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CINNAMATE HYDROXYLATION AND THE ENZYMES LEADING FROM PHENYLPYRUVATE TO *p*-COUMARATE SYNTHESIS IN GRAPEFRUIT TISSUES

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Abstract—Cinnamic acid was converted to *p*-coumaric acid in grapefruit tissue slices. The rate of the conversion appeared to be much faster in young fruit than in mature fruit. It was shown also that tyrosine is synthesized mainly from *p*-hydroxyphenylpyruvate by tyrosine : 2 ketoglutarate aminotransferase. The absence of phenylalanine hydroxylase and tyrosine ammonia-lyase suggested strongly that tyrosine is not an effective precursor for the biosynthesis of flavonoids in grapefruit.

INTRODUCTION

BECAUSE young grapefruit accumulate large amounts of naringenin glycosides, their tissues are very useful and convenient plant materials for studies of the enzyme chemistry and the biological regulatory systems of flavonoid synthesis.[1] In earlier work[1,2] we showed that young grapefruit possesses L-phenylalanine ammonia-lyase (PAL) (E.D. 4.3.1.5) and L-phenylalanine:2-ketoglutarate aminotransferase (PKA) (E.C. 2.6.1.5) both of which are reported to be essential in the biosynthesis of *trans*-cinnamate from phenylpyruvate.[3–8] It was observed also that *in vitro* glutamate dehydrogenase (GDH) (L-glutamate-NAD(P) oxidoreductase, E.C. 1.4.1.2) plays an important role in the conversion of phenylpyruvate to phenylalanine by supplying glutamate which serves as the amino group donor.[2] More important, the correlation observed between PAL activity and naringenin glycoside accumulation during fruit development suggested that PAL is an element of an enzymic regulator system that controls the biosynthesis of flavonoids in grapefruit.[1]

In this paper further systematic studies of the overall reactions involved in the synthesis of *p*-coumarate from phenylpyruvate were carried out to elucidate the role of PAL in grapefruit. The presence of cinnamate hydroxylase (E.C. 1.14.3) was demonstrated for the first time in grapefruit. Also, the biosynthesis of tyrosine via *p*-hydroxyphenylpyruvate by tyrosine:2-ketoglutarate aminotransferase (TKA) (E.C. 4.3.1.5) was demonstrated. The role of tyrosine in flavonoid synthesis in grapefruit is discussed.

RESULTS

Attempts to detect cinnamate hydroxylase activity in extracts of acetone powders or extracts obtained directly from fresh tissues were inconsistent. Consequently, we adopted a

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tissue slice technique[9] in which ^{14}C-labelled cinnamate was used, and we found consistent conversion of supplied labelled cinnamate to *p*-coumarate. Four radioactive spots were found on a radiochromatogram. Two of them were identified as cinnamate and *p*-coumarate, while the others were not identified. The uptake of the substrate, ^{14}C-labelled cinamate, into the tissues was first detected after 2 hr of incubation. It reached a peak at about 6 hr and the maximal level (approximately 45% of the total radioactivity found in tissues) remained fairly constant throughout the period of incubation. The formation of *p*-coumarate was found to increase linearly as a function of incubation time when labelled *p*-coumarate was expressed on the basis of total radioactivity found. These results indicate that tissues of young grapefruit (10-g size) possess cinnamate hydroxylase activity. On the other hand, tissues obtained from 200-g size fruit converted cinnamate to *p*-coumarate in only trace amounts after 48 hr of incubation.

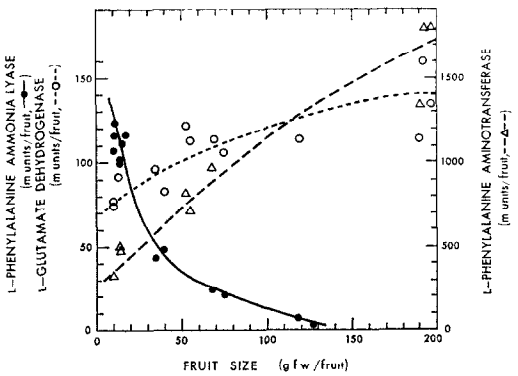

FIG. 1. CHANGES IN ACTIVITIES OF PAL,[1] PKA AND GDH DURING GRAPEFRUIT DEVELOPMENT.

