringenin,[2, 5] which may well be the simplest (biosynthetically) plant flavanone. In earlier work[9] we identified the main cinnamic acid, coumarin, and flavonoid aglycones of grapefruit and showed how these constituents fit into a logical biosynthetic sequence (based on existing knowledge) leading from *p*-coumaric acid to coumarins, psoralens, flavanones, flavones, dihydroflavonols and flavonols. Recent work indicates that chalcones, which are in equilibrium with flavanones, are the more immediate precursors for flavones and dihydroflavonols than are the flavanones.[8]

* A laboratory of the Western Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture.

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[2] J. W. KESTERSON and R. HENDRICKSON, *Naringin, A Bitter Principle of Grapefruit*, Florida Agr. Expt. Sta. Tech. Bull. 511A, Gainesville (1957).

[3] R. HENDRICKSON and J. W. KESTERSON, *Hesperidin In Florida Oranges*, Florida Agr. Expt. Sta. Tech. Bull. 684, Gainesville (1964).

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In this paper we explore the relationship between naringenin glycoside accumulation and L-phenylalanine ammonia-lyase activity in the developing grapefruit.

RESULTS

Trans-cinnamic acid was identified as a product of the enzymic action of an acetone powder extract on L-phenylalanine by the procedures of Koukol and Conn.[10] Boiled extracts did not convert L-phenylalanine to *trans*-cinnamic acid. The pH optimum for PAL activity was broad, with the maximum at 9·3. Total PAL activity per fruit was highest in the very young fruit, 123·5 mμmoles/min in a 10·85 g fruit. Activity decreased sharply as the fruit increased in size during the early months of growth, 2·11 mμmoles/min in a 127·5 g fruit (Fig. 1), and

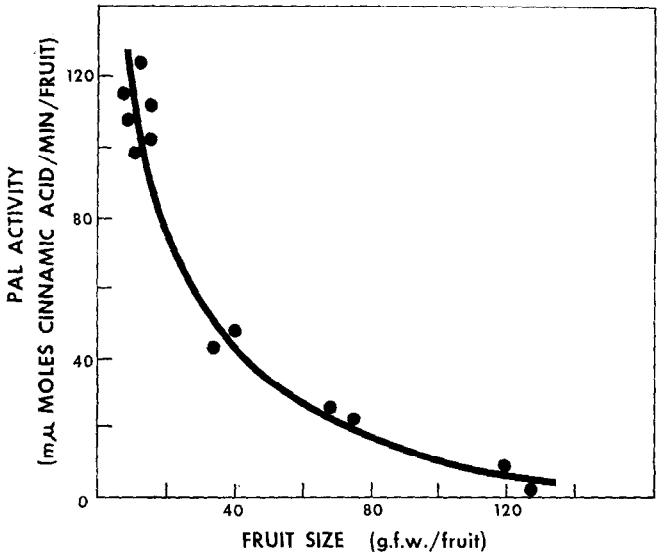

FIG. 1. PAL ACTIVITY OF DEVELOPING GRAPEFRUIT.

only a trace of activity in 190 and 394 g fruits. Young grapefruit (10·8 g/fruit) also had substantial chalcone-flavanone isomerase activity whereas neither young nor older grapefruit had measurable tyrosine ammonia-lyase activity.

Total naringin (naringenin 7-neohesperidoside) plus naringenin 7-rutinoside (hereafter referred to as naringenin glycosides) content per fruit increased sharply during early growth (up to 15–20 per cent of mature weight). The increase became much more gradual during subsequent growth, Fig. 2. Similar results have been reported by others for grapefruit,[2, 6, 7] and for the flavanone glycoside contents of other citrus fruits.[1, 3, 4]

A plot of the rate of naringenin glycoside accumulation (mμmoles/hr/g f.w.; X-axis) v. PAL activity (mμmoles cinnamate/hr/g f.w.; Y-axis) yielded the least squares line: $Y = 3{\cdot}19X - 6{\cdot}68$ (equation 1). The confidence limits of the slope of the line at the 0·01 probability level are $\pm 0{\cdot}36$ and the correlation coefficient for the rate of naringenin glycoside accumulation and PAL activity is 0·999, indicating a very high degree of correlation.

[10] J. KOUKOL and E. E. CONN, *J. Biol. Chem.* **236**, 2692 (1961).

