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Microsomal Flavonoid 3′-Monooxygenase from Maize Seedlings[1]

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ABSTRACT

Identification of flavonoid 3′-monooxygenase establishes another reaction in the biosynthesis of flavonoid compounds in maize (*Zea mays* L.). The flavonoid 3′-hydroxylase was obtained as a microsomal enzyme preparation by buffer extraction of 5 day old maize seedlings and ultracentrifugation. Seedlings were exposed to light 24 hours prior to enzyme extraction. The extraction buffer required the addition of sucrose or glycerin and dithiothreitol to obtain an active hydroxylase that retained its activity on storage at −70°C. Enzymic activity required O_2 and NADPH, was optimum at pH 8.5 and 30°C, and could be inhibited 79% by carbon monoxide. Carbon monoxide inhibition could be reduced to 21% by irradiation of the samples with 450 nanometer light during incubation. Kaempferol, a flavonol; naringenin, a flavanone; and apigenin, a flavone, all served as substrates for the hydroxylase. Treatment of the microsomal enzyme preparation, previously reduced with sodium dithionite, with carbon monoxide gave a 455 nanometer absorption peak which disappeared on oxidation of the preparation with the formation of a 420 nanometer peak. These results suggest a cytochrome P-450 type monooxygenase enzyme. The concentration of cytochrome P-450 was 0.21 nanomoles per milligram protein. Identification of the monooxygenase provides further biochemical information about a biosynthetic sequence for which the genetics have been studied intensely.

The principal anthocyanins found in maize (*Zea mays* L.) are Pg-gl[2] and Cy-gl (3). These compounds differ structurally in that an additional hydroxyl group is found at the 3′-position in the B-ring of Cy-gl. Structures for these and the other substrates and products considered in this paper are given in Table I. The timing of the addition of this hydroxyl group remains a point of discussion since there is evidence for this to occur either at the 9- or 15-carbon stage of synthesis. Hess (12) observed the incorporation of substituted cinnamic acids, without further modification, into anthocyanin and proposed the 'cinnamic acid starter hypothesis', which suggested hydroxylation occurred at the 9-carbon level. Subsequently, Fritsch and Grisebach (9) observed 3′-hydroxylation of naringenin and eriodictyol by a microsomal preparation obtained from *Haplopappus gracilis*. Similar 3′-hydroxylation was observed in microsomal preparations obtained from *Matthiola incana* (7), snapdragon (*Antirrhinum majus*) (8), and cell cultures of parsley (*Petroselinum hortense* Hoffm.) (2, 11). Although the enzyme preparation had been suggested to be a heme-containing monooxygenase (7), Hagmann *et al.* (11) identified the activity as a Cyt P-450 containing enzyme. The 3′-hydroxylases referred to here all required O_2 and NADPH as the electron donor. The only report of hydroxylase activity at the 9-carbon level in any of the preparations was in that from *H. gracilis* (9).

Although the genetics of anthocyanin biosynthesis in maize has been studied extensively, the only enzyme activities to be demonstrated have been chalcone synthase (5) and UFGT (13). The subject of this report is the extraction and partial characterization of flavonoid 3′-hydroxylase, a microsomal enzyme involved in anthocyanin biosynthesis in maize.

MATERIALS AND METHODS

Plant Materials. Seeds were geminated in the dark at 30°C, on filter paper saturated with 1 mM $CaSO_4$, and grown for a period of 4 d after which they were kept under cool-white fluorescent light for 24 h prior to enzyme extraction. The seedlings were washed in distilled H_2O and separated into roots and the shoots, which included mesocotyl minus roots and any further growth beyond the first internode. Unless otherwise indicated the seed was of a uniform, defined genetic background which included all the genetic factors necessary for anthocyanin synthesis.

Chemicals. Chemicals used in this study were readily available commercially with the exception of eriodictyol which was obtained from Sarsynthese[3], Avenue du President J.-F. Kennedy,

Table I. *Chemical Structures of the Flavonoid Compounds Involved in the Hydroxylation Reaction*

Compound	Hydroxylation	Basic Structure
4-Keto-2,3-dihydro-		
Naringenin[a]	5,7,4′	
Eriodictyol[a]	5,7,3′,4′	
4-Keto-2,3-dehydro-		
Apigenin[b]	5,7,4′	
Luteolin[b]	5,7,3′,4′	
Kaempferol[c]	3,5,7,4′	
Quercetin[c]	3,5,7,3′,4′	
4-Deoxy-2,3-dehydro-		
Pelargonidin[d]	3,5,7,4′	
Anthocyanidin	3,5,7,3′,4′	

[a] Flavanone. [b] Flavone. [c] Flavonol. [d] Anthocyanidin.

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[2] Abbreviations: Pg-gl, pelargonidin 3-*O*-glucoside; Cy-gl, cyanidin 3-*O*-glucoside; UFGT, uridinediphosphoglucose:flavonoid glucosyltransferase; Ches, 2(*N*-cyclohexylamino)ethane sulfonic acid; NEM, *N*-ethylmaleimide; PCMB, *p*-chloromercuribenzoate.

[3] Mention of a trademark, proprietary product, or vendor does not constitute a guarantee of warranty by the United States Department of Agriculture or the University of Missouri and does not imply approval to the exclusion of other products or vendors that may also be suitable.

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Enzyme Preparation. Plant material (7.5 g) was homogenized for 10 s in a Waring Blendor containing 40 ml of 0.05 M Bicine buffer (pH 8.5) (containing 0.8 M sucrose and 5.0 mM DTT) and 1.8 g Polyclar AT. The homogenate was then filtered through Pellon and the filtrate centrifuged for 15 min at 26,000*g*. The resulting supernatant (crude extract) was brought to 0.03 M $MgCl_2$, stirred for 20 min, and centrifuged at 190,000*g* for 30 min. The pellet constituted the microsomal fraction (4). All the steps were carried out at 4°C as nearly as possible.

Hydroxylase Assay. The assay contained 0.372 mM NADPH, 50 mM Bicine buffer (pH 8.5) (+ 2.0 mM DTT), 0.35 mM kaempferol and enzyme (about 200 μg protein) in a total volume of 200 μl. Reaction mixtures were thoroughly mixed, incubated at 30°C for 30 min with shaking in open Eppendorf centrifuge tubes, and the reactions terminated as described below. The reactions, in which kaempferol and apigenin were converted to quercetin and luteolin, were terminated by the addition of 800 μl of a 2:1 (v/v) mixture of chloroform:methanol (containing 1% HCl) and mixing. The resulting biphasic solutions (6) were then centrifuged for 30 s at 15,000*g* which yielded the quercetin and luteolin in the 400 μl methanolic upper phase. The assay for the conversion of naringenin to eriodictyol was terminated by addition of the reaction mixture to a C_{18} μBondapak Sep-Pak column. The eriodictyol bound on the Sep-Pak was then eluted with successive fractions composed of 1.5 ml of water, 0.5 ml of methanol, and 0.5 ml of methanol:acetic acid (9:1, v/v). The latter fraction was analyzed for eriodictyol by HPLC. Specific activity of the hydroxylase is defined as pmol of product formed per min per mg protein.

Apparent K_m values were determined for NADPH and kaempferol by linear regression analysis. In determining the K_m value for kaempferol, two levels of protein (197 and 394 μg/sample) were used in separate determinations, kaempferol concentrations ranging from 8.7 to 349 μM and NADPH at 372 μM. The K_m value for NADPH was determined at a 35 μM concentration of kaempferol, 240 μg of protein, and a range of concentrations of NADPH from 12 to 125 μM.

Identification of the Reaction Products. Reaction products were identified using a Waters model 244 HPLC equipped with a C_{18} μBondapak column and a model 440 absorbance detector. Quercetin and kaempferol could be separated by injection of an aliquot of the Folch partition, described above, followed by elution with methanol:acetic acid:water (30:10:60; v/v/v), and detection at 365 nm. The resulting k' values were 3.2 and 4.0 for quercetin and kaempferol, respectively. Apigenin and luteolin were separated in a manner similar to that for quercetin and kaempferol using a 340 nm detector with the resulting k' values of 2.7 and 4.5 for apigenin and luteolin, repectively. Naringenin and eriodictyol could be separated by injection of an aliquot from the sep-pak followed by elution with methanol:acetic acid:water (15:10:75; v/v/v) and detection at 280 nm. The resulting k' values were 3.2 and 6.2 for eriodictyol and naringenin, respectively.

Inhibitors. Inhibitors were added to the reaction mixture at 2.5 and 5.0 mM concentrations with the exception of ferric chloride (10–100 μM), NEM (20–40 μM), and Cyt *c* (10–40 μM). The exclusion of O_2 was accomplished by vigorously flushing the reaction mixture with N_2 gas prior to and after adding the substrate, then sealing the tube and incubating the sample. Inhibition of the hydroxylase reaction by CO and the subsequent effect of light on this CO-inhibition were assayed in the following manner. The assay mixtures, which were prepared minus kaempferol, were thoroughly saturated with CO (30 s), kaempferol was added, and the resulting solutions were saturated with CO (30 s). The separate samples, plus a standard assay sample, were incubated under light of 450 nm wavelength or in the dark. Light of this wavelength was obtained by a combination of light filters which had a maximum transmittance at 443 nm and 5% less transmittance at 450 nm.

pH Optimum Determination. The optimum pH for the hydroxylase was determined by adjusting the pH of the reaction mixtures using Hepes from 6.5 to 7.5, Bicine from 7.8 to 8.8, and Ches from 9.3 to 9.8.

Carbon Monoxide Difference Spectra of the Microsomes. Difference spectra, in the 400 to 500 nm range, were obtained for the microsomal preparation by the method of Omura and Sato (16). Microsomal protein was suspended (about 2 mg/ml) in the extraction buffer, and both sample and blank cuvettes (1 cm light path) were reduced by the addition of sodium dithionite and base line absorbance was recorded. CO was then bubbled

Table II. *Flavonoid 3′-Hydroxylase Activity and Electron Donor Requirement*

Enzyme Fraction	Addition[a]	Specific Activity[b]
Crude	None	0
	NADPH	66.96
Microsomal	None	0
	NADPH	200.88
	NADPH generating system	177.12
	NADH	0

[a] Electron donor added at 372 μM or equivalent level. [b] Specific activity = pmol of product/mg protein·min.

Table III. *Effect of Inhibitors and Sulfhydryl Reagents on Flavonoid 3′-Hydroxylase Activity*

Assay medium is described in "Materials and Methods".

Addition	Concentration	Activity	Control
	mM	*pmol/min*	*%*
Control		40.6	100
EDTA	5.0	46.5	115
Diethyldithiocarbamate	5.0	40.6	100
Potassium cyanide	5.0	43.0	106
NaN_3	5.0	35.8	88
α,α′-Dipyridyl	5.0	30.6	75
p-Chloromercuribenzoate	5.0	26.2	26
Ferric chloride	0.094	0	0
N-Ethylmaleimide	2.5	0	0
NADP/NADPH	0.186/0.372	34.3	84
NADP/NADPH	0.372/0.372	27.7	68
NADP/NADPH	0.744/0.372	17.0	42
Cyt *c*	0.01	29.1	71
Cyt *c*	0.02	8.7	22
Cyt *c*	0.03	0	0

Table IV. *Flavonoid 3′-Hydroxylation: Inhibition by CO and its Reversal by Light, and the O_2 Requirement*

Kaempferol was the substrate, other assay conditions are described in the text.

Treatment	Activity[a]	Control
		%
None	109.8	100
Carbon monoxide, light[b]	86.5	79
Carbon monoxide, dark[b]	23.2	21
Nitrogen, air[c]	25.8	23

[a] Activity, pmol of quercetin produced per min. [b] Samples were flushed with CO for 30 s prior to and after adding the substrate. [c] Sample was flushed with N_2 and sealed for the incubation to exclude air.

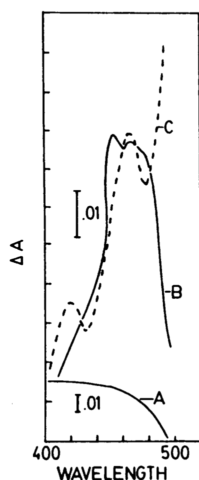

FIG. 1. CO difference spectra of maize microsomes. Curve A, dithionite-reduced microsomes: both the sample and reference cells contained a microsomal suspension (2 mg protein per ml in 0.05 M Bicine buffer [pH 8.5] containing 0.8 M sucrose + 5 mM DTT). Curve B, spectrum obtained after treating the sample cell with CO; curve C, spectrum after treating the sample cell (CO-treated) with air.

through the sample cuvette for 2 min and the difference spectrum recorded, after which air was vigorously bubbled through the sample and the spectrum recorded. Cyt P-450 content in the enzyme preparations was determined from the CO-difference spectrum by the method of Omura and Sato (16) using 91 mM^{-1} as the extinction coefficient. CO was produced by heating zinc metal and calcium carbonate (20 mmol:10 mmol) according to the method of Weinhouse (18).

Protein Assay. Protein was analyzed by the method of Bradford (1) using BSA, fraction V, as the standard. Microsomal preparations were homogenized in a Potter-Elvehjem tissue grinder prior to analysis.

RESULTS

Microsomal preparations were used to determine the properties of the corn seedling flavonoid 3′-hydroxylase described here. Although hydroxylase activity could be assayed in the crude extract, the specific activity was 3-fold higher in the microsomal precipitate (Table II). The addition of sucrose and DTT to the extraction medium resulted in a near doubling of hydroxylase activity over that observed when these were omitted (data not shown). Shoots of the young seedlings were used as the source of the enzyme, although the hydroxylase was also found at a lower concentration in the roots. The hydroxylase could also be found at a very low level in the aleurone layer of immature seeds that had been harvested 20 d after pollination and stored at −70°C. No hydroxylase could be extracted from other tissues of maize, *e.g.* aleurone of mature seeds, dry seeds, and albino seedlings grown in the greenhouse, nor could activity be detected when the seedlings were frozen in liquid N_2 and the resulting powder extracted for enzyme. The electron donor requirement could be satisfied with either NADPH or an NADPH generating system (Table II). Hydroxylation was inhibited by NADP (Table III) and no activity was observed when NADH was added as the electron donor (Table II). Catalytic activity was readily measured when kaempferol was used as the substrate but no activity could be detected when the corresponding flavanonol, dihydrokaempferol, was used as the substrate. Qualitative evidence was obtained for the hydroxylation of naringenin and apigenin to eriodictyol and luteolin, respectively. Apparent K_m values of 7.15 μM and 5.8 μM were determined for kaempferol and NADPH, respectively. No activity could be detected, by a previously published method (14), for hydroxylation at the 9-carbon level (*p*-coumaric acid to caffeic acid).

Enzyme activity was linear over a 60 min incubation period at 30°C. The reaction was also linear with added enzyme up to 300 μg protein per sample. The optimum pH for the hydroxylase reaction was determined to be 8.5 with a sharp drop in activity on either side of the optimum. Deletion of DTT from the assay mixture had little effect on activity if DTT had been added to the extraction medium. The hydroxylase was stable when stored at −70°C if sucrose had been added to the extraction medium. Enzyme activity could be destroyed by heating the preparation in a boiling water bath for 10 min.

The effects of a series of chelating agents and inhibitors are summarized in Table III. NaN_3, α,α',-dipyridyl, and PCMB produced limited inhibition, while $FeCl_3$ and NEM inhibited the reaction totally. Addition of Cyt *c* to the assay medium at 10, 20, and 30 μM levels resulted in 28, 78, and 100% inhibition, respectively, of the hydroxylation reaction. Exclusion of O_2 by vigorously flushing the reaction mixture with N_2 gas resulted in a 77% decrease in activity (Table IV). Exposure of the assay mixtures to CO prior to incubation resulted in 79% inhibition of the hydroxylation found in the nontreated samples. This CO inhibition could be reduced to 21% when the CO-exposed sample was incubated under light of 450 nm wavelength (17). The absorbance spectra in Figure 1 show the effects of reduction of the microsomal enzyme with sodium dithionite (curve A), the effects of CO on the reduced sample (curve B), and the effect of air on the CO-treated sample (curve C). The reduced enzyme preparation gave a base line trace, whereas the CO-treated enzyme showed major absorption peaks at 455 and 468 nm. The air-treated preparation showed major absorption peaks at 420 and 468 nm, but the 455 nm absorption peak had disappeared. Calculation of the concentration of Cyt P-450, by the method of Omura and Sato (16) using 91 cm^{-1} mM^{-1} as the extinction coefficient resulted in 0.21 nmol/mg of protein.

DISCUSSION

Flavonoid 3′-hydroxylase, an important enzyme in the biosynthesis of flavonoid compounds in maize, has been isolated and partially characterized from maize (*Zea mays* L.). The anthocyanins are the major compounds of this type that are synthesized in maize. This enzyme has been studied in other plant species, *e.g. H. gracilis* (9), *M. incana* (7), snapdragon (*A. majus*) (8) and parsley (*P. hortense*) (2, 11) and the results of its activity *in vivo* are well known in maize (3). The maize hydroxylase is very similar to the hydroxylases studied in other species in that all are microsomal type preparations, required NADPH as an electron donor, are inhibited by NADP, and require O_2 in the reaction. Although there are differences in the substrates utilized by the enzymes from the several sources, this may be a reflection of the extent of characterization of the various enzymes. The maize hydroxylase appeared to be most active with kaempferol, with an apparent K_m for kaempferol of 7.15 μM, but did hydroxylate apigenin and naringenin to luteolin and eriodictyol, respectively, making it active on flavonols, flavones, and flavanones. The pH profile observed for the maize hydroxylase, over a range of 6.5 to 9.8, had an optimum at 8.5, dropping off sharply on either side of 8.5, which is one unit higher than that recorded for the other plant hydroxylases considered above. Degradation of the added NADPH occurred at the lower end of this pH range. The oxidized form of the electron donor, $NADP^+$ is a competitive inhibitor of NADPH-Cyt reductase (15), which is involved in electron transfer in the hydroxylation reaction. Addition of KCN, which typically inhibits heme enzymes (10), was shown to enhance hydroxylation by the maize enzyme. Not surprisingly, Cyt *c* inhibited hydroxylation since it can serve as an electron acceptor in the microsomal electron system. The most obvious

difference between enzymes from other sources and the maize hydroxylase is in the effect of NEM (2.5 mM), which is totally inhibitory to the maize enzyme, but enhances the activity of the parsley enzyme at a 1 mM concentration (11). The Cyt P-450 complex includes a reductase function (11); thus, inhibition of hydroxylation by NEM was an expected result. Although the results of the assay of those inhibitors that are common to all the investigations vary somewhat, the differences are minimal.

The effect of added Cyt *c* on hydroxylase activity coupled with the effect of CO and light on activity strongly suggest that the maize enzyme is a Cyt P-450 monooxygenase. The CO-difference spectral data, the reversal of CO-inhibition by light, and the requirement for O_2 observed for the maize hydroxylase meet three of the requirements, given by West (19), that characterize a reaction catalyzed by a Cyt P-450 dependent oxygenase.

Characterization of this hydroxylase should facilitate the study of gene action in maize. This information coupled with available mutants should be useful in identifying the structural gene for the enzyme, and permit the study of modifying genes that are abundant in maize.

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