PKA and GDH activities were followed during grapefruit growth and development as was PAL activity previously.[1] The results are shown in Fig. 1. The data on PAL activity obtained previously[1] are cited also for comparison. The changes in GDH and PKA activities, shown as total activity per fruit, differed significantly from those of PAL activity. Total activity of PKA and GDH per fruit was lowest in the very young fruit, 325 m units and 75 m units in 10-g size, respectively. Both activities, however, rose steadily with increasing fruit weight throughout fruit development. On the other hand, PAL activity, highest in the young fruit, declined sharply during the first few weeks of growth and the decrease continued as the fruit increased in size, although the rate became much slower as the fruit grew larger. It was found also that PKA activity was almost 10-fold higher than the activities of either PAL or GDH during the earlier months of growth.

Figure 2 illustrates how activities of PAL, PKA and GDH correlate with the declining rate of accumulation of naringenin glycosides during development of grapefruit. The results obtained in the study and the data on naringenin glycosides obtained previously[1] are plotted on a graph expressing the rate of naringenin glycoside accumulation vs. activities of these enzymes. The rate of glycoside accumulation and PAL activity have a very high degree of correlation[1] whereas activities of PKA and GDH do not. PKA and GDH activities remained high even after the rate of glycoside accumulation declined sharply.

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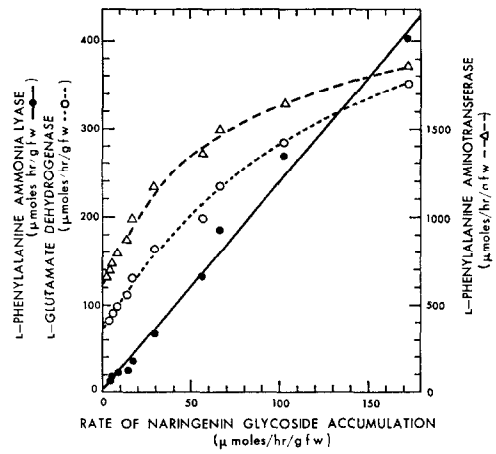

FIG. 2. THE CORRELATION BETWEEN ACTIVITIES OF PAL, GDH AND PKA, AND THE DECLINING RATE OF NARINGENIN GLYCOSIDE ACCUMULATION IN GRAPEFRUIT.

Two possible pathways of tyrosine biosynthesis were investigated. One was from L-phenylalanine catalyzed by phenylalanine hydroxylase and the other was from *p*-hydroxyphenylpyruvate catalyzed by tyrosine:2-ketoglutarate aminotransferase (TKA). Phenylalanine hydroxylase activity was studied using ^{14}C-labelled phenylalanine by a procedure similar to that used for the detection of cinnamate hydroxylase activity. The results showed that neither extracts of acetone powders obtained from fruit at various stages of maturity nor albedo tissue slices from young fruit were able to convert the labelled phenylalanine to tyrosine.

The biosynthesis of tyrosine via *p*-hydroxyphenylpyruvate seems to represent an important pathway in grapefruit. It was found that extracts of the acetone powder obtained from 10-g size fruit contained a relatively high activity of TKA. The results of radioisotope experiments showed that approximately 25% of the labelled tyrosine was converted to *p*-hydroxyphenylpyruvate during the 2 hr of incubation. Also, it was found that 10-g size contained about 400 m units of TKA.

It was not clear, however, whether the synthesis of tyrosine occurred via *p*-hydroxyphenylpyruvate through the action of TKA or through the action of PKA of broad substrate specificity. To clarify this, PKA was purified approximately 53-fold by DEAE- and CM-cellulose column chromatography (Table 1) and its substrate specificity was studied. PKA was found to be highly specific for phenylpyruvate, attacking *p*-hydroxyphenylpyruvate at a rate which was only 6% of the rate at which it attacked phenylpyruvate.