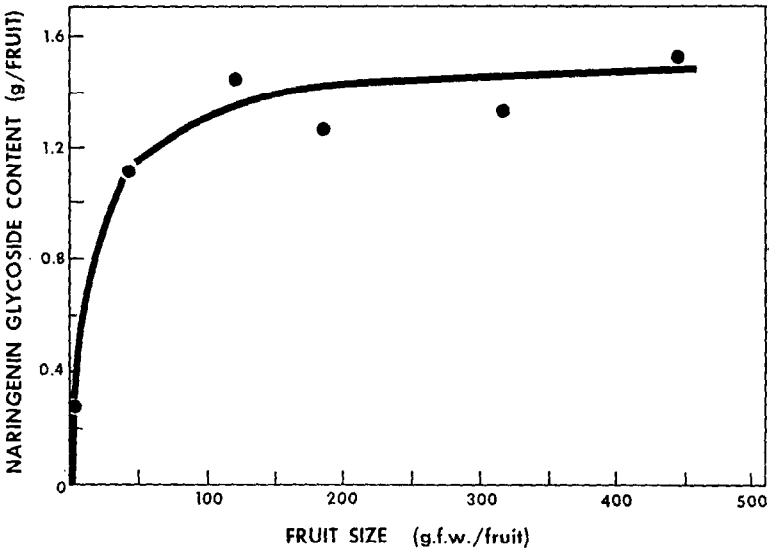

FIG. 2. NARINGENIN GLYCOSIDE CONTENT OF DEVELOPING GRAPEFRUIT.

DISCUSSION

L-Phenylalanine ammonia-lyase[10] (PAL) is the first enzyme in the hydroxycinnamic acid pathway of phenolic biosynthesis in plants. In dicotyledonous plants it appears to be the sole link between the shikimic acid pathway and pathways leading to lignins, coumarins, flavonoids, etc. In monocotyledonous plants L-tyrosine ammonia-lyase[11] provides an additional link. PAL has been shown to be an inducible enzyme in certain plant tissues such as potato tubers, where it appears to be controlled by both feedback repression of enzyme synthesis and feedback inhibition of enzyme activity by cinnamate.[12] Thus, PAL appears to be important in the regulation of phenolic biosynthesis in some plants.

Because L-phenylalanine is a precursor of naringin,[13] PAL activity should be present in grapefruit tissues during periods of active naringenin glycoside synthesis. Our results show that young grapefruit do indeed possess appreciable PAL activity. Moreover, a linear relationship exists between PAL activity and the rate of naringenin glycoside accumulation ($Y = 3{\cdot}19X - 6{\cdot}68$, equation 1) and the relationship holds over a range wherein the rate of naringenin glycoside accumulation decreases from 172 to 1·69 mμmoles/hr/g fresh weight. Because naringenin glycoside accumulation was measured under actual field growing conditions the values calculated for the rate of accumulation are, of necessity, overall averages rather than specific rates for a single set of conditions. Also, PAL activity as determined in our assay procedure is really a measure of potential activity as differentiated from actual *in situ* activity, although these activities are probably proportional. Consequently, we view the molar ratio of *in vitro* PAL activity to rate the of *in situ* naringenin glycoside accumulation of 3·19 (equation 1) primarily as evidence that grapefruit have *potential* PAL activity sufficient to account for the observed rate of naringenin glycoside accumulation in the intact fruit.

[11] A. C. NEISH, *Phytochem.* **1**, 1 (1961).
[12] Cf. K. R. HANSON, M. ZUCKER and E. SONDHEIMER, in *Phenolic Compounds and Metabolic Regulation* (edited by B. J. FINKLE and V. C. RUNECKLES), p. 68, Appleton-Century-Crofts, New York (1967).
[13] J. F. FISHER, *Phytochem.* **7**, 769 (1968).

The observed decrease in PAL activity as the fruit increases in size does not appear to be caused by an inhibition of PAL activity in the older fruit, since activities were additive when extracts from young and older fruits were mixed. Consequently, the decrease in activity per fruit appears to be the result of lower enzyme levels in the older fruit. Naringenin glycosides are known to comprise the bulk of the total cinnamate derived phenols in grapefruit[5,9] (the fruit has a very low lignin content). Therefore, the close relationship between the rate of naringenin glycoside accumulation (synthesis) and PAL activity observed in this study strongly suggests that PAL is an element of an enzyme-regulator system that controls the synthesis of naringenin glycosides (and other cinnamate derived phenols) in grapefruit.

We previously reported[6] that naringenin glycoside accumulation occurs mainly during the period of fruit growth associated with extensive cell division, as opposed to the period of cell enlargement.[14] This suggested that naringenin glycoside synthesis occurs mainly early in the life-cycle of each group of new cells and indicated a regulatory system in which the synthesis is turned on in newly divided cells and is gradually turned off (or is reduced to very low levels) as the cells age. (We do not mean to imply that cell division depends on naringenin glycoside synthesis nor that regulation of synthesis is absolutely dependent on cell age.) Our current results, which show a sharp decrease in PAL activity per fruit as the fruit increases in size during the first several months of growth, are in agreement with this hypothesis. As the fruit develops (after the early logarithmic growth phase) the number of new cell divisions per fruit must drop sharply and eventually become very small. This would result in a rapid decrease in PAL activity during this period, as observed.

