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# MALONYL-COENZYME A: ANTHOCYANIDIN 3-GLUCOSIDE MALONYLTRANSFERASE FROM FLOWERS OF *CALLISTEPHUS CHINENSIS*

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**Key Word Index**—*Callistephus chinensis*; Asteraceae; anthocyanin biosynthesis; malonylated anthocyanins; malonyltransferase.

**Abstract**—In flowers of *Callistephus chinensis* containing malonylated pelargonidin 3-glucoside, an enzyme was demonstrated which catalyses the transfer of the malonyl moiety from malonyl-CoA to the 3-glucosides of pelargonidin, cyanidin and delphinidin and with a considerably lower reaction rate also to cyanidin 3,5-diglucoside. Besides malonyl-CoA, a range of other dicarboxylic acids serve as acyl donors but to a lesser extent. The enzyme exhibits a sharp pH-optimum at 7.0 and a temperature optimum of 50°. The reaction does not require additional co-factors and is inhibited by divalent cations and by several inhibitors of enzymic reactions, in particular by *p*-chloromercuribenzoate. The possible significance of malonylation of anthocyanins for their transport into the vacuole is discussed.

## INTRODUCTION

Whereas aromatic acylation of anthocyanins has long been known [1], aliphatic acylation was at first thought to be rare. In recent years, however, the occurrence of anthocyanins acylated with aliphatic acids, such as acetic acid and in particular malonic acid, has increasingly been reported [2–4]. These rather labile acyl groups were often overlooked during earlier studies due to their hydrolysis under the usual extraction procedures based on methanolic HCl.

In flowers of *Callistephus chinensis*, earlier chemicogenetic investigations indicated the presence of aliphatic acylated anthocyanins, but the acyl group was not identified [5]. Very recently, one of these acylated anthocyanins was definitively identified as pelargonidin 3-*O*-(6″-malonyl)glucoside [3].

Up to now, enzymes catalysing acylation of anthocyanins could only be demonstrated in flower extracts of *Silene dioica* [6] and *Matthiola incana* [7]. Both enzymes specifically transfer hydroxycinnamic acids. We now report the first demonstration and the characterisation of an enzyme in flower extracts of *Callistephus chinensis* which catalyses the malonyl transfer from malonyl-CoA to the sugar moiety of anthocyanins.

## RESULTS

Incubation of pelargonidin 3-glucoside with malonyl-CoA and the crude flower extract resulted in the formation of one product with a higher retention time ($R_t$ 4.8 min) on HPLC than the substrate ($R_t$ 4.2 min). In incubations with boiled enzyme or without malonyl-CoA, no product was formed. The product was identified as pelargonidin 3-*O*-(6″-malonyl)glucoside by comparison with an authentic sample by HPLC, by TLC in different solvents and by alkaline hydrolysis.

Because the crude extract still contained some anthocyanins, which might interfere with the reaction and the quantitative analysis of the reaction mixture by HPLC, it was passed through Sephadex G-50. This purification step caused no loss in enzyme activity, although the protein content of the eluate amounted only to 0.2 mg × ml$^{-1}$. Under standard conditions the reaction was linear with time up to 15 min and with protein concentration up to 5 μg protein (Sephadex G-50 eluate). The enzyme reaction exhibited an optimum at pH 7.0 in 0.1 M KPi buffer. This pH was chosen for standard conditions but with 0.05 KPi. Product formation was not influenced by the lower ion concentration which is, however, more suitable for the HPLC column.

The acylation reaction showed a saturation with malonyl-CoA at a concentration of 75 nmol. With considerably higher amounts (25 fold) no decrease in reaction rate was observed. For pelargonidin 3-glucoside as substrate, the optimal concentration was between 50 and 100 nmol. With 140 nmol the reaction rate decreased to 70% of the optimum. This inhibition can hardly be explained as due to an impurity in the substrate, because its purity determined by HPLC was at least 98%.

The enzyme preparation could be stored at −20° for several weeks without appreciable loss of enzyme activity. Moreover, flowers could be frozen in liquid nitrogen and stored at −70° without loss of extractable enzyme activity. The considerable stablity of the enzyme is further underlined by the temperature optimum of the reaction. The acylation reaction exhibited an optimum as high as 50° with half maximal velocities at 30° and 80°. But even at 0° about 17% of the optimal value was observed.

Divalent ions have an inhibitory effect on the reaction (Table 1). Moreover, some common inhibitors of enzymic reactions affected the acylation reaction differently. *Para*-chloromercuribenzoate is the strongest and potassium cyanide the weakest inhibitor (Table 1).

