BIOLOGIA PLANTARUM (PRAHA)

12 (2) : 98—104, 1970

Hydroxylation Reactions in Roots of Maize (*Zea mays* L.)

TATIANA PŠENÁKOVÁ*, P. KOVÁCS, M. PŠENÁK and J. KOLEK

Department of Plant Physiology, Institute of Biology, Slovak Academy of Sciences and Biochemistry and Microbiology Department, Faculty of Pharmacy, Komenský's University, Bratislava

Received March 8, 1969

Abstract. The authors succeeded in demonstrating accumulation of tyrosine and p-coumaric acid in three day-old roots of maize (*Zea mays* L.) fed with L-phenylalanine and cinnamic acid. Phenylpyruvic acid applied under the same conditions gave rise to phenylalanine, indicating the presence of the corresponding transferase activity. Even simultaneous application of inhibitors of transaminase activity — hydroxylamine and isonicotinic acid hydrazide — did not result in the formation of p-hydroxyphenylpyruvic acid.

Higher plants contain a great number of various phenolic materials. It is very important to study the mechanism of origin of these substances both from the point of view of their qualitative presence and of their function as regulators of growth processes. Mono- and polyphenolic materials together with the corresponding phenoloxidases play an important role in some complex processes *e.g.* in biological oxidations, in host-parasite relationships and in metabolic regulation (RUBIN 1960, FARKAS and KIRÁLY 1962, FINKLE and RUNECKLES 1967).

There is a close relation between phenolic substances and aromatic amino acids; this relation arises both from the similarity of the mechanism of their formation and from the known possible conversion of these aromatic acids into some important phenolic substances. Hydroxylation is the key reaction in the conversion of aromatic substances into phenolic compounds. The hydroxylation on the level of phenylpropane compounds (of the type C_6-C_3 unit) has been studied by several authors (NAIR and VINING 1965, SATO 1966, RUSSELL and CONN 1967, ENGELSMA 1965, MCGALLA and NEISH 1959, WALKER 1964, KOVÁCS and JINDRA 1965, VAUGHAN and BUTT 1967). The entrance of hydroxyl groups into the structure of phenolic substances can occur as well on the level of C_6-C_3-C_6 type (diarylpropane unit) for instance with flavonoids (Grisebach 1967).

Until now these hydroxylation reactions have not been studied in the root

* *Address:* Dubravská cesta 26, Bratislava Czechoslovakia.

system of plants, especially of maize. In the present paper, hydroxylation of phenylalanine, cinnamic acid and phenylpyruvic acid was observed in three day-old maize roots fed with these substances and the results are presented.

Material and Methods

Material

We used 3 cm long roots of maize seedlings of the variety "Český bílý koňský zub". The maize seeds were soaked in tap water for 24 hours at laboratory temperature and afterwards transferred to moist filter paper and germinated for 72 hours in an incubator at 25° in the dark.

For each experiment, 3 g of isolated roots were vacuum infiltrated with 0·5% solution of phenylalanine and cinnamic acid and 0·1% solution of phenylpyruvic acid (pH of the solution was adjusted with 20% sodium carbonate to 6·5). An oil vacuum pump was used for infiltration: the air was exhausted for 3 minutes and the pump was then detached and the air was slowly introduced during 3 minutes. This process was repeated three times. The roots were washed after infiltration with distilled water and kept on wet filter paper in an incubator at 30° for 5 hours. Control samples serving for the determination of infiltrated phenylalanine and cinnamic acid were not incubated. After the incubation period, the roots fed with phenylalanine and cinnamic acid were extracted with 30 ml of 80% ethanol by homogenization in a mortar; roots fed with phenylpyruvic acid were homogenized. with 30 ml of 96% ethanol. The homogenates were extracted 12 hours at 0°. After centrifugation (5000 × g for 10 minutes), the supernatants were concentrated in vacuum at 20° to 2 ml.

Amino Acid Determination

Amino acids were determined after chromatographic separation using standard technique (Whatman No 1, three times repeated development in the system n-butanol-acetic acid-water (4 : 1 : 5), descending technique). Ninhydrine reagent was used for detection (Heillman and Barrolier 1957). After detection, the spots were eluted in 5 ml of methanol (30 minutes in the dark) and the resulting color was measured on a spectrophotometer K-51 at 495 nm.

Evidence and Determination of p-Coumaric Acid

The hydroxylation product of cinnamic acid was identified by paper chromatography and thin layer chromatography.

We used Whatman No 1 chromatographic paper and for ascending development the following systems were applied: a) isopropanol-ammonia-water (8 : 1 : 1, v/v), b) 2% acetic acid, c) 10% acetic acid, d) benzene-acetic acid-water (40 : 10 : 1, v/v), e) n-butanol-ammonia-water (40 : 5 : 5, v/v), f) 0·05 M phosphate buffer.

Chromatograms were detected with diazotized sulphanilic acid (Nair and Vining 1965). The resulting red spots of p-coumaric acid were eluted with 5 ml of water and quantitatively estimated at 445 nm. Cinnamic acid was detected with 1% solution of potassium permanganate (Bland and Logan 1967).

A layer of potato starch or silica gel 0·4 mm thick was prepared in the following way: 10 g of starch (silica gel) and 1·4 g of calcium sulphate powder were thouroughly mixed in 25 ml of water; the mixture was layered on a plate 15 × 15 cm which was dried at laboratory temperature for 15 hours. We used the following developing system; toluene-ethyl formate-formic acid (5 : 4 : 1, v/v, system — e). To achieve good separation, saturation of the developing chamber with vapours of the system used was necessary.

Chromatographic Analysis of Aromatic α-keto Acids

These acids were analysed in free form and also in the form of corresponding quinoxalinols which were prepared according to NIELSEN (1963). When free acids were analysed, the following systems were used: a) isopropanol-ammonia-water (8 : 1 : 1, v/v), b) benzene-propionic acid-water (2 : 2 : 1, v/v), c) n-butanol-acetic acid-water (4 : 1 : 5, v/v); p-hydroxyphenylpyruvic acid was detected with diazotized sulphanilic acid (NAIR and VINING 1965).

When quinoxalinol derivatives of keto acids were chromatographed, the following systems were used: a) methanol-chloroform-0·1 N sodium hydroxide (5 : 5 : 1, v/v), b) ethanol-chloroform-0·1 N sodium hydroxide (5 : 5 : 1, v/v), c) ethanol-0·1 N sodium hydroxide (10 : 1, v/v). Before the developing procedure, the chromatographic paper was sprayed with 0·1 N sodium hydroxide and dried in the air. Quinoxalinols of phenylpyruvic acid and p-hydroxyphenylpyruvic acid were identified under ultraviolet light using standards. The spots were intensified by spraying with 0·1 N sodium hydroxide.

Spectrophotometric Measurements

For analyses in the ultraviolet zone of light, we used a Unicam SP-800 spectrophotometer with 10 mm cells. The spectrum of tyrosine was measured after chromatographic separation and elution with 3 ml of ethanol. The homogeneity of the originating p-coumaric acid was evaluated using the same method. The chromatographic paper was washed before use several times with 70% acetone. The amount of infiltrated cinnamic acid was determined according a standard curve at 268 nm. After chromatographic separation and determination of the site of cinnamic acid on a guide strip of chromatogram by detection with potassium permanganate, the corresponding spots of cinnamic acid were eluted with 3 ml of ethanol. The extinction of eluates was measured at 268 nm.

Determination of Proteins

Proteins were determined according to LOWRY *et al.* (1951) using crystalline bovine serum albumin as standard protein.

Results

Results obtained on hydroxylation of phenylalanine are given in Table 1 and Fig. 1.

Table 2 and Fig. 2 represent results obtained on hydroxylation of cinnamic acid.

TABLE 1
INFILTRATION EXPERIMENTS with phenylalanine

Protein (mg)	μmoles infiltrated phenylalanine	μmoles infiltrated phenylalanine/mg protein	μmoles formed tyrosine/mg protein	μmoles formed tyrosine/μmole infiltrated phenylalanine
15·6	7·8	0·503	0·033	0·064

The hydroxylation product of cinnamic acid — p-coumaric acid — was also identified by paper chromatography using various developing systems and detection reagents as well as by thin layer chromatography. The results obtained are given in Tables 3 and 4.

TABLE 2
INFILTRATION EXPERIMENTS with cinnamic acid

Protein (mg)	μmoles infiltrated cinnamic acid	μmoles infiltrated cinnamic acid/mg protein	μmoles formed p-coumaric acid/mg protein	μmoles formed p-coumaric acid/μmole infiltrated cinnamic acid
15·6	13·5	0·87	0·14	0·155

When free aromatic α-keto acids and their quinoxalinol derivatives were chromatographed, it appeared that the hydroxylation on the level of phenylpyruvic acid did not take place in the observed material under the given

Fig. 1.

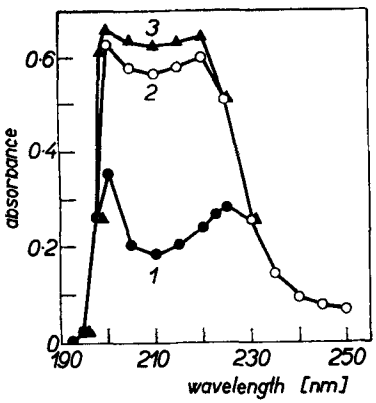

Fig. 2.

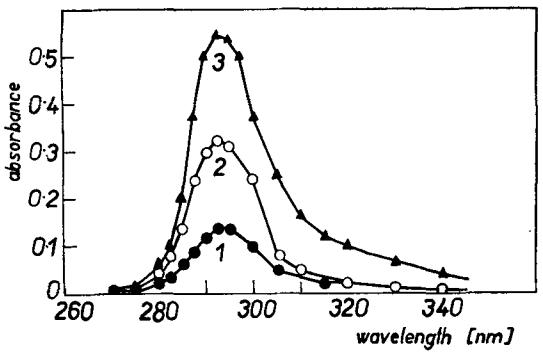

Fig. 1. Absorption spectra of tyrosine: 1 — authentic sample, 2 — tyrosine from control and 3 — from phenylalanine fed roots.

Fig. 2. Absorption spectra of p-coumaric acid: 1 — authentic sample, 2 — p-coumaric acid from control and 3 — from cinnamic acid fed roots.

TABLE 3

SOME PROPERTIES of the product as compared with p-coumaric acid

Test	Compound	
	p-coumaric acid	product
R_f values in solvent a.	0·42	0·42
b.	0·40	0·40
c.	0·50	0·50
d.	0·47	0·47
Colour with reagent 1.	red	red
2.	white on violet background	white on violet background
Fluorescence in u. v. light		
$-NH_3$	—	—
$+NH_3$	dark blue	dark blue

Reagents: 1 — diazotized sulphanilic acid
2 — potassium permanganate

conditions. p-Hydroxyphenylpyruvic acid did not originate in experimental samples which were incubated with phenylpyruvic acid, nor was the infiltrated phenylpyruvic acid present.

We tried to determine whether transamination of phenylpyruvic acid takes place in the material studied. For this reason we determined the content of phenylalanine in samples which were fed with the corresponding acid, using samples fed with water as control. Chromatographic analysis showed

TABLE 4

SOME PROPERTIES of product as compared with p-coumaric acid, caffeic acid and ferulic acid on thin layer chromatography

Test	Compound			
	p-coumaric acid	caffeic acid	ferulic acid	product
R_f values in solvent e	0·82	0·69	0·90	0·82
Fluorescence in u. v. light $-NH_3$	—	blue	blue	—
$+NH_3$	dark blue	blue	blue	dark blue

that the experimental sample contains 0·05 μmoles of phenylalanine/1 mg of protein, more than in control samples. Although this result indicates the conversion of phenylpyruvic acid into phenylalanine, we used some inhibitors of transaminases to suppress the possible competition for this substrate between transaminase and the corresponding hydroxylase of phenylpyruvic acid. As inhibitors, we used 0·1 M solution of hydroxylamine, and hydrazide of isonicotinic acid, which were infiltrated into the material together with sodium phenylpyruvate. In these experiments, phenylalanine was not formed

and when quinoxalinols of aromatic α-keto acids were chromatographed, the presence of phenylpyruvic acid was demonstrated. p-Hydroxyphenylpyruvic acid was not found even under these conditions.

Discussion

The results obtained show that hydroxylation of phenylalanine and cinnamic acid takes place in 3 day-old roots of maize (*Zea mays* L.) variety "Český bílý koňský zub". Comparing reciprocal ratios of the originating products to the respective substrates — μmoles Tyr/μmoles Phe, respectively μmoles p-coumaric acid/μmoles cinnamic acid, as well as amount of originating products to 1 mg of proteins (tables 1 and 2) — it is evident that hydroxylation of cinnamic acid is more intensive. This finding agrees with the opinion of other authors (BATE-SMITH and SWAIN 1965, MCCALLA and NEISH 1959) concerning the secondary importance of phenylalanine hydroxylation in production of phenolic compounds in plants. It is very probable that the primary change in phenylalanine in the material studied is deamination, with simultaneous origin of cinnamic acid which undergoes intense hydroxylation.

When seeking evidence for hydroxylation of phenylpyruvic acid in the presence of transaminase inhibitors, we obtained negative results; this result is in agreement with the fact that hydroxylation on the level of phenylpyruvic acid has not as yet been demonstrated. When phenylpyruvic acid was infiltrated accumulation of phenylalanine took place; this indicates the presence of phenylpyruvic acid transaminase.

Another recent investigation deals with isolation, purification and separation of phenylalanine transminase and deaminase as well as the study of some properties of these enzymes from roots of 3 day-old maize plants (PŠENÁKOVÁ *et al.* 1969).

References

BATE-SMITH, E. C., SWAIN, T.: Recent development in the chemotaxonomy of flavonoid compounds. — Lloydia **28** : 313—331, 1965.

BLAND, D. E., LOGAN, A. F.: The metabolism of phenylalanine and cinnamoyl compounds. — Phytochemistry **6** : 1075—1083, 1967.

ENGELSMA, G.: Photoinduced hydroxylation of cinnamic acid in gherkin hypocotyls. — Nature **208** : 1117—1119, 1965.

FARKAS, G. L., KIRÁLY, Z.: Role of phenolic compounds in physiology of plant disease and disease resistance. — Phytopath. Z. **44** : 105—150, 1962.

FINKLE, B. J., RUNECKLES, V. C.: Phenolic Compounds and Metabolic Regulation, Appleton Century Crofts, New York, 1967.

GRISEBACH, H.: Biosynthetic Patterns in Microorganisms and Higher Plants. — John Wiley and Sons, Inc., New York, 1967.

HEILLMAN, J., BARROLIER, J., WATZKE, E.: Beitrag zur Aminosäurebestimmung auf Papierchromatogrammen. — Z. Physiol. Chem. **309** : 219—220, 1957.

KOVÁCS, P., JINDRA, A.: Biosynthesis of alkaloids. On the transformation of tyrosine to 3,4-dihydroxyphenylalanine in *Papaver somniferum* L. plants. — Experimentia **21** : 18—19, 1965.

LOWRY, O. H., ROSENBROUGH, N. J., FARR, A. L.: Protein measurement with Folin phenol reagent. — J. biol. Chem. **193** : 265—275, 1951.

MCCALLA, D. R., NEISH, A. C.: Metabolism of Phenolpropanoid compounds in *Salvia*. — Canad. J. Biochem. Physiol. **37** : 531—547, 1959.

NAIR, P. M., VINING, L. C.: Phenylalanine hydroxylase from spinach leaves. — Phytochemistry **4** : 401—411, 1965.

NAIR, P. M., VINING, L. C.: Cinnamic acid hydroxylase in spinach. — Phytochemistry **4** : 161—118, 1965.

NIELSEN, K. H.: Paper chromatographic determination of aromatic α-ketoacids. — J. Chromat. **10** : 463—472, 1963.

PŠENÁKOVÁ, T., KOVÁCS, P., PŠENÁK, M.: Transaminierung und Desaminierung von Phenylalanin in Wurzeln von *Zea Mays*. — Phytochemistry **8** : 2277 — 2280, 1969.

RUBIN, B. A.: Ideji V. I. Palladina i sovremennoje sostojanije učenija o dychaniji rastenij. — Ukr. biochim. Ž. **32** : 295—613, 1960.

RUSSELL, D. W., CONN, E. E.: The cinnamic acid 4-hydroxylase of pea seedlings. — Arch. Biochem. Biophys. **122** : 256—258, 1967.

SATÔ, M.: Conversion by the isolated chloroplasts of p-coumaric acid to caffeic acid. — Phytochemistry **5** : 385—389, 1966.

VAUGHAN, P. F. T., BUTT, V. S.: Electron-donor requirements in the hydroxylation of p-coumaric acid. — Biochem. J. **104** : 65—66P, 1967.

WALKER, J. R. L.: Properties of apple polyphenoloxidase. — Austr. J. biol. Sci. **17** : 360—370, 1964.

TATIANA PŠENÁKOVÁ, PETER KOVÁCS, MIKULÁŠ PŠENÁK a JOZEF KOLEK, Biologický ústav SAV, Oddelenie fyziologie rastlín a Katedra biochémie a mikrobiológie Farmaceutickej fakulty Univerzity Komenského Bratislava: **Hydroxylačné reakcie v koreni kukurice *Zea Mays* L.** — Biol. Plant. **12** : 98—104, 1970.

V trojdňových koreňoch kukurice *Zea mays* L. sa po infiltrácii L-fenylalanínu a kyseliny škoricovej podarilo dokázať nahromadenie ich hydroxylačných produktov, t. j. tyrozínu a kyseliny p-kumárovej. Za rovnakých podmienok použitá kyselina fenylpyrohroznová viedla k vzniku fenylalanínu, čo poukazuje na prítomnosť príslušnej amínotransferázovej aktivity. Ani súčasná aplikácia inhibítorov transaminačných reakcií — hydroxylamínu a hydrazidu kyseliny izonikotínovej — nemala za následok tvorbu kyseliny p-hydroxyfenylpyrohroznovej.