The lower levels of PAL in the older fruit could be the result of a decrease in the rate of synthesis or activation and/or an increase in the rate of degradation or inactivation of the enzyme. In potato tuber slices development of PAL activity has been shown by Zucker[15] to involve synthesis of PAL protein, and the decrease in PAL activity that begins after about 24 hr appears to be caused by the synthesis of a protein that degrades or inactivates PAL. In our laboratory, Thorpe[16] has recently demonstrated similar (though not identical) behavior of PAL activity in grapefruit callus originating from fruit albedo tissue. Havir and Hanson[17] have suggested the possibility of the reversible altering of PAL activity by the action of a specific dehydrogenase or transaminase acting on the carbonyl group of the active site of PAL. Thus, in addition to induction, feedback repression, and feedback inhibition there is also evidence of a PAL inactivation mechanism in plant tissues. While the mechanism by which naringenin glycoside accumulation is regulated in grapefruit remains to be determined present knowledge of PAL certainly implicates it as a potential element in that regulatory system and warrants further research on its specific role.

The absence of L-tyrosine ammonia-lyase activity in grapefruit tissues is in agreement with previous reports that this enzyme is found in monocotyledons but not in dicotyledons.[18] Its absence suggests that in grapefruit *p*-coumaric acid (which is known to be present[9]) is synthesized from cinnamic acid via a cinnamate 4-hydroxylase system. Enzymes of this type have been reported in spinach leaves[19] and pea seedlings.[20]

The presence of chalcone-flavanone isomerase (CFI) activity confirms the presence of this

[14] Cf. J. M. BAIN, *Australian J. Botany* **6**, 1 (1958).
[15] M. ZUCKER, *Plant Physiol.* **43**, 365 (1968).
[16] T. A. THORPE, private communication.
[17] E. A. HAVIR and K. R. HANSON, *Biochemistry* **7**, 1904 (1968).
[18] M. R. YOUNG, G. H. N. TOWERS and A. C. NEISH, *Can. J. Botany* **44**, 341 (1966).
[19] P. M. NAIR and L. P. VINING, *Phytochem.* **4**, 161 (1965).
[20] D. W. RUSSELL and E. E. CONN, *Archs Biochem. Biophys.* **122**, 256 (1967).

enzyme in grapefruit. Shimokoriyama[21] previously reported evidence of CFI activity in the peel of several other citrus fruits. More recently CFI has been isolated from soybean seedlings (*Soja hispida*)[22] and some of its properties reported.[23] The presence of CFI in grapefruit during the period of active flavanone accumulation is in good agreement with the observed high PAL activity and with the overall pathway: L-phenylalanine → *trans*-cinnamate → *p*-coumarate → → → naringenin chalcone* ⇄ naringenin*.

EXPERIMENTAL

Plant Material

Fruit from several mature grapefruit trees (*Citrus paradisi* Macf. var. Marsh; rootstock, sour orange) growing in the desert region of California were used. Grapefruit of uniform size and representative of the main population of fruit on the trees (March bloom) were picked at roughly monthly intervals beginning in late April, frozen, and held at −20° until analyzed.

Extraction and Analysis of Naringenin Glycosides

Whole fruits were blended in MeOH and the tissue exhaustively extracted. The extracts were quantitatively analyzed for naringin plus naringenin 7-rutinoside (herein referred to collectively as naringenin glycosides) by a specific TLC method.[24] Average weight per fruit was also determined for the twelve fruit used for the flavanone determination (twenty-four fruit used for the youngest stage). Naringenin glycoside content was plotted as a function of fruit size (weight), Fig. 2. The increase in naringenin glycoside content per fruit was read from this plot for each 5-g incremental increase in fruit size beginning at the 10-g stage. The average rate of naringenin glycoside accumulation was then calculated for each of these intervals using the above values and the observed time for each corresponding 5-g increase in fruit size to occur under actual growing conditions. The mean temperature during this period was 29°. These calculated average rates (ranging from 172 to 1·69 mμmoles/hr/g f.w.) were plotted along the X-axis to obtain equation 1.