TABLE 1. PURIFICATION OF L-PHENYLALANINE:2 KETOGLUTARATE AMINOTRANSFERASE

Treatment	Total vol. (ml)	Total protein (mg)	Sp. act. (m unit/mg protein)	Recovery (%)	Relative act.
Extract	220	1562	1·81	100	1
$(NH_4)_2SO_4$ ppt and dialysis	17	170	10·3	61·8	5·7
DEAE-cellulose column	60	17·5	51·1	31·6	28·2
CM-cellulose column	47	4·3	95·6	14·5	52·8

DISCUSSION

The role of *p*-coumaric acid as a precursor for the biosynthesis of flavonoids and lignins is well known.[4,10–12] In dicotyledonous plants, the biosynthesis of *p*-coumaric acid via cinnamic acid seems to represent the sole pathway to *p*-coumarate since these plants do not generally possess appreciable TAL activity.[11] This is indeed the case in grapefruit as shown by our earlier experiments in which TAL activity was found to be absent from the fruit.[1] The presence of cinnamate hydroxylase provides additional evidence to confirm the cinnamate route.

Cinnamate hydroxylase appears to be widespread in higher plants.[13–15] However, this enzyme is so unstable that no one has succeeded in isolating it; consequently, its properties and its mechanism of action are not fully understood. It seems that *in vitro* the enzyme requires the external reducing agents, tetrahydrofolic acid and NADPH.[13,14] In addition, data on quantitive analyses of the enzyme in higher plants are very limited. Shimada *et al.*[15] observed an increase in its activity paralleling the progress of lignification in bamboo shoots, suggesting that this enzyme along with PAL and TAL perform an important role in lignin biosynthesis. Our results show the presence of significant cinnamate hydroxylase activity in young grapefruit but only traces of activity in the mature fruit when compared on a unit weight of tissue basis. This lowering of activity with increased age of the fruit corresponds with a lowering in the rate of naringenin glycoside accumulation. Since L-phenylalanine is a known precursor of naringin,[16] the presence of cinnamate hydroxylase activity during periods of active flavonoid biosynthesis supports the cinnamate route to *p*-coumarate in grapefruit.

The absence of measurable phenylalanine hydroxylase activity suggests that the biosynthesis of tyrosine via phenylalanine is not an important pathway in grapefruit. This enzyme is reported to be absent from shoots of bamboo and asparagus[15] and present in small but measurable amounts in spinach leaves,[17] *Salvia splendens*[3] *Triticum vulgare*[18] and *Fagopyrum tataricum*.[18] It appears likely, however, that the activity of phenylalanine hydroxylase in higher plants is generally low and insignificant.[12,19] In this respect higher plants differ from animals where tyrosine is formed readily from phenylalanine.[20,21]

In grapefruit, tyrosine synthesis from *p*-hydroxyphenylpyruvate appears to be an active pathway as shown by the presence of substantial TKA activity in the young fruit. The possibility that tyrosine might be synthesized from *p*-hydroxyphenypyruvate by PKA was ruled out by substrate specificity tests. A 10-g size fruit contained approximately 400 m units of TKA and 500 m units of PKA, but the purified PKA obtained from the same size fruit reacted with *p*-hydroxyphenylpyruvate at a rate only 6% of that of phenylpyruvate. These results which are in agreement with earlier reports,[12,22] indicate that

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tyrosine and phenylalanine are synthesized through different channels by two distinct enzymes.

