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BIOSYNTHESIS OF NARINGIN AND PRUNIN IN DETACHED GRAPEFRUIT

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Key Word Index—*Citrus paradisi*; Rutaceae; grapefruit; biosynthesis; radiolabel; flavonoids; naringin, prunin.

Abstract—[^{14}C]Acetate and [^{14}C]phenylalanine were fed to detached immature grapefruit (*Citrus paradisi* Macfad.) fruits and two radiolabelled flavanone glycosides were detected. TLC analysis and enzymatic hydrolysis studies indicated that the two labelled flavonoids were naringin (naringenin 7-*O*-β-neohesperidoside) and prunin (naringenin 7-*O*-β-glucoside). Prunin, which has not been previously shown to be present in grapefruit, was then detected in immature peel. This indicates that immature grapefruits are capable of biosynthesizing flavonoids from simple precursors and suggests that multiple-glycosylation of flavanones may occur by the addition of discrete single sugar units.

INTRODUCTION

The flavonoids are an important class of plant secondary metabolites, which have been the subject of intense biochemical studies in recent years [1]. Despite this effort, there is still much to be learned about their biosynthesis and bioregulation. Citrus species are of much interest because they accumulate large amounts of flavanone glycosides, early intermediates in the flavonoid biosynthetic pathway [2, 3]. Grapefruit is particularly intriguing because it accumulates the bitter flavonoid naringin, which when in excess adversely affects the quality of the fruit [4–6]. Detached young grapefruit leaves from a mature plant were able to convert [^{14}C]phenylalanine to [^{14}C]naringin, while older leaves did not [7]. This indicates the flavanone glycosides accumulate during periods of rapid cell division and growth.

Very little research has been conducted on the bioregulatory controls of flavonoid metabolism in citrus. The exact chemical nature of these controls is still unknown, as are the details on location of the exact sites of synthesis and subsequent translocation of the products throughout the plant. In order to add to our understanding of flavonoid metabolism, we are currently examining the sites of flavonoid biosynthesis in grapefruit. In this paper, we demonstrate that immature fruit can biosynthesize naringin and prunin from phenylalanine or acetate.

RESULTS

It has been a matter of general interest to determine whether developing fruit are biosynthetically capable of making their constituents, or whether they serve as a sink for compounds made elsewhere in the plant, or a combination of both. The percentage of radioactivity incorporated into MeOH extracts of detached young grapefruits which were fed either [1-^{14}C] acetate or [^{14}C] phenylalanine through the stem or directly to the fruit tissue itself is shown in Table 1. Two radiolabelled peaks were detected when extracts were analysed by TLC on silica gel plates developed with solvent system 1. Replicates of each of the experiments shown in Table 1 were run (data not shown). All experiments showed the two labelled peaks by TLC, but the replicates generally had lower levels of radioactivity incorporated. Further analysis using the three other TLC solvent systems indicated that the R_fs of the two labelled compounds corresponded to those of the flavanone glycosides naringin and prunin. [^{14}C] Acetate fed directly to the fruit tissue without the stem attached was also converted to naringin, but the total amount of radio label taken up was much less than that in the fruit with the stem attached.

This is the first report of prunin in grapefruit. The higher sensitivity of instruments detecting radiolabel allows for the detection of much lower levels of prunin than the methods previously used. An aliquot of the SepPak fraction of the [1-^{14}C] acetate-fed fruit+stem was then subjected to reverse phase HPLC. Analysis of the collected fractions by liquid scintillation counting showed one large radioactive peak with the same R_f as naringin and prunin, which did not separate under these conditions. The fractions containing naringin and prunin were pooled and concentrated. Re-analysis by TLC again showed the corresponding radioactive peaks next to the naringin and prunin standards.

When a portion of the SepPak fraction was treated with naringinase and subsequently analysed by TLC, the enzyme completely converted the radioactive naringin peak to prunin and the aglycone naringenin (Table 2). The commercial naringinase used has both α-rhamnosidase and β-glucosidase activities, which accounts for the increase in the amount of the labelled prunin in these experiments. Treatment of another portion of the extract by β-glucosidase converted the radioactive prunin to naringenin, but did not affect the radioactive naringin.

Prunin has not been shown to be present in grapefruit in previous studies, yet when extracts of peel and juice

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Table 1. Radiolabel incorporated into naringin and prunin by three detached immature grapefruits

	Fruit + stem	Fruit + stem	Fruit (no stem)
Radiolabel	phenylalanine	HOAc	HOAc
cpm fed ($\times 10^7$)	1 1	1 1	1 1
cpm extracted (% of cpm fed)	2 79	2 97	1.62
cpm naringin (% of cpm fed)	1 16	0.73	0 16
cpm prunin (% of cpm fed)	0.09	0 08	0 04

Table 2. TLC analysis of enzymatically hydrolized MeOH extracts of [^{14}C]HOAc-fed immature grapefruit

	Radioactivity (%)		
Treatment	Naringin	Prunin	Naringenin
None	90	10	0
Naringinase	0	40	60
β-Glucosidase	90	0	10
R_f*	0.5	0.75	1 0

*Solvent system 1.