Acetone Powders and PAL Assays

Acetone powders (AP) were prepared from frozen whole grapefruit by finely grinding twice in −20° acetone, washing with excess cold acetone and vacuum drying. The AP was held at −20° under vacuum and exhibited no loss in PAL activity during 1 month storage. Extracts of the AP with buffer had high absorption at 290 nm which interfered with the PAL assay. Therefore, the following special procedure was used to reduce the phenolic content and increase the enzyme concentration of the enzyme extracts: 200 mg AP plus 200 mg insoluble PVP were mixed with 5 ml of 0·1 M borate buffer, pH 8·0 (0·01 M in dithiothreitol). Extraction proceeded for 30 min in an ice bath with occasional stirring, followed by centrifugation at 12,000 *g* at 0° for 10 min. The procedure was repeated once and the pooled extracts were mixed with 2·0 g Sephadex G-25 (coarse), held for 1 hr at 0°, and centrifuged in a special centrifuge tube (Pharmacia Fine Chemicals, Inc.). The supernatant was the enzyme extract. Activities of replicate extracts varied by less than 3 per cent and enzyme activity was proportional to the weight of AP extracted. With low-activity samples, up to 1 g of AP (and appropriate amounts of PVP and Sephadex) was used to increase sensitivity.

PAL activity was assayed by measuring the increase in absorbance at 290 nm and 25·5°. The 4·0 ml reaction mixture contained $6{\cdot}67 \times 10^{-3}$ M L-phenylalanine, 0·1 M glycine buffer, pH 9·2, and 0·08 to 0·8 unit of enzyme. The blank contained buffer and enzyme. The pH optimum was found to be broad with the maximum at pH 9·3. One unit of PAL activity is defined as the amount of enzyme that catalyzes the production of 1 m μmole of cinnamic acid per minute under these conditions. Enzyme concentrations as low as 0·01 unit could be detected. *Trans*-cinnamic acid was identified as a product of the enzyme catalyzed reaction by the isolation, spectral, and chromatographic procedures of Koukol and Conn.[10]

AP's were prepared in triplicate from ten fruits each for the 10-g size fruits, three fruits each for the 14-g fruits, and in duplicate from single fruits for the larger size fruits. The PAL activity of developing grapefruit, 10-g through 394-g size, were determined. Fruits of 190- and 394-g size gave only a trace of PAL activity. PAL activity (mμmole/min/fruit) was plotted against fruit size, Fig. 1. PAL activity for each incremental increase in fruit size was read from this plot, the average activity for each interval calculated, the units converted (the range was 561–2·18 mμmoles/hr/g f.w.), and plotted along the Y-axis to obtain equation 1.

*As aglycones or glycosides.

[21] M. SHIMOKORIYAMA, *J. Am. Chem. Soc.* **79**, 4199 (1957).
[22] E. WONG and E. MOUSTAFA, *Tetrahedron Letters* 3021 (1966).
[23] E. MOUTSAFA and E. WONG, *Phytochem.* **6**, 625 (1967).
[24] J. E. FISHER, H. E. NORDBY and T. J. KEW, *J. Food Sci.* **31**, 947 (1966).

Test for Inhibitor of PAL Activity

An enzyme extract from a 10·8-g size fruit with high PAL activity (0·8 unit) was mixed with a equal volume of extract from a 119·3-g size fruit with low activity (0·04 unit). The PAL activity of the mixture was equivalent to 96 per cent of the sum of activities of the two extracts.

Other Enzymes

Extracts used to test for L-tyrosine ammonia-lyase (TAL) activity were prepared using 500 mg AP in the same manner as for PAL. TAL activity was assayed by measuring the increase in absorbance at 333 nm and 25·5°. The reaction system contained L-tyrosine in place of L-phenylalanine, and 0·4 ml enzyme extract. None of the three grapefruit samples tested (sizes: 10·67, 34·48, and 127·5 g/fruit) exhibited TAL activity whereas all had PAL activity.

Chalcone-flavanone isomerase activity was tested using isoliquiritigenin (2′,4′,4-trihydroxychalcone) as substrate (1000 μg) in 40 ml reaction mixture 0·1 M in tris buffer, pH 8·0, and containing 3·0 ml enzyme extract or 3·0 ml of boiled extract. The enzyme extract was prepared by extracting 1 g AP from 10·8-g size grapefruit with 0·1 M tris buffer, pH 8·0 and treating in the same manner as before. Reaction time was 5 min at 25·5°. Liquiritigenin was identified as a product of the enzyme system by TLC, whereas no liquiritigenin was present in the control system. In addition, the enzyme system had several other spots not present in the control.

Acknowledgements—We thank Mr. D. M. Metzler for technical assistance and Dr. J. Furr, USDA Date and Citrus Station, Indio, California, for the fruit.