The absence of TAL and phenylalanine hydroxylase suggests strongly that tyrosine is not an effective precursor for the biosynthesis of flavonoids in grapefruit. In some monocotyledonous plants, tyrosine is an important intermediate in the formation of lignin and related phenylpropanoid compounds.[4,11,23,24]

Quantitative data on the analysis of enzyme activities and naringenin glycosides during the early stages of fruit development provide additional supporting evidence of the key role played by PAL in the biosynthesis of flavonoids in grapefruit. In relation to naringenin glycoside accumulation, the pattern of change in PAL activity was altogether different from that of glutamate:oxaloacetate aminotransferase,* PKA and GDH. Among these enzymes PAL was the only one whose activity correlated well with the declining rate of glycoside accumulation.[1] In fact, activities of the other enzymes remained high even after glycoside accumulation had slowed drastically. Halbrock *et al.*[25] have shown that in developing parsley plants, in addition to PAL, the activities of chalcone–flavanone isomerase, UDP–apiose synthetase, and a glucosyl- and an apiosyltransferase correlate with the concentrations of flavone glycosides. These enzymes are involved in steps subsequent to PAL in the biosynthesis of apiin. As flavonoid biosynthesis slows the activities of the enzymes of the pathway should diminish correspondingly. In developing intact grapefruit PAL is the first enzyme in the pathway whose activity parallels the slowdown in flavonoid accumulation. In earlier experiments, we demonstrated the biosynthesis of *trans*-cinnamate from phenylpyruvate using a cell-free extract of tissues from very young grapefruit.[2] PAL, PKA and GDH activities were all sufficient to allow synthesis of significant amounts of *trans*-cinnamate. As the fruit develops further the undiminished activities of PKA and GDH show that the capacity to produce phenylalanine does not drop even though flavonoid synthesis is slowing. Consequently, the level of PAL activity appears to be the rate-limiting factor in the production of *trans*-cinnamate in older fruit.

In a number of other cases, PAL activity has been shown to increase paralleling the increase in overall quantity of phenylpropanoid compounds.[8,26–29] On the other hand, in some cases there is no direct correlation between PAL activity and the formation of phenylpropanoids,[30,31] suggesting that there might be alternative pathways from shikimic acid to polyphenols. Our results support the view that PAL under normal growth conditions plays a key role in flavonoid biosynthesis in intact grapefruit and this role may involve a regulatory as well as a synthetic aspect.

EXPERIMENTAL

Acetone powders were prepared by the procedure described previously[1] from various sizes of grapefruit sampled during the 1969 season from a single tree (*Citrus paradisi* Macf. var. Marsh) growing the desert region of California. DL-phenylalanine 3-^{14}C was purchased from New England Nuclear Corp., Boston

* Unpublished data.

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Massachusetts and cinnamic aid 3-^{14}C was obtained from Schwarz Bioresearch, Inc. Orangeburg, New York.

Cinnamate hydroxylase activity was demonstrated by using thinly sliced albedo tissues and ^{14}C-labelled cinnamate. Conversion of cinnamate to *p*-coumarate was followed as a function of incubation time. Approximately 1 g of tissue slices (2 mm thick) obtained from 10-g size fruit was incubated in 20 ml of a liquid tissue culture medium[9] minus orange juice and casamino acid, but including 5×10^{-4} M *trans*-cinnamate and a suitable dilution of labelled cinnamate. The incubation was carried out in a 120-ml flask under dark on a gyrotory shaker. The slices were sampled periodically, washed with H_2O, and ground with H_2O by a Polytron. The homogenate was then acidified and passed through a 1·0 × 10·0 cm Dowex 50 (H^+form) column. The effluent obtained was brought to dryness under reduced pressure and the residue was redissolved in 1 ml H_2O. An aliquot (30 μl) was spotted on a precoated cellulose TL plate, and the plate was developed with 1-BuOH–$(NH_4)_2CO_3$ buffer.[32] Cinnamate and coumarate were identified by reference to R_fs of authentic samples. The radioactive spots were located and their relative concentrations were estimated by a Vanguard Automatic Chromatogram Scanner.