sacs of immature grapefruits were treated with β-glucosidase, naringenin was released (Table 3). This indicates the presence of one (or more) naringenin glucoside(s). Under our HPLC conditions, standard prunin coeluted with narirutin, the 7-*O*-β-rutinoside of naringenin. However, when the narirutin/prunin peak was bulk-purified by HPLC and subsequently subjected to silica gel TLC, the two compounds were separated. The presence of prunin was confirmed by HPLC on a amino-propyl column, which separated naringin, narirutin and prunin, and by re-analysis on the C_{18} column. The isolated peak had the same R_t and absorbance spectra as standard prunin All attempts at detecting prunin from mature peel failed. Crude estimates of the prunin levels can be made from the β-glucosidase treatments. In 20 mm diameter fruit, prunin was present at 1/17th the level of naringin and 1/3rd the level of narirutin By the time the fruit is 60 mm in diameter, the prunin level has dropped to only 1/30th the level of naringin and 1/5th the level of narirutin In mature fruit, β-glucosidase treatment released comparable levels of naringenin at 1/24th the level of naringin and 1/4th the level of narirutin. These ratios only reflect the average of three experiments of fruit harvested from the same tree at the same time. The ratios may be subject to change due to genetic and environmental factors.

DISCUSSION

The data presented here show that young grapefruit are fully capable of biosynthesizing complex secondary metabolites like the flavanone glycosides from simple precursors. Previously, only young leaves from mature trees have been shown to have this capability [7]. Enzyme activities in several major metabolic pathways have been detected in citrus fruit including the flavonoid pathway [6], the tricarboxylic acid pathway [8], respiratory pathways [9], nucleotide synthesis [10], and the ability to fix carbon dioxide [11] This indicates that the growing and developing fruit has the ability to biosynthesize many of its components. While the fruit may serve as a sink for materials transported from the rest of the plant, the tissues within the fruit itself contribute a large portion to its final composition. The regulation of this ratio may be an important factor in the determination of the final amount of naringin in the mature fruit

This is the first report of the presence of prunin in grapefruit and many have important ramifications towards piecing together the sequence of the last steps of flavonoid biosynthesis in citrus. Little is known about the regulation of the later stages in flavonoid biosynthesis

Table 3. Total naringenin glucosides in immature and mature grapefruit determined by β-glucosidase hydrolysis and HPLC

Tissue		Amount (μmol/g fr wt)		
Size of fruit (mm)	Tissue analysed	Narirutin	Naringin	Naringenin*
20	Whole	87	572	34
60	Green peel	86	468	16
60	Green juice sacs	50	148	13
75	Yellow peel	44	299	12
75	Yellow juice sacs	21	70	8
	R_t (min)	24 6	26 0	33 0

*Naringenin present only after hydrolysis with β-glucosidase

and metabolism. Treatment of mature grapefruit peel with β-glucosidase released naringenin, indicating the presence of naringenin glucosides, but prunin itself could not be detected. This suggests that there are other as yet unidentified glucosides of naringenin in citrus. Several different naringenin glucosides have been shown to exist in other plant families [12].

It is still unknown whether glucosylation and rhamnosylation occur before or after the naringenin chalcone is closed to the flavanone. Most glycosyl-transferases examined from other sources have been shown to act on the flavonoid form and not the chalcone form [1]. In citrus however, there is some conflicting evidence in the literature on this subject. Recently, cell cultures of grapefruit were shown to be able to glucosylate exogenously added naringenin and hesperetin, even though the cultures did not accumulate flavanones by themselves [13]. The activity of a flavanone–chalcone isomerase, which acts on the aglycone, has been detected in acetone extracts of grapefruit [14]. On the other hand, a chalcone cyclase isolated from grapefruit would only convert naringin chalcone to naringin, but would not convert naringenin chalcone or prunin chalcone [15]. It may be that this enzyme is present in addition to flavanone-chalcone isomerase. This may be the point at which the biosynthetic pathway, leading to the formation of other flavonoids and the anthocyanins, diverges from the pathway that leads to flavanone glycoside accumulation. Prunin accumulation in the young fruit may be a by-product of the first pathway, while glycosylation of chalcones lead to the formation of naringin. The substrate specificities of the glycosyl-transferases need to be studied in order to determine if there is one set of broadly specific transferases, which act on both chalcones and flavanones, or several different specific transferases.

EXPERIMENTAL

For the detached studies, branches with immature fruit (15 mm in diameter) were removed from potted Marsh and Ruby Red dwarfs kept in the greenhouse. The plant branches were immersed in H_2O and the tissues of interest were cut underwater with a sharp blade. The fresh cut ends were carefully placed in microtubes containing either: 5 μCi [1-^{14}C]NaOAC (358.6 mCi/mmol) or 2.5 μCi [U-^{14}C]phenylalanine (493 mCi/mmol), H_2O added to make a total vol. of 50 μl. The detached fruits were placed in the hood with the fan on and under constant illumination by fluorescent light at room temp. After most of the initial soln was absorbed, another vol. of H_2O was added and allowed to be absorbed. This was repeated several times before the tissue was transferred to a vial containing H_2O and allowed to metabolize for 24–72 hr under the conditions described above. The tissues were then cut into small segments and homogenized with a Tissumizer with MeOH (3 × vol to wt). After centrifugation the supernatent was decanted, and residue extracted again. The supernatants were combined and a small amount of H_2O was added (typically 1/10 of the vol), and the soln passed through a C_{18} SepPak to remove the highly non-polar material. The elutate (hereafter called the SepPak fraction) was then conc by evapn and analysed by TLC and/or HPLC.

TLC was performed on silica gel fluorescent plates. Plates were developed in the following ascending solvent systems at room temp. (1) EtOAc–MeCOEt–HCO_2H–H_2O (5:3:1:1); (2) *n*-BuOH–HOAc–H_2O (4:1·5); (3) $CHCl_3$–EtOAc–HCO_2H (5:13:2); (4) nitromethane–MeOH (5:2); (5) *n*-PrOH–EtOAc–H_2O (3:2:1). Standards were visualized under UV light, radiochromatograms were detected by a TLC linear analyser.

HPLC was performed on two equivalent systems, one for radioactive studies and one for cold studies. Absorbance at 282 nm was monitored by a UV/VIS detector for the radioactive studies and by a diode-array detector for the cold studies. The HPLC columns used were C_{18} reverse phase (0.4 mm × 25 cm). Chromatographs were developed over a 30 (or 55) min linear gradient beginning with a MeOH–0.01 M H_3PO_4 (1:4) and ending with MeOH at a flow rate of 1 ml per min. One ml fractions were collected and the total radioactivity in each was determined by liquid scintillation counting. Fractions containing naringin and prunin were pooled, concentrated and analysed by TLC.

Enzymatic hydrolysis with naringinase and β-glucosidase was used to confirm the identity of the radioactive peaks in the MeOH extract. A small amount of sample (typically 5000–10 000 cpm of labelled material or 1–2 mg of unlabelled material) was added to a 100 mM HOAc solution, buffered at pH 4 for naringinase or pH 5 for β-glucosidase. One mg of the appropriate enzyme was added, and the mixture was incubated in a closed vial overnight at room temp. The protein was pptd by the addition of trichloroacetic acid, removed by centrifugation, and the sample analysed by TLC or HPLC.

For the isolation of prunin, 2.5 g of immature grapefruit peel (var. 'Hudson Foster') of 6 cm in diameter, was ground up and extracted (× 4) with MeOH. The MeOH was evapd off and the remaining liquid passed through a C_{18} SepPak. The initial elutate was discarded and the material retained on the SepPak was eluted with MeOH. After conc by evapn, the sample was analysed by HPLC using the 55 min gradient. Full spectra of eluted peaks were obtained from the diode array detector system. The prunin, narirutin and naringin peaks were collected from several chromatographic runs and conc by evaporation. The conc sample was spotted on a silica gel TLC plate and developed with solvent system 1. The area of the plate corresponding to the prunin standard was carefully scraped off and the silica powder extracted (× 5) with MeOH. After concn, the sample was then re-analysed by HPLC on the C_{18} column. In addition, samples were analysed on a amino-propyl HPLC column, which was developed isocratically using a MeOH–CH_2Cl_2 (2:3). MeOH extracts from 20 mm fruit and 75 mm mature yellow fruit peel were similarly analysed.

REFERENCES

1. Ebel, J. and Hahlbrock, K. (1982) in *The Flavonoids: Advances in Research* (Harborne, J. B. and Mabry, T. J., eds), p. 641. Chapman & Hall, New York.
2. Albach, R. F. and Redman, G. H. (1969) *Phytochemistry* **8**, 127.
3. Wong, E. (1968) *Phytochemistry* **7**, 1751.
4. Kesterson, J. W. and Hendrickson, R. (1953) *Fla. Agric. Exp. Stn. Tech. Bull.*, 511.
5. Hagen, R. E., Dunlap. W. J. and Wender, S. H. (1966) *J. Food Sci.* **31**, 542.
6. Hasegawa, S. and Maier, V. P. (1981) *Proc. Int. Citriculture* p. 914.
7. Fisher, J. F. (1968) *Phytochemistry* **7**, 769.
8. Roe, B., Davis, P. L. and Brummer, J. H. (1984) *Phytochemistry* **23**, 713.
9. Brummer, J. H. (1986) *ACS Symp. Series No.* 317, *Biogeneration of Aromas* p. 275.

10. Tomlinson, P T and Lovatt, C. J. (1987) *J Am Soc Hort. Sci.* **112**, 529.
11. Moreshet, S. and Green, G. C., (1980) *J. Exp Botany* **120**, 15.
12 Bohm, B A (1975) in *The Flavonoids* (Harborne, J B., Mabry, T. J., and Mabry, H., eds), p. 560. Academic Press, New York.
13 Lewinsohn, E., Berman, E., Mazur, Y and Gressel, J. (1986) *Phytochemistry* **25**, 2531.
14 Hasegawa, S. and Maier, V. P. (1970) *Phytochemistry* **9**, 2483
15. Raymond, W. R. and Maier, V P (1977) *Phytochemistry* **16**, 1535