Phytochemistry, 1976, Vol. 15, pp. 1865–1868. Pergamon Press. Printed in England.

UDP-GLUCOSE: CYANIDIN 3-*O*-GLUCOSYLTRANSFERASE FROM RED CABBAGE SEEDLINGS

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(*Received* 9 *June* 1976)

Key Word Index—*Brassica oleracea*; Cruciferae; red cabbage; flavonoids; cyanidin; glucosyltransferase; biosynthesis.

Abstract—An enzyme, catalysing the glucosylation of cyanidin at the 3-position using uridine diphosphate-D-glucose (UDPG) as glucosyl-donor, has been isolated and purified about 50-fold from young red cabbage (*Brassica oleracea*) seedlings. The pH optimum for this reaction was *ca* 8 and no additional cofactors were required. The reaction was inhibited by cyanidin (above 0.25 mM) and by very low concentrations of the reaction product cyanidin-3-glucoside (5 μM). The K_m values for UDPG and cyanidin were 0.51 and 0.4 mM respectively. In addition to cyanidin the enzyme could also glucosylate the following compounds at the 3-position: pelargonidin, peonidin, malvidin, kaempferol, quercetin, isorhamnetin, myricetin and fisetin. In contrast, cyanidin-3-glucoside, cyanidin-3-sophoroside, cyanidin-3,5-diglucoside, apigenin, luteolin, naringenin and dihydroquercetin were not glucosylated.

INTRODUCTION

Glycosides are of widespread occurrence in plants. They contain a monosaccharide or an oligosaccharide residue attached to an aglycone moiety. Particularly striking is the great variation of flavonoid glycosides [1,2]. In the past 20 years the presence of various glucosyltransferases has been demonstrated. These enzymes catalyse the transfer of the sugar moiety from donors such as UDPG, dTDPG, UDP-D-galactose and UDP-D-glucuronic acid to such acceptors as simple hydroxyphenols [3], phenolic acids [4], sterols [5] and flavonoids [6–8]. However, earlier attempts to demonstrate the *in vitro* glucosylation of anthocyanidins were unsuccessful. For example, the glucosylation of pelargonidin and pelargonidin-3-glucoside using extracts from the petals of *Impatiens balsamina* was not achieved, although the extracts contained enzymes catalysing the glucosylation of hydroquinone and flavonols [9]. Recently, preliminary work with extracts from *Haplopappus gracilis* cell suspension cultures had indicated the glucosylation by UDPG of cyanidin at the 3-position [10]. In this paper, we report the partial purification and properties of a glucosyltransferase from red cabbage (*Brassica oleracea*) seedlings, which catalyses the glucosylation of cyanidin and some flavonols at the 3-position with UDPG as glucose donor.

RESULTS

Purification of the enzyme

Six-day old seedlings were extracted with KPi buffer and the crude extract treated with Dowex ion-exchanger to remove phenolic compounds. The enzyme was further purified by $(NH_4)_2SO_4$ fractionation and by chromatography on DEAE-cellulose and hydroxylapatite columns. The purification procedure, which resulted in a preparation having a sp. act. *ca* 50 times greater than that of the extract after Dowex treatment, is summarized in Table 1. All kinetic experiments described here were undertaken using the enzyme obtained after hydroxylapatite chromatography.

Product identification

The product of the enzymic glucosylation of cyanidin was identified as cyanidin-3-*O*-glucoside by co-chromatography with an authentic sample using the solvent systems described in the experimental section, as well as by spectrophotometric analysis [11]. The glucosylation of other anthocyanidins and flavonols by the enzyme preparation led also to the respective 3-glucosides (Table 2). These were identified by their R_f values and UV

Table 1. Purification of cyanidin 3-*O*-glucosyltransferase from red cabbage seedlings

Purification stage	Volume (ml)	Protein (mg)	Specific activity (μmol cyanidin-3-glucoside/hr/mg protein)	Extent of purification
Dowex	330	619	0.078	—
Sephadex G-25 eluate	30	84	0.091	1.2
DEAE-cellulose eluate	17	21	0.56	7
Hydroxylapatite eluate	3.7	2.8	3.64	46

* On leave of absence from National Research Centre, El-Dokki, Cairo, Egypt.