PKA and GDH activity were extracted from acetone powders by the procedure described previously.[1] PKA activity was assayed spectrophotometrically by a coupled reaction with glutamate dehydrogenase by measuring decrease in NADH concentration. A reaction mixture, in a total of 4 ml containing $1{\cdot}25 \times 10^{-3}$ M each of phenylpyruvate, L-glutamate and NH_4Cl, 0·075 M Tris buffer at pH 8·0, $1{\cdot}25 \times 10^{-4}$ M of NADH, 10^{-5} M pyridoxal phosphate, 1 ml of the enzyme preparation and 4 units of glutamate dehydrogenase was incubated at 23°. Changes in absorbancy at 340 nm were measured by a Cary 15 spectrophotometer.

GDH activity was assayed also by following the decrease in NADH. Reaction mixtures consisted of 5×10^{-3} M each of 2-ketoglutarate and NH_4Cl, $1{\cdot}25 \times 10^{-4}$ M NADH, 0·075 M Tris buffer at pH 8·0, and 1 ml of the enzyme preparation in a total of 4·0 ml.

One unit of GDH and PKA activity is defined as the amount that catalyzes the formation of 1 μmole of product in 1 min under the conditions used. In all cases, the rate of product formation was linear with time.

PKA was purified by the DEAE cellulose column chromatographic method described in a previous publication.[2] The PKA fraction obtained from the column, which contained 895 m units and 17·5 mg proteins, was purified further on a 2·5 × 20 cm CM cellulose column which had been equilibrated with 0·01 M phosphate buffer at pH 7·0. The column was eluted with a linear gradient formed between 150 ml of 0·01 M potassium phosphate buffer, pH 7·0 and 150 ml of the same buffer solution containing 0·8 M NaCl. The effluent was collected in 10-ml fractions. PKA activity was collected in fractions 10–20.

The presence of TKA was demonstrated by the following procedure. The enzyme was extracted from 1 g of the acetone powder obtained from 10-g size fruit with 20 ml of 0·1 M potassium phosphate buffer, pH 7·0 containing 200 mg of presoaked insoluble PVP. The mixture was centrifuged at 20,000 *g* for 10 min and the supernatant was passed through a 2·5 × 40 cm G-25 Sephadex column. The effluent was then concentrated by lyophilization and used as the enzyme source. TKA activity was detected with ^{14}C-labelled tyrosine as substrate by observing the formation of labelled *p*-hydroxyphenylpyruvate. A reaction mixture consisted of 0·1 M potassium phosphate buffer at pH 8·0, $1{\cdot}5 \times 10^{-3}$ M each of 2-ketoglutarate and tyrosine containing a suitable dilution of ^{14}C-tyrosine, 10^{-5} M pyridoxal phosphate, and 0·05 mg protein in a total of 0·5 ml. After 2 hr of incubation at 30°, samples were spotted on a precoated cellulose TL plate and the plate was developed with butanol–formate (95:5, saturated with water). The radioactive spots were detected and their relative activities were measured by a Vanguard Automatic Chromatogram Scanner. Quantitative analysis of TKA activity was assayed spectrophotometrically by the coupled reaction with glutamate dehydrogenase by measuring decrease in NADH. The procedure was similar to that used for PKA except that *p*-hydroxyphenylpyruvate was used as substrate.

Phenylalanine hydroxylase activity was examined with ^{14}C-labelled phenylalanine. Labelled phenylalanine was incubated with extracts of acetone powders in the presence of NADPH and tetrahydrofolic acid by the procedure of Nair and Vining,[13] and also with thinly sliced albedo tissues by a method similar to that used for cinnamate hydroxylase assay. The reaction product was then examined for the production of labelled tyrosine by a TLC method with two solvent systems; *t*-BuOH–methyl ethyl ketone–acetone–MeOH–H_2O–NH_4OH (40:20:20:1:14:5) and phenol–H_2O (3:1). The radioactive spots were examined with a Vanguard Automatic Chromatogfam Scanner.

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Key Word Index—Citrus paradisi; Rutaceae; grapefruit; cinnamate hydroxylase; tyrosine; 2-ketoglutarate aminotransferase; phenylalanine hydroxylase; tyrosine ammonia lyase; phenylalanine ammonia lyase.