Phytochemistry, 1971, Vol. 10, pp. 2649 to 2652. Pergamon Press. Printed in England

SHORT COMMUNICATION

HYDROXYLATION OF KAEMPFEROL, DIHYDROKAEMPFEROL AND NARINGENIN BY A PHENOLASE PREPARATION FROM SPINACH BEET

ROSEMARY J. ROBERTS and P. F. T. VAUGHAN

Department of Biochemistry, The University, Glasgow, W.2, Scotland

(*Received* 17 *December* 1970)

Abstract—An assay, based on the colorimetric determination of 3′,4′-dihydroxyflavonoids, has been developed to study the hydroxylation of kaempferol, naringenin and dihydrokaempferol catalysed by a phenolase from leaves of spinach beet (*Beta vulgaris* L. ssp. vulgaris). K_m values of 1·6, 2·2 and 52·0 × 10^{-4} M were obtained for naringenin, kaempferol and dihydrokaempferol, respectively, and the maximal velocities for these substrates were 8, 9 and 125 nmoles product/min/m-unit enzyme. For comparison, K_m values of 2·2, 1·0 and 16·7 × 10^{-4} M were determined for eriodictyol, quercetin and dihydroquercetin with maximal velocities of 0·037, 0·025 and 0·037 nmoles oxygen consumed/min/m-unit enzyme, respectively. These results are discussed in relation to the suggestion that dihydrokaempferol is a better substrate for hydroxylation at the 3′-position than kaempferol.

INTRODUCTION

PREVIOUS qualitative studies[1] demonstrated that purified spinach beet (*Beta vulgaris* L. ssp vulgaris) phenolase[2] catalysed the hydroxylation of 4′-hydroxyflavonoids in the 3′-position. Dihydrokaempferol has been shown[3] to be a better substrate for hydroxylation at the 3′-position than kaempferol in excized buckwheat seedlings. In the present study the kinetic parameters K_m and V_{max} were determined for the 4′-hydroxyflavonoids kaempferol, dihydrokaempferol and naringenin to see whether the differences observed in the feeding experiments were reflected at the enzyme level.

RESULTS

1 μmole kaempferol, dihydrokaempferol and naringenin were separately incubated with 66 m-units* enzyme, ascorbate (10 μmoles), $(NH_4)_2SO_4$ (1500 μmoles), ethylenediaminetetraacetic acid (10 μmoles), bovine serum albumin (200 μg), and Na_2HPO_4 (100 μmoles)/(citric acid, 40 μmoles) buffer over the range pH 4·0–7·5 in a total volume of 3 ml under air at 30°. Ethylenediaminetetraacetic acid was added to prevent the copper catalysed oxidation of ascorbate.[4] Bovine serum albumin was added to decrease the amount of reaction inactivation and thus to maintain the initial rate of reaction over a longer period of time (Fig. 1a). The reaction was terminated at 0, 5 and 10 min after the addition of 4′-hydroxyflavonoid by the transfer of 0·5 ml samples into 1·0 ml acetic acid (20% v/v) in a

* 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°.

[1] P. F. T. VAUGHAN, V. S. BUTT, H. GRISEBACH and L. SCHILL, *Phytochem.* **8**, 1373 (1969).
[2] P. F. T. VAUGHAN and V. S. BUTT, *Biochem. J.* **113**, 109 (1969).
[3] L. PATSCHKE, W. BARZ and H. GRISEBACH, *Z. Naturforsch.* **216**, 45 (1966).
[4] V. S. BUTT and M. HALLAWAY, *Arch. Biochem. Biophys.* **92**, 24 (1961).

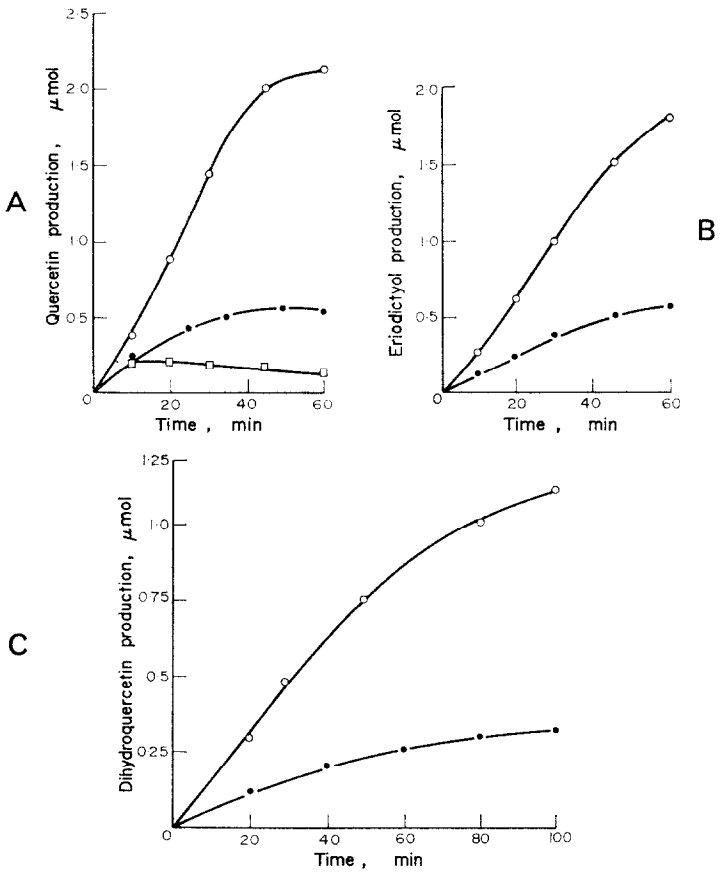

FIG. 1. TIME COURSE OF OXYGEN CONSUMPTION AND 3′,4′-DIHYDROXYFLAVONOID PRODUCTION. Oxygen consumption (○) and 3′,4′-dihydroxyflavonoid production (●) were determined during the hydroxylation of 1 μmole kaempferol (a), naringenin (b) and dihydrokaempferol (c) under the conditions described in the text. (□) Quercetin production in the absence of bovine serum albumin.

centrifuge tube. The amount of product formed was determined spectrophotometrically as described in the Experimental section.

Maximal activity for each substrate was observed between pH 5·2 and 5·6 with a second peak of lower activity at pH 7·0–7·2. All further incubations were therefore carried out at pH 5·3.

A time course study for the hydroxylation of kaempferol (1 μmole) incubated with 120 m-units enzyme under standard conditions of assay, showed that appreciable inhibition of hydroxylation was observed when 0·5 μmoles of quercetin had been produced (Fig. 1a). Oxygen consumption, however, was at all times greater than quercetin production and did not show reaction inactivation until 2·0 μmoles had been consumed. Similar effects were observed with naringenin (1 μmole) and dihydrokaempferol (1 μmole; Fig. 1b and c), although in the case of dihydrokaempferol reaction inactivation was less apparent.

Determination of the initial rate of hydroxylation in the presence of a range of concentrations of kaempferol, dihydrokaempferol and naringenin revealed that maximal rate was obtained with 0·17 mM kaempferol and 0·66 mM naringenin. Substrate inhibition was

observed at higher concentrations with both these compounds. Maximal rate was not achieved with 1·7 mM dihydrokaempferol. Lack of material prevented this range from being extended and so it is not known whether substrate inhibition would be observed at higher concentrations.

Plots of the reciprocal of rate of product formation against the reciprocal of substrate concentration[5] for kaempferol and naringenin were non-linear at high concentrations, indicating substrate inhibition, whereas a similar plot for dihydrokaempferol yielded a straight line. Using this method Km values of 1·6, 2·2 and 52×10^{-4} M and V_{max} values of 8, 9 and 125 nmole produced/min/m-unit enzyme were obtained for naringenin, kaempferol and dihydrokaempferol, respectively.

Determination of the initial rate of oxygen consumption for a range of concentrations of quercetin, dihydroquercetin and eriodictyol showed that maximal rate of catechol oxidase activity was obtained with 0·13 mM quercetin. Maximum rate was not observed with 0·17 mM dihydroquercetin nor with 0·13 mM eriodictyol. There was no indication of substrate inhibition at the concentrations of 3′,4′-dihydroxyflavonoids used.

Plots of the reciprocal of rate of oxygen consumption against the reciprocal of substrate concentration for eriodictyol, quercetin and dihydroquercetin yielded straight lines from which K_m values of 2·2, 1·0 and $16·7 \times 10^{-4}$ M and V_{max} values of 0·037, 0·025 and 0·037 nmoles oxygen consumed/min/m-unit enzyme were obtained for eriodictyol, quercetin and dihydroquercetin, respectively.

DISCUSSION

The observation that oxygen consumption was in excess of that required to account for 3′,4′-dihydroxyflavonoid production suggested that hydroxylation of 4′-hydroflavonoids was accompanied by catechol oxidase activity. The similarity between the K_m values for naringenin and eriodictyol and for kaempferol and quercetin supports the view that the corresponding 3′,4′-dihydroxyflavonoid would compete effectively with 4′-hydroxyflavonoid for the enzyme active site. The lower K_m value for dihydroquercetin compared with dihydrokaempferol implies that even more favourable competition would occur in this case.

The occurrence of catechol oxidase activity during hydroxylation of 4′-hydroxyflavonoids in the presence of ascorbate was similar to the hydroxylation of *p*-coumanic acid.[6] A further similarity between the hydroxylation of kaempferol, naringenin and *p*-coumanic acid under these conditions was the greater sensitivity of *o*-dihydric phenol production than oxygen consumption to reaction inactivation. The relative lack of reaction inactivation observed with dihydrokaempferol is probably due to smaller amounts of dihydroquercetin that had accumulated compared with quercetin and eriodictyol.

Wood and Ingraham[7] in a study on the hydroxylation of [1-^{14}C]phenol by mushroom phenolase in the presence of ascorbate, found that, following reaction-inactivation, radioactivity became covalently bound to protein. They concluded that the newly formed *o*-quinone underwent a Michael addition with a free amino group on the enzyme. The protection observed in the presence of bovine serum albumin is therefore probably due to a competition between free amino groups on the enzyme and foreign protein for *o*-quinone.

The 25-fold increase in the K_m value for dihydrokaempferol compared with kaempferol or naringenin suggests that in a mixture of these hydroxyflavonoids, dihydrokaempferol

[5] H. Lineweaver and D. Burk, *J. Am. Chem. Soc.* **56**, 658 (1934).
[6] P. F. T. Vaughan and V. S. Butt, *Biochem. J.* **111**, 32P (1968).
[7] B. J. B. Wood and L. L. Ingraham, *Nature, Lond.* **205**, 291 (1965).

would not be used efficiently as a substrate. The 15-fold increase in the V_{max} value for dihydrokaempferol compared with kaempferol or naringenin however implies that if dihydrokaempferol accumulated in plant tissue as the sole 4′-hydroxyflavonoid it would be effectively hydroxylated in the 3′-position.

These studies would therefore support the suggestion made previously[3] that dihydrokaempferol is apparently a better substrate for hydroxylation at the 3′-position than kaempferol, provided that dihydrokaempferol is the only 4′-hydroxyflavonoid present. The pronounced reaction inactivation observed as quercetin accumulates in the absence of bovine serum albumin is also consistent with the view that kaempferol is a poor substrate for hydroxylation.

In order to clarify this system further it would be necessary to determine the nature and concentrations of flavonoids present in spinach leaves, and to compare the substrate specificities of phenolases in spinach and buckwheat.

EXPERIMENTAL

Substrates. Kaempferol was purchased from the Sigma (London) Chemical Co. Ltd., London, S.W.6. Quercetin was a product of Koch-Light Laboratories Ltd., Colnbrook, Bucks. Dihydrokaempferol, dihydroquercetin, naringenin and eriodictyol were supplied by Prof. H. Grisebach.

Determination of 3′,4′-dihydroxyflavonoids. Samples (0·5 ml) containing up to 0·167 μmole of 3′,4′-dihydroxyflavonoid were added to 1 ml acetic acid (20% v/v). 0·5 ml $NaNO_2$ (20% w/v) and 0·5 ml Na_2MoO_4 (20% w/v) were then added, the mixture shaken and allowed to stand for 1 min. 1 ml 5 M NaOH was added and the red colour developed immediately was measured at 520 nm by a modification of the method of Arnow.[8] A linear relationship between extinction and 3′,4′-dihydroxyflavonoid was observed over this range, and each batch of measurements was accompanied by a standard sample of 0·167 μmole 3′,4′-dihydroxyflavonoid. E^{mM}_{1cm} of 1·20, 1·05 and 1·73 were obtained for quercetin, dihydroquercetin and eriodictyol respectively using this method.

Determination of oxygen consumption. Oxygen consumption during the hydroxylation of kaempferol, dihydrokaempferol and naringenin was determined under standard conditions of incubation. Catechol oxidase activity was determined by measuring oxygen consumption under standard conditions of incubation in which the 4′-hydroxyflavonoids were replaced by the respective 3′,4′-dihydroxyflavonoids. Oxygen consumption was measured using conventional Warburg technique.

Acknowledgements—The authors thank Professors J. N. Davidson, F.R.S.; R. M. S. Smellie for their support throughout this work; and H. Grisebach for the generous donation of dihydrokaempferol, dihydroquercetin, naringenin and eriodictyol.

[8] L. E. ARNOW, *J. Biol. Chem.* **118**, 531 (1937).