Table 2. Substrate specificity of cyanidin 3-glucosyltransferase from red cabbage seedlings

Substrate	Specific activity (product cpm/mg protein)	% Activity with reference to cyanidin
Pelargonidin	68600	37
Cyanidin	184000	100
Peonidin	77300	42
Malvidin	18300	10
Kaempferol	123000	67
Quercetin	145000	79
Isorhamnetin	103000	56
Myricetin	191000	104
Fisetin	30400	16

The substrate specificity of the enzyme was tested by incubating the above substrates (28 nmol) with 20 μl of the purified enzyme in Pi buffer, pH 6.5, for 10 min. The reaction was terminated by addition of 10 μl HOAc and the products identified and the activity determined by PC. No detectable activity was found with the following substrates: apigenin, luteolin, naringenin, dihydroquercetin, cyanidin-3-glucoside, cyanidin-3-sophoroside and cyanidin-3,5-diglucoside.

properties. Authentic samples were available in the case of malvidin-, quercetin- and isorhamnetin-3-glucosides.

Properties of the purified enzyme

No loss in activity was observed after the enzyme had been stored at 4° for several weeks in the presence of 0.02% NaN_3. The enzyme showed lesser stability when stored at −20° and was unstable in solutions containing low concentrations of protein. This instability could be eliminated by addition of bovine serum albumin to the assay system; the optimum concentration was found by titration experiments to be *ca* 2 mg/ml (Table 3). Unless otherwise stated, all experiments were performed in the presence of this addition.

The pH optimum for the glucosylation of cyanidin was about pH 8 (Fig. 1). Whereas only cyanidin-3-glucoside was produced from cyanidin and UDP-D-[U-^{14}C]-glucose when reactions were carried out in KPi buffer, small but significant amounts of radioactivity could also be detected in cyanidin-3-sophoroside and cyanidin-3-sophoroside-5-glucoside when the reactions were allowed to proceed in glycine–NaOH buffer (pH 7.5–8.5). Due to the known instability of cyanidin at pH 8 and also from studies of a similar glucosyltransferase isolated from *Haplopappus gracilis* cell suspension cultures (unpublished results), the assay reactions were normally carried out at pH 6.5 in Pi buffer.

The synthesis of cyanidin-3-glucoside in KPi buffer pH 6.5 was proportional to protein concentration up to only 8 μg protein per assay (125 μl) of the purified enzyme. The glucosylation of cyanidin at this pH also showed an abnormal time-course (Fig. 2); initially the

Table 3. Effect of BSA and cyanidin-3-glucoside on the initial rate of cyanidin glucosylation

Addition	Concentration	% Control activity*
None	—	100
Bovine serum albumin	1 mg/ml	216
	2 mg/ml	190
	3 mg/ml	215
	4 mg/ml	217
Cyanidin-3-glucoside	24 μM	37
	12 μM	63
	6 μM	72

* Reaction carried out at pH 6.5 for 10 min.

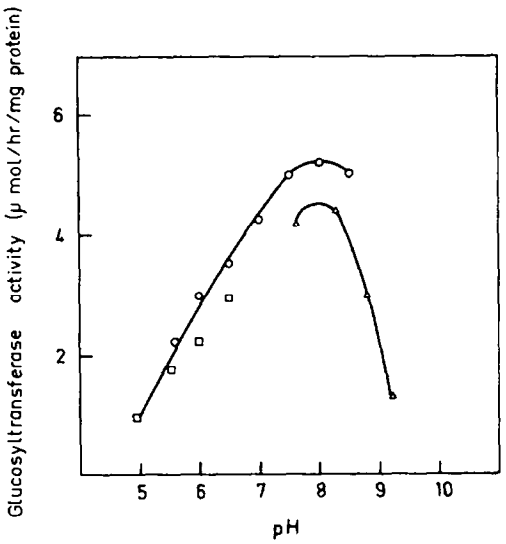

Fig. 1. Effect of pH on the cyanidin: 3-*O*-glucosyltransferase activity of the purified enzyme. Cyanidin and UDPG-[^{14}C] were incubated with the purified enzyme (15 μg protein) under conditions given in the Experimental section using 0.12 M concentrations of the following buffers: Glycine–NaOH (△—△), KH_2PO_4–K_2HPO_4 (●—●) and K_2HPO_4–citric acid (□—□).

reaction proceeded rapidly but its rate fell to zero after 10–15 min. The nature of these kinetics was studied more closely. The reaction was severely inhibited by low concentrations of the product cyanidin-3-glucoside (Table 3), and its production during the reaction probably led to the complete inhibition of the glucosylation after only a short period. Since it seemed likely that this product was acting as a competitive inhibitor against cyanidin, attempts were made to alleviate this inhibition by increasing the initial cyanidin concentration. This was however found to be unfavourable since the enzyme was progressively inhibited by increasing cyanidin concen-

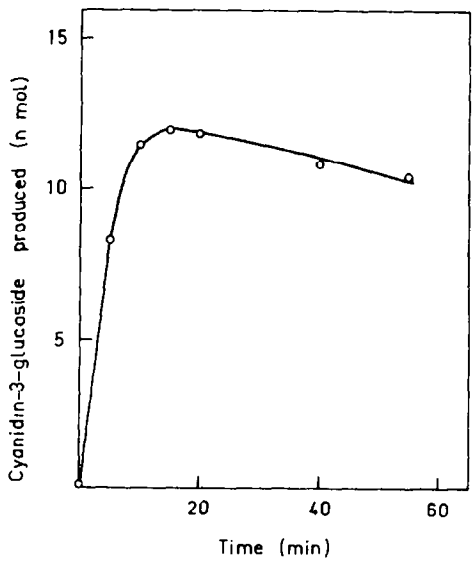

Fig. 2. Time-course of cyanidin-3-glucoside formation. The purified enzyme (23 μg protein) was incubated at pH 6.5 under the conditions described in the Experimental section.

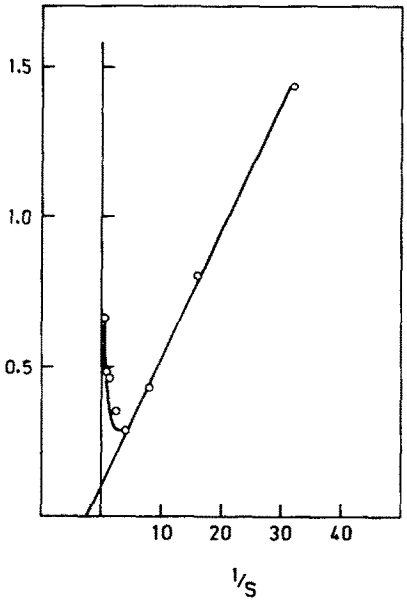

Fig. 3. Lineweaver–Burk plot of enzyme activity (v) and cyanidin concentration (s). The UDPG concentration was 2 mM. v is expressed as μmol cyanidin-3-glucoside/hr/mg protein; s is expressed as mmol/l.

trations above the optimum level of 0.25 mM. The K_m for cyanidin, calculated from the Lineweaver–Burk plot (Fig. 3), was 0.4 mM. It is therefore clear that the observed abnormal time-course and protein dependence of this reaction can be partially, if not totally, explained by the fact that the assay reactions must be performed under conditions when the enzyme is not saturated with respect to cyanidin. All reactions were thus performed using an optimum cyanidin concentration (0.22 mM) and an incubation time of 5–10 min. Under such conditions, the K_m for UDPG was 0.51 mM, as determined from a Lineweaver–Burk plot, which was linear in the concentration range used (0.3–3.0 mM).

The enzyme, at this level of purification, was not specific for cyanidin alone. As shown in Table 2, the enzyme could also glucosylate at pH 6.5 pelargonidin, peonidin, malvidin, kaempferol, quercetin, isorhamnetin, myricetin and fisetin at the 3-position. In contrast, apigenin, luteolin, naringenin, dihydroquercetin, cyanidin-3-glucoside, cyanidin-3-sophoroside and cyanidin-3,5-diglucoside were not glucosylated.

DISCUSSION

Rubrobrassicin, the major anthocyanin of red cabbage, was first isolated in 1936 by Chmielewska [12]. Subsequent work has indicated that this pigment is a mixture of three or four acylated cyanidin-3-sophoroside-5-glucosides. The exact nature of the acylating group is still unclear; *p*-coumaric, ferulic and sinapic acids have all been suggested as acylating groups [13–15]. Information about the biosynthesis of cyanidin has been obtained through tracer studies [16] and some evidence is also available from experiments with *Haplopappus gracilis* cell cultures concerning the enzymes involved in the cyanidin pathway [17]. Work was now undertaken to elucidate how cyanidin is glucosylated to produce cyanidin-3-sophoroside-5-glucoside.

When cyanidin was incubated with UDP-D-[U-^{14}C]-glucose in the presence of a crude extract from red cabbage seedlings, cyanidin-3-[U-^{14}C]-glucoside was the main product; no radioactivity was detected in cyanidin-5-glucoside. The enzyme catalysing this reaction was purified about 50-fold by $(NH_4)_2SO_4$ fractionation and chromatography on DEAE-cellulose and hydroxylapatite columns. The characterization of the partially-purified enzyme was hindered by kinetic properties which are somewhat unusual for flavonoid glucosyltransferases. The enzyme was unstable in solutions of low protein concentration and was severely inhibited by the substrate cyanidin above an optimum concentration of 0.25 mM. Moreover, the reaction was potently inhibited by very low (5–25 μM) concentrations of the product cyanidin-3-glucoside. Reactions were therefore carried out under non-saturating conditions with respect to cyanidin and using short incubation periods. The above kinetic properties pose interesting questions as to how this reaction may be controlled *in vivo*. Product inhibition by cyanidin-3-glucoside could perhaps be reduced or eliminated by the further glucosylation of this compound, provided that the new product does not inhibit the reaction. A second alternative is that the product is channeled into a different compartment of the cell as has been earlier suggested [17].

Many enzyme preparations have been described which catalyse the transfer of the sugar moiety of a sugar nucleoside diphosphate to the 7-hydroxyl group [7] or to the 3-hydroxyl group [6,8,9] of flavonoid acceptor molecules. Whereas these preparations, where tested, were unable to catalyse the glucosylation of anthocyanidins, preliminary work has detected an UDPG: cyanidin 3-*O*-glucosyltransferase in crude extracts from *Haplopappus gracilis* cell suspension cultures [10]. We report here for the first time the purification of an UDPG: cyanidin 3-*O*-glucosyltransferase. However, as found with the enzymes mentioned above, the red cabbage enzyme was not specific for one acceptor molecule, since it could also catalyse the glucosylation of a range of flavonoid compounds possessing an OH group at the 3 position. One notable exception was the lack of 3-*O*-glucosylation of dihydroquercetin. This supports the conclusion drawn from tracer experiments that glucosylation is the last step in flavonoid glucoside [18] and cyanidin glucoside [17] formation. Interestingly, the glucosylation of other anthocyanidins was also appreciable. There are indications that isorhamnetin is a constituent of red cabbage [11]. Preliminary experiments have detected an *S*-adenosylmethionine 3′,4′-dihydroxyflavonoid-3′-*O*-methyltransferase in these seedlings, which methylates quercetin to isorhamnetin (J. E. Poulton, unpublished results). It is interesting that this product was then glucosylated by the red cabbage 3-*O*-glucosyltransferase at a reasonable rate under the experimental conditions described. As found with the two separate glucosyltransferases from *Petroselinum hortense* cell cultures [7,8], the red cabbage enzyme had a distinct positional specificity, since glucosylation could not be effected at the 7-position.

The biosynthesis of the more highly glucosylated derivatives, cyanidin-3-sophoroside and -3-sophoroside-5-glucoside, was not studied here. It appears likely, from the substrate specificity of the red cabbage enzyme (Table 1) and from rutin biosynthesis using extracts from *Phaseolus aureus* [6], that more than one glucosyltransferase

is involved in this biosynthetic pathway. It is therefore surprising that the red cabbage enzyme, after hydroxylapatite chromatography, catalysed the formation of these compounds from cyanidin and UDPG in glycine–NaOH buffer but not in KPi buffer. It is not known whether this is a real property of the enzyme or whether the enzyme preparation is contaminated by other glucosyltransferases. The results obtained so far indicate that the glucosylation proceeds via the pathway cyanidin → cyanidin-3-glucoside → cyanidin-3-sophoroside → cyanidin-3-sophoroside-5-glucoside.

EXPERIMENTAL

Red cabbage (*Brassica oleracea*, var. *rubra*, Frührot) seeds were purchased from a local merchant and plants were raised under continuous illumination in boxes on 2 sheets of Whatman 3 MM paper, which were kept moist.

Flavonoids were from our laboratory collection. Cyanidin-3-glucoside, -3,5-diglucoside and -3-sophoroside were authentic samples. UDP-D-[U-^{14}C]-glucose was purchased from the Radiochemical Centre, Amersham, Great Britain.

Chromatographic methods. Descending PC on Schleicher and Schüll (2043 b) paper was used. The solvent system used for assays was 1% HCl. Other solvent systems used for identification purposes were (I) BAW (*n*-BuOH–HOAc–H_2O, 4:1:5), (II) HOAc (HOAc–conc HCl–H_2O, 15:3:82) and (III) Bu/HCl (*n*-BuOH–2 M HCl, 1:1).

Buffer solns. The following were used: (A) 0.1 M KPi buffer, pH 7.7, containing 14.5 mM β-mercaptoethanol, (B) 20 mM KPi buffer, pH 7.7, containing 1.45 mM β-mercaptoethanol, (C) 20 mM KPi buffer, pH 6.7, containing 1.45 mM β-mercaptoethanol and 0.02% NaN_3.

Enzyme purification. Unless otherwise stated, all steps were carried out at 4°. Six-day-old seedlings (180 g) were washed with cold H_2O and homogenised in a mortar with 12 g PVP, 80 g quartz sand and 310 ml buffer A. The resultant liquid was centrifuged for 20 min at 10000 *g*. To the supernatant liquid (350 ml), 50 g Dowex 1 × 2 (pre-equilibrated with buffer A) were added and stirred for 15 min. The ion-exchanger was filtered off under suction and this process was then repeated. The resultant liquid was brought to 40% satn by the addition of $(NH_4)_2SO_4$ over a period of 15 min, during which the pH of the soln was continuously adjusted to 6.9–7.1 by adding small vols of dil KOH soln. The mixture was allowed to stand for a further 25 min at 0° and then centrifuged at 10000 *g* for 20 min. The supernatant liquid was brought to 80% satn by the further addition of $(NH_4)_2SO_4$ over a period of 25 min, with adjustment of the pH as before. The mixture was allowed to stand for 20 min at 0° and the ppt was collected by centrifuging at 10000 *g* for 20 min. This was redissolved in buffer B (13 ml total vol) and was found to contain the majority of the glucosyltransferase activity. This fraction was chromatographed on a column (3 × 25 cm) of Sephadex G-25, which had been previously equilibrated with buffer B. The resultant filtrate (27 ml) was applied to a DEAE-cellulose column (2 × 6 cm), which had been equilibrated with buffer B. After washing the column with buffer B, elution was continued with a linear 20–300 mM KPi gradient (140 ml), pH 7.7, containing 1.45 mM β-mercaptoethanol. The glucosyltransferase was eluted from the column after the Pi buffer concn had reached *ca* 150 mM. The most active fractions were combined, concd by ultrafiltration to 17 ml and dialysed for 18 hr against 2 l. of buffer B. The dialysed soln was applied to a hydroxylapatite column (2 × 5 cm), which had been pre-equilibrated with buffer C. After washing with buffer C, elution was continued with a linear 20–200 mM KPi buffer gradient (100 ml, pH 6.7), containing 1.45 mM β-mercaptoethanol and 0.02% NaN_3. Fractions possessing glycosyltransferase activity, which were eluted after the buffer concn had reached 30 mM, were pooled (40 ml) and concd by ultrafiltration to 3.7 ml. This fraction was stored at 4° until used for the kinetic experiments described.

Enzyme assay. The assay mixture contained 250 nmol UDP-D-[^{14}C]-glucose (containing 100000 dpm), 28 nmol cyanidin (in ethyleneglycolmonomethylether), 250 μg BSA and 15 μmol KPi (pH 6.5), to which was added 5–20 μl of the glucosyltransferase preparation to give a final vol of 125 μl. The reaction mixture was incubated at 30° for up to 20 min and the reaction terminated by addition of 10 μl HOAc. Chromatography was carried out as quickly as possible by methods given above.

Protein estimation. Protein content of crude preparations was determined by the biuret method, using crystalline BSA as standard [19]. After the DEAE-cellulose stage, all determinations were made according to the method of ref. [20].

Acknowledgements—This work was supported by Deutsche Forschungsgemeinschaft (SFB 46) and by Fonds der Chemischen Industrie. The authors thank Dr. G. Hrazdina, Cornell University, Ithaca, N.Y. for reference samples of cyanidin-3,5-diglucoside and cyanidin-3-sophoroside.

N. S. and J. P. thank the Alexander von Humboldt-Stiftung for the award of Fellowships.

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