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HYDROXYLATION OF FLAVONOIDS BY A PHENOLASE PREPARATION FROM LEAVES OF SPINACH BEET

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Abstract—An enzyme preparation from leaves of spinach beet (*Beta vulgaris* L. ssp. *vulgaris*) which catalyses the hydroxylation of *p*-coumaric acid to caffeic acid is also able to catalyse the hydroxylation of naringenin, dihydrokaempferol and kaempferol at the 3′-position to eriodictyol, dihydroquercetin and quercetin, respectively.

INTRODUCTION

AN ENZYME preparation which catalyses the hydroxylation of *p*-coumaric acid to caffeic acid was extracted from leaves of spinach beet (*Beta vulgaris* L. ssp. *vulgaris*) and purified.[1] Hydroxylation of 4′-hydroxyflavonoids *in vitro* have so far not been investigated with enzyme preparations from higher plants. In view of the interest attached to the stage at which the oxygenation pattern of the B-ring of flavonoids is determined, whether by hydroxylation of cinnamic acid or a C_{15}-intermediate, the ability of this enzyme to catalyse the hydroxylation of 4′-hydroxyflavonoids in the B-ring was examined.

RESULTS

In experiment 1A (Fig. 1), 90 m-units[2] of enzyme (purified to stage III) was incubated under air at 30° with kaempferol (1 μmole), ascorbate (10 μmoles), $(NH_4)_2SO_4$ (1500 μmoles) and Na_2HPO_4 (100 μmoles)/citric acid (40 μmoles) buffer, pH 5·3, in a total volume of 3 ml. In experiment 1B (Fig. 1), the partially purified enzyme was replaced by 90 m-units of enzyme purified further by chromatography on a CM-cellulose column.

In experiments 2A and 2B (Fig. 2), 64 m-units of the purified enzyme were used, and kaempferol was replaced by naringenin (1 μmole) under the conditions of experiment 1A.

In experiments 3A and 3B (Fig. 3), 68 m-units of the purified enzyme were used, with dihydrokaempferol (1 μmole) as substrate under the conditions of experiment 1A.

[1] P. F. T. VAUGHAN and V. S. BUTT, *Biochem. J.*, in press.

[2] 1 unit of enzyme activity is defined as the amount of enzyme catalysing the formation of 1 μmole caffeic acid per min from 10 μmoles *p*-coumaric acid incubated with ascorbate (10 μmoles), ammonium sulphate (1500 μmoles) and Na_2HPO_4 (100 μmoles)/citric acid (40 μmoles) buffer, pH 5·3, in a total volume of 3 ml under air at 30°.

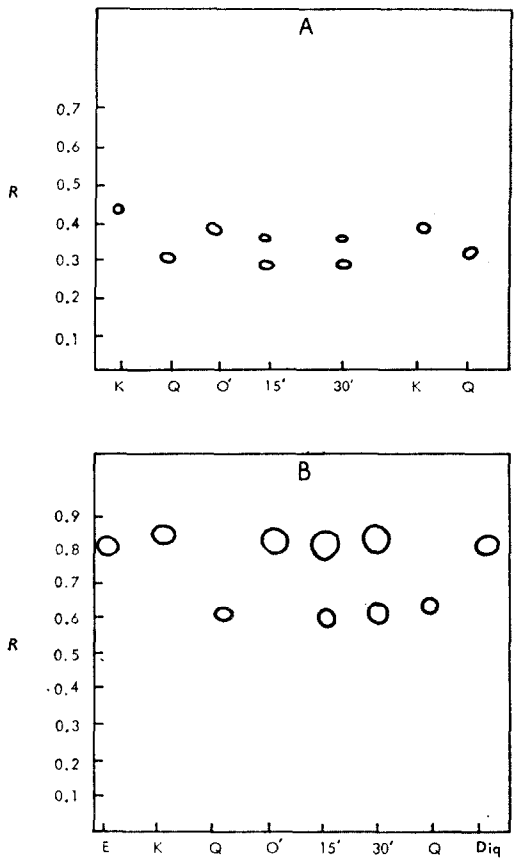

FIG. 1. THIN-LAYER CHROMATOGRAM AFTER INCUBATION WITH KAEMPFEROL AS SUBSTRATE.
K, kaempferol; Q, quercetin; Diq, dihydroquercetin; E, eriodictyol. 0, 15 and 30 refer to incubation period (min). A, developing solvent: toluene/ethyl formate/formic acid (50:40:10). B, developing solvent: chloroform/ethyl acetate/formic acid (50:40:10).

TABLE 1. R_f VALUES AND COLOURS OF SUBSTRATES AND PRODUCTS ON TLC*

Compound	Solvent system†		Colour	
	A	B	Without reagent	With sodium molybdate
Kaempferol	0·44	0·82	Yellow-green	—
Quercetin	0·34	0·61	Yellow (A) Yellow-grey (B)	—
Naringenin	0·42	0·85	Pink-brown (B)	Pink (A)
Eriodictyol	0·33	0·72	Grey (B)	Brown (A)
Dihydroquercetin	—	0·76	Buff (B)	—

* Silica gel with sodium acetate.[3]
† Key: A = toluene/ethyl formate/formic acid (50:40:10).[3]
B = chloroform/ethyl acetate/formic acid (50:40:10).[3]
Spray reagent: 0·1 % aqueous sodium molybdate.

[3] E. STAHL and P. J. SCHORN, *Hoppe-Seyler's Z. Physiol. Chem.* **325**, 263 (1961).

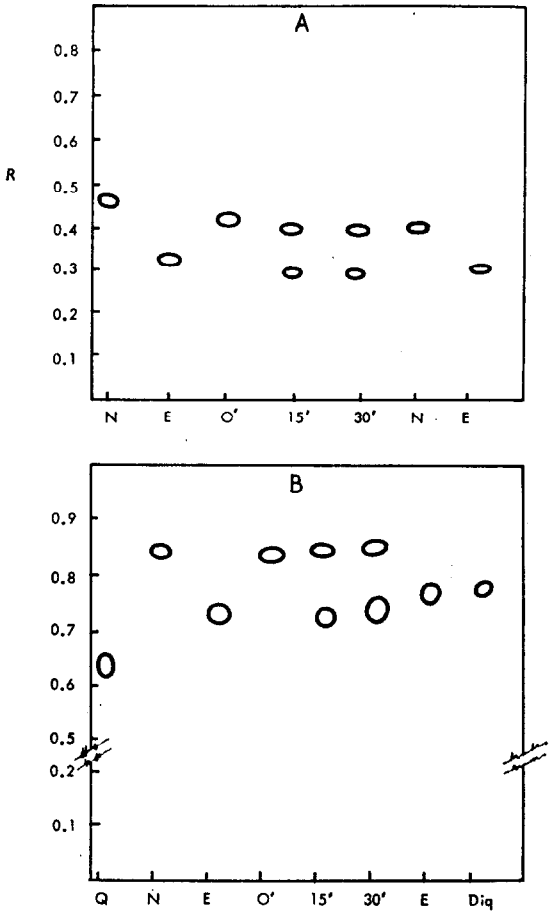

FIG. 2. THIN-LAYER CHROMATOGRAM AFTER INCUBATION WITH NARINGENIN AS SUBSTRATE.
N, naringenin; E, eriodictyol; Q, quercetin; Diq, dihydroquercetin. 0, 15 and 30 refer to incubation period (min). Developing solvents in A and B as in Fig. 1A and 1B respectively.

TABLE 2. R_f VALUES AND COLOURS OF DIHYDROKAEMPFEROL AND DIHYDROQUERCETIN ON WHATMAN NO. 1 PAPER

Compound	Solvent system* C	Solvent system* D	Colour with 1% ethanolic $FeCl_3$
Dihydrokaempferol	0·84	0·95	Mauve
Dihydroquercetin	0·73	0·87	Grey

* Key: C = water-saturated phenol;[4]
D = *n*-butanol/acetic acid/water (6:1:2).[5]

[4] S. H. WENDER and T. B. GAGE, *Science* **109**, 287 (1949).
[5] G. G. NORDSTRÖMAND and T. SWAIN, *J. Chem. Soc.* 2764 (1953).

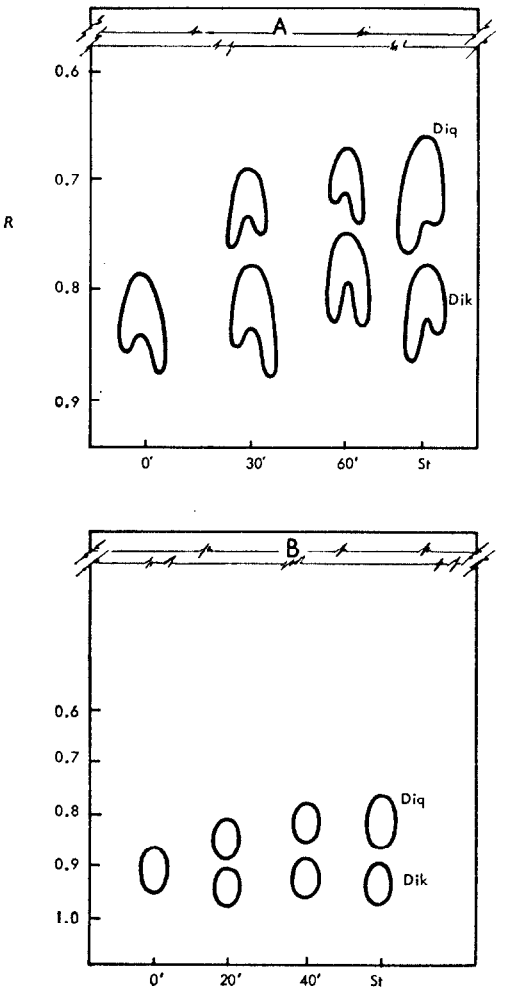

FIG. 3. PAPER CHROMATOGRAM AFTER INCUBATION WITH DIHYDROKAEMPFEROL AS SUBSTRATE. Dik, dihydrokaempferol; Diq, dihydroquercetin. 0, 20, 30, 40 and 60 refer to incubation period (min). A, developing solvent: water-saturated phenol. B, developing solvent: *n*-butanol/acetic acid/water (6:1:2).

In each experiment, the incubation was terminated by addition of 5 ml of ether after the period of time indicated in the figures. Ether extraction was repeated three times and the combined extracts were worked up and chromatographed on thin-layer plates or on paper as described in the Experimental section. The R_f values and the colour with and without spray reagents of the substrates and products are recorded in Tables 1 and 2.

Successful separation of kaempferol and quercetin, of naringenin and eriodictyol, and of dihydrokaempferol and dihydroquercetin were achieved (Tables 1 and 2). All hydroxylation products were compared with authentic substances. Quercetin was established as the product of kaempferol hydroxylation by its R_f in the two solvents and the yellow or yellow-grey colour of the unsprayed spots, which were quite distinct from the R_f values and colours of dihydroquercetin and eriodictyol.

Eriodictyol was established as the product of naringenin hydroxylation by its R_f in the two solvents and the colours of the spots before and after spraying with sodium molybdate. The R_f values for eriodictyol and dihydroquercetin are similar, but the two compounds are readily distinguished by the colours of their spots.

The appearance of dihydroquercetin with dihydrokaempferol as substrate was established by its R_f in the two solvents and the characteristic colour with ferric chloride.

DISCUSSION

The present results prove that the enzyme preparation from spinach beet catalyses the introduction of a hydroxyl group into the 3′-position of the B-ring in a number of 4′-hydroxyflavonoids. The enzyme shows high specificity for the position of hydroxylation since no further hydroxylation at the 5′-position in ring B or in positions 6 or 8 in ring A were observed. On the other hand the substrate specificity is rather low. In addition to *p*-coumaric acid various 4′-hydroxyflavonoids with different oxidation stages of the heterocyclic ring (flavanone, 3-hydroxyflavonone, flavonol) can serve as substrates. The qualitative results in these experiments give no indication of the substrates most rapidly hydroxylated. It had been shown earlier that in excised buckwheat seedlings dihydrokaempferol is apparently a much better substrate for hydroxylation at the 3′-position than kaempferol.[6]

The question remains open whether this enzyme is concerned in the biosynthesis of flavonoids with a 3′,4′-hydroxylation pattern. If enzymes with such low substrate specificity are involved in flavonoid biosynthesis it is possible that the relative levels of enzyme and substrate will determine whether hydroxylation of a particular flavonoid will proceed. On the other hand, according to a hypothesis by Hess, the hydroxylation pattern in ring B is already determined at the cinnamic acid stage and a specific enzyme selects a certain cinnamic acid from a given cinnamic acid pool for flavonoid biosynthesis.[7] If this hypothesis is correct, hydroxylation of C_{15} intermediates would play no significant role in flavonoid biosynthesis. In support of this assumption, Hess[8] has found the same isozyme pattern for phenolases in flower buds of nine pure lines of *Petunia hybrida*, even though these lines contained the genes of B-ring "hydroxylation" in various combinations. Quantitative studies on the properties of the enzyme(s) from spinach beet and similar enzymes from other plants would be necessary to clarify this question further.

EXPERIMENTAL

Reference Compounds

Quercetin was a commercial product (Fa. C. Roth, Karlsruhe). Quercetin and kaempferol[9] were reduced with sodium-dithionite to dihydroquercetin[10] and dihydrokaempferol,[11] respectively. Naringenin was obtained[12] by acid hydrolysis of 4,2′, 4′, 6′-tetrahydroxychalcone-2′-glucoside.[13] Erodictyol[14] was obtained in a similar manner from 3,4,2′, 4′, 6′-pentahydroxychalcone-2′-glucoside.[15]

[6] L. PATSCHKE, W. BARZ and H. GRISEBACH, *Z. Naturforsch.* **21b**, 45 (1966).
[7] D. HESS, *Biochemische Genetik*, p. 89 ff, Springer-Verlag, Berlin (1968).
[8] D. HESS, *Z. Pflanzenphysiol.* **56**, 295 (1967).
[9] R. ROBINSON and J. SHINODA, *J. Chem. Soc.* 1937, 1980 (1925).
[10] J. C. PEW, *J. Am. Chem. Soc.* **70**, 3031 (1948); T. A. GEISSMAN and H. LISCHNER, *J. Am. Chem. Soc.* **74**, 3001 (1952).
[11] W. E. HILLIS, *Australian J. Sci. Res.* Ser. A. **5**, 379 (1952); T. TSUKAMATO and T. TOMINAGA, *J. Pharm. Soc. Japan* **73**, 1179 (1953).
[12] H. GRISEBACH and W. BILHUBER, *Z. Naturforsch.* **22b**, 750 (1967).
[13] G. ZEMPLEN and R. BOGNAR, *Ber. Dtsch Chem. Ges.* **75**, 1040 (1942).
[14] T. A. GEISSMAN, *J. Am. Chem. Soc.* **62**, 3258 (1940).
[15] L. PATSCHKE and H. GRISEBACH, *Z. Naturforsch.* **20b**, 1039 (1965).

Chromatography of the Reaction Mixture

The ether extracts from each tube were combined and evaporated to dryness, and the residue was dissolved on 0·1 ml of ethanol. Thin-layer plates were prepared from a slurry of 40 g of silica gel G in 70 ml of 0·3 M sodium acetate.[3] The plates were allowed to dry at room temperature overnight, and then at 37° for 2·5 hr before use. 10-μl samples of the ethanolic solution from experiments with kaempferol and naringenin were applied to each plate.

100-μl samples of the ethanolic solutions from experiments with dihydrokaempferol were applied to Whatman No. 1 paper, and developed by descending chromatography.