Table 1. Influence of divalent ions and inhibitors on acyltransferase activity

| Additions | Acyltransferase activity (%) |
|---|---|
| none | 100 |
| 1 mM $CaCl_2$ | 80 |
| 1 mM $MgCl_2$ | 86 |
| 1 mM $MnCl_2$ | 72 |
| 1 mM $CoCl_2$ | 91 |
| 1 mM $CuSO_4$ | 20 |
| 1 mM $FeSO_4$ | 55 |
| 1 mM $ZnCl_2$ | 63 |
| 1 mM $ZnSO_4$ | 54 |
| 1 mM KCN | 74 |
| 2 mM EDTA | 66 |
| 1 mM Diethylpyrocarbonate | 55 |
| 2 mM Diethyldithiocarbamate | 64 |
| 0.2 mM *p*-Chloromercuribenzoate | 36 |

Tests on the substrate specificity revealed that delphinidin, cyanidin and pelargonidin 3-glucoside are equivalent substrates for the acyltransferase, but cyanidin 3,5-diglucoside is acylated at a low rate of 10% compared to the 3-glucosides. The 3-sambubiosides of the above anthocyanidins did not serve as substrates. Several CoA-esters have been tested as acyl donors for the three 3-glucosides and cyanidin 3,5-diglucoside. The CoA-esters derived from cinnamic acid as 4-coumaroyl- and caffeoyl-CoA failed, as well as the CoA-esters from acetic and *n*-propionic acid. The best donor was malonyl-CoA. With this acyl donor 27 nmol malonylated Pg 3-*O*-glucoside were formed with a 10 min incubation at 30°. With glutaryl-CoA the reaction rate amounted to 50% compared to malonyl-CoA, with succinyl-CoA to 40% and with methylmalonyl-CoA only to 30%.

## DISCUSSION

Acyltransferases catalysing the transfer of hydroxycinnamic acids to flavonol glucosides or anthocyanins, respectively, have been reported in pea seedlings [8] and in flowers of *Silene dioica* [6] and *Matthiola incana* [7]. Malonyltransferases for flavone and flavonol glycosides have been isolated from irradiated parsley cell cultures [9, 10] and for isoflavones from *Cicer* seedlings [11]. We have now demonstrated for the first time an acyltransferase, which catalyses the transfer of the malonyl residue from malonyl-CoA to the sugar moiety of anthocyanidin 3-glucosides. In agreement with the malonyltransferases mentioned above, the enzyme from *Callistephus* flowers is associated with the soluble fraction and is very stable to temperature and to storage. But it has a lower pH-optimum than the other malonyltransferases.

The enzyme exclusively catalyses the transfer of dicarboxylic acids. The fact that malonyl-CoA is the most efficient acyl donor, corresponds to the presence of malonylated anthocyanins in *Callistephus* flowers [3]. The reaction rate with other dicarboxylic acids decreased as the length of the $CH_2$-chain between the two carboxyl groups increased. The maximal $CH_2$-chain length between the two carboxyl groups which allows acyl donor activity of the CoA-esters could not be determined, because the appropriate CoA-esters were not available. With regard to acceptor specificity, anthocyanidin 3-glucosides but not 3-sambubiosides can be acylated by the enzyme. The latter compunds are, of course, not present in *Callistephus* flowers [5]. Surprisingly, however, anthocyanidin 3,5-diglucosides, which occur in acylated form in the flowers, are only acylated at a much lower rate than the respective 3-glucosides. Nevertheless, acylation seems to be the last step in the biosynthesis of malonylated 3,5-diglucosides, because the enzyme catalysing 5-*O*-glucosylation uses anthocyanidin 3-glucosides as substrate but not the acylated 3-glucoside (Teusch, unpublished).

The malonylated flavonoid glycosides of parsley, which are synthesized in the cytoplasm, are exclusively located within the vacuole [12]. This fact and the observation of a pH-dependent conformational change of malonylated apigenin 7-*O*-glucoside suggested that malonylation may facilitate the transport of flavonoid glycosides through the tonoplast into the vacuole [13]. It has long been known that anthocyanins accumulate in the vacuoles. Up to now, however, it has not been clear whether malonylation is of importance for the vacuolar location of anthocyanins.

## EXPERIMENTAL

*Plant material.* The investigations included the anthocyanin-containing line 06 of *Callistephus chinensis* (genotype ChCh, FF, AA, GG, rr, mm). The plant material was cultivated in the experimental garden of our institute. Chemicogenetic and enzymatic investigations proved that gene M controls the 5-*O*-glucosylation [5, 14, 15]. The multiple alleles of the R-locus (R, r', r) are responsible for the degree of hydroxylation of the B-ring. Gene Ch controls chalcone-isomerase activity [16]. Genes A, F and G concern anthocyanin synthesis after dihydroflavonol formation. Gene F governs the activity of dihydroflavonol-4-reductase [17]. The actions of the genes A and G are still unknown.

*Chemicals and references.* All CoA-esters of aliphatic acids and UDPG were purchased from Sigma. 4-Coumaroyl- and caffