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BIOSYNTHESIS OF *TRANS*-CINNAMATE FROM PHENYLPYRUVATE AND L-GLUTAMATE BY CELL-FREE EXTRACTS OF GRAPEFRUIT

SHIN HASEGAWA and V. P. MAIER

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

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Abstract—In addition to L-phenylalanine ammonia-lyase (E.C. 4.3.1.5), young grapefruit (*Citrus paradisi* Macf.) possess phenylalanine–2-ketoglutarate aminotransferase (E.C. 2.6.1.5) and glutamate dehydrogenase (L-glutamate:NAD(P) oxidoreductase, E.C. 1.4.1.2) activities sufficient to account for the biosynthesis of an appreciable amount of *trans*-cinnamate from phenylpyruvate and L-glutamate. This biosynthesis was demonstrated *in vitro* using a cell-free extract of young fruit tissues.

INTRODUCTION

BECAUSE flavanones are early intermediates in flavonoid biosynthesis,[1] citrus fruits with their high concentration of flavanone glycosides are a very useful and convenient plant system for studies of the enzyme chemistry and regulatory mechanisms of the flavonoid pathway. The grapefruit is particularly useful in this regard because it primarily accumulates glycosides of the simple flavanone naringenin. In earlier work we observed a direct relationship between L-phenylalanine ammonia-lyase (E.C. 4.3.1.5) (PAL) activity and the rate of naringenin glycoside accumulation in developing grapefruit.[2] It was also observed that 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 suggested that PAL is an element of an enzyme-regulator system that controls synthesis of naringenin glycosides in grapefruit.

In this paper two additional enzymes reported to be essential in the bio-synthesis of *trans*-cinnamate[3–8] were studied. Appreciable amounts of L-phenylalanine–2-ketoglutarate aminotransferase (PKA) (E.C. 2.6.1.5) and glutamate dehydrogenase (GDH) (L-glutamate–NAD(P) oxidoreductase, E.C. 1.4.1.2) were found in young grapefruit tissues. The importance of these enzymes to flavonoid biosynthesis in grapefruit tissues is discussed.

RESULTS

In reaction mixtures containing L-glutamate and phenylpyruvate, 2-ketoglutarate was identified as a product of the PKA catalyzed reaction by the TLC method. The product of

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

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the reaction had an R_f (0·61) indentical to that of 2-ketoglutarate. After 18 hr of incubation approximately 30% of the glutamate was converted to 2-ketoglutarate and there was a corresponding decrease in phenylpyruvate (R_f 0·90). In the reverse reaction the production of phenylpyruvate from L-phenylalanine and 2-ketoglutarate was detected by the spectral method of Scandurra and Cannella.[9] Phenylpyruvate production was linear with time for the initial 30 min of incubation. Boiled enzymic preparations failed to produce either 2-ketoglutarate from L-glutamate and phenylpyruvate or phenylpyruvate from L-phenylalanine and 2-ketoglutarate. PKA activity of a grapefruit roughly 10 g in size was 320 mμmole/min/fruit.

The presence of GDH in the grapefruit tissue was shown by identifying L-glutamate as a reaction product from 2-ketoglutarate. The product of the reaction had R_f values identical to those of L-glutamate in two solvents (0·23 and 0·47). The reaction was also followed by measuring the decrease in absorbance at 340 nm and it was found that the initial rate of the

TABLE 1. BIOSYNTHESIS OF *trans*-CINNAMATE FROM PHENYLPYRUVATE AND L-GLUTAMATE

Expt.	Incubation time*		
	2 hr	18 hr	Conversion‡
A. Complete†	0·30 μmole	0·45 μmole	12%
B. Complete system minus NADH and NADH regenerating system	0·32	0·60	15
C. Complete system but limited glutamate (0·1 μmole)	0·25	0·68	17
D. Complete system but limited glutamate (0·1 μmole) and minus NADH and NADH regenerating system	—	Trace	—

* After 2 and 18 hr incubation at 30°, 4 ml of the reaction mixture was taken for analysis of cinnamate.

† The complete system contained 10 μmole phenylpyruvate, 10 μmole L-glutamate, 1 μmole pyridoxal-5-phosphate, 500 μmole Tris buffer, pH 8·0, 10 μmole NH_4Cl, 1 μmole NADH, 10 μmole ethanol, 5 units alcohol dehydrogenase and 1 ml of the enzymic preparation in a total vol. of 10 ml.

‡ Based, after 18 hr incubation, on the amount of cinnamate formed from phenylpyruvate originally present in the reaction mixture.

reaction was linear with time under the conditions used. Boiled enzymes did not convert 2-ketoglutarate to L-glutamate. GDH activity of a grapefruit roughly 10 g in size was 77 mμmole/min/fruit.

The production of *trans*-cinnamate from phenylpyruvate and L-glutamate by grapefruit tissue extracts was demonstrated under various conditions. The results are shown in Table 1. The conversion of phenylpyruvate to *trans*-cinnamate was 12% for the complete system, 15% for the complete system minus NADH and the NADH regenerating system and 17% for the complete system which had only a limited amount of L-glutamate. Only a trace amount of *trans*-cinnamate was produced in the reaction mixture which contained a limited amount of L-glutamate in the absence of NADH and the NADH regenerating system.

DISCUSSION

While PKA,[6] GDH,[7,8] and PAL[4] have been reported previously in plant tissues, their coupled activities in a single plant cell-free extract have not been reported before, to our

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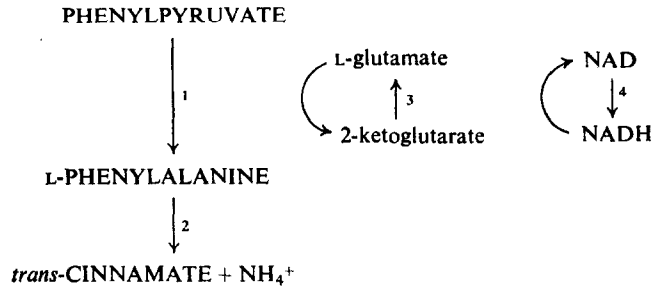
knowledge. Activities of these enzymes have been demonstrated, however, in bamboo tissue slices[10] by following conversion of exogenous phenylpyruvate to *p*-coumarate via phenylalanine and presumably cinnamate. We have shown that grapefruit tissues possess PKA, GDH, and PAL in sufficient amounts to account for appreciable *trans*-cinnamate synthesis from phenylpyruvate. This biosynthesis was demonstrated *in vitro* by using a cell-free extract of young grapefruit tissues (Fig. 1). In this system the PKA catalyzes the production of L-phenylalanine from phenylpyruvate with L-glutamate, the amino donor, being converted to 2-ketoglutarate. The L-phenylalanine is then converted to *trans*-cinnamate and ammonium ion by the catalytic action of PAL. L-Glutamate is regenerated from 2-ketoglulurate and ammonium ion by GDH with the concomitant oxidation of NADH to NAD. Consequently, the net reaction consumes 1 mole each of phenylpyruvate and NADH and produces 1 mole of *trans*-cinnamate. Exogenous alcohol dehydrogenase and ethanol were used to regenerate NADH from NAD.

This work demonstrates, at the enzyme level, the sequence in grapefruit tissue by which phenylpyruvate, the first aromatic compound formed in the shikimic acid pathway, can be



1. L-Phenylalanine:2-ketoglutarate aminotransferase.
2. L-Phenylalanine ammonia-lyase.
3. Glutamate dehydrogenase.
4. Alcohol dehydrogenase (added).

FIG. 1. BIOSYNTHESIS OF *trans*-CINNAMATE IN GRAPEFRUIT TISSUE EXTRACTS.

converted via L-phenylalanine to *trans*-cinnamate. Since grapefruit tissues were previously shown to lack tyrosine ammonia-lyase activity[2] the above pathway appears to be the primary route to the cinnamic acids in this tissue. Since the young fruits accumulate very substantial quantities of flavanone glycosides and only small amounts of the cinnamic acids, coumarins and lignins it would appear that a very large proportion of the cinnamic acid produced by these enzymes is metabolized further to the flavonoids. (Phenylalanine has been shown to be converted to flavonoids in young grapefruit leaves by ^{14}C tracer studies.)[11] The high activities of PKA, GDH and PAL, the presence of chalcone-flavanone isomerase,[1] and the direct relationship between PAL activity and the rate of naringenin glycoside accumulation in developing grapefruit[2] are in accord with this view.

The conversion of phenylpyruvate to *trans*-cinnamate via L-phenylalanine in grapefruit extracts requires an amino donor in the PKA catalyzed reaction. In our system, L-glutamate could play that role, but ammonium ion could not (Table 1). Thus, the complete system which contained sufficient glutamate (Expt. A) produced 0·45 μmole of cinnamate in 18 hr,

[10] T. HIGUCHI and M. SHIMADA, *Phytochem.* **8**, 1185 (1969).
[11] J. F. FISHER, *Phytochem.* **7**, 769 (1968).

whereas the system (Expt. D) with only limited glutamate (0·1 μmole and 10 μmole NH_4Cl) and without a glutamate-regenerating pathway produced only a trace of cinnamate. Since we have not yet studied the specificity of PKA, the involvement of amino donors other than L-glutamate *in vivo* should not be ruled out. The L-phenylalanine aminotransferase of *Phaseolus aureus* in the reverse reaction (phenylalanine to phenylpyruvate) utilized as amino receptors pyruvate and to some extent glyoxylate and oxalacetate in addition to 2-keto-glutarate.[6]

Further experiments pointed out the importance of GDH in *trans*-cinnamate synthesis and showed that the grapefruit extract contained GDH activity adequate to regenerate sufficient L-glutamate from 2-ketoglutarate to supply the PKA catalyzed reaction. In this case the amino donor was ammonium ion, but NADH was also required. Thus (Expt. C) the system with limited glutamate (0·1 μmole) but with NADH and the NADH regenerating pathway produced 6·8 times more cinnamate than the amount of glutamate originally present. However, the same system minus NADH and the NADH regenerating pathway (Expt. D) produced only a trace of cinnamate. These results show that regeneration of L-glutamate by GDH was taking place during the reaction. In the fruit, L-glutamate could be recycled in this manner from 2-ketoglularate and ammonium ion provided NADH was available. The high activity of GDH measured in grapefruit extracts, suggests that the tissues possess more than sufficient activity for this purpose.

The phenylpyruvate to L-phenylalanine step in the synthesis of *trans*-cinnamate is a point at which the products of other metabolic systems can interact and influence *trans*-cinnamate synthesis. Either amino donors must be available in amounts equimolar with phenylpyruvate or NADH must be available for recycling the donor.

EXPERIMENTAL

Material

Acetone powders were prepared by the procedure described previously[2] from approximately 10-g size grapefruit sampled in May from a tree (*Citrus paradisi* Macf. var. Marsh) growing in the desert region of California. The powders were stored at −20° until used.

Extraction of Enzymes

10 g of acetone powder plus 5 g of presoaked insoluble PVP were mixed with 100 ml of 0·1 M phosphate buffer, pH 7·0, containing 5×10^{-3} M of dithiothreitol. The residue after centrifugation was re-extracted with 50 ml of the same solution and the pooled extracts were passed through two columns (4·5 × 50 cm) of Sephadex G-25, coarse, to remove phenolic and other type compounds which interfere with enzymic reactions. Proteins were precipitated from the effluent with 0·9 saturation with $(NH_4)_2SO_4$, dissolved in a minimal portion of 0·05 M phosphate buffer, pH 7·0, and dialyzed against water at 2°. Unless otherwise stated, this dialysate which contained approximately 5 mg protein/ml was used as the enzyme preparation.

Partial Purification of PKA

The dialysate of the $(NH_4)_2SO_4$ precipitate was fractionated on a DEAE cellulose column (2·5 × 30 cm) equilibrated to pH 8·0 with 0·01 M Tris buffer. Proteins were eluted by a linear gradient system. A mixing chamber had 250 ml of 0·01 M Tris buffer, pH 8·0, and a reservoir had 250 ml of the same buffer containing 0·8 M NaCl. The effluent was collected in 10-ml fractions. The PKA activity was poorly retained in the column and was eluted in fractions 10–20, whereas the PAL was collected in fractions 67–78. All the preparative procedures were carried out below 5°.

Detection of PKA Activity

The presence of PKA was shown by measuring either the production of 2-ketoglutarate from L-glutamate and phenylpyruvate or the formation of phenylpyruvate from L-phenylalanine and 2-ketoglutarate. To identify 2-ketoglutarate as an enzymic product, the reaction was carried out as follows: the mixture contained $1{\cdot}5 \times 10^{-3}$ M each of L-glutamic acid and phenylpyruvic acid, 10^{-5} M of pyridoxal-5-phosphate, 0·05 M

Tris buffer, pH 8·0, and 1 ml of the enzyme preparation in a total vol. of 4·0 ml. After 18 hr of incubation at 30°, the mixture was brought to pH 2 with HCl, extracted with ethyl acetate, evaporated to dryness under reduced pressure and dissolved in methanol. The extract was analyzed for 2-ketoglutarate and residual phenylpyruvate on a TL plate of cellulose. The plate was developed with H_2O satd. *n*-BuOH–HCO_2H (95:5), and sprayed with a 0·1% solution of bromphenol blue in 95% ethanol saturated with Na_2CO_3.

In a reverse reaction, the enzymic formation of phenylpyruvate was followed by the method of Scandurra and Cannella.[9] To prevent the formation of *trans*-cinnamate the enzyme used in this experiment was free of PAL activity. The reaction mixture held at 30° contained $1·5 \times 10^{-3}$ M each of L-phenylalanine and 2-ketoglutaric acid, 10^{-5} M of pyridoxal-5-phosphate and the partially purified enzyme in a vol. of 10·0 ml. 1 ml samples were withdrawn at 10-min intervals. To the sample 2 ml of 1·75 N NaOH were added and its absorbance measured at 318 nm.

Detection of GDH Activity

GDH was detected by following the reductive amination of 2-ketoglutarate by measuring either the decrease in absorbance at 340 nm or the production of L-glutamate by TLC. The incubation mixture contained, in a total vol. 4 ml, 5×10^{-3} M each of 2-ketoglutaric acid, NH_4Cl and NADH, and 1 ml of the enzymic preparation. The reaction was carried out at pH 8·0 and 30°. After a 2-hr incubation, the mixture was acidified with HCl and transferred to the top of a 1·5 × 2·0 cm column of Dowex 50 (H^+ form). The column was washed thoroughly with water and was eluted with 0·2 M NH_4OH. The effluent was analyzed for glutamate on a TL plate of cellulose. Separate plates were developed with *n*-BuOH–HOAc–H_2O (4:1:5, upper phase), R_f 0·23, and MeOH–H_2O–pyridine (40:10:2) R_f 0·47, and sprayed with ninhydrin.

Biosynthesis of trans-*Cinnamate from Phenylpyruvate and* L-*Glutamate*

The coupled activities of PKA, GDH and PAL were studies using the reaction system given in Table 1 (footnote 2). After incubation the reaction mixture was brought to pH 2 with HCl, extracted with ethyl acetate, evaporated to dryness under vacuum, and the residue dissolved in measured vol. of methanol. Cinnamate content was determined by TLC analysis on silica gel (F-254) layers using C_6H_6–EtOAc–H_2O–HOAc (200:47:15:1). Cinnamate was detected as a shadow under short wavelength u.v. light and amounts were estimated by comparison with knowns. The enzymic production of cinnamate was carried out under the various conditions given in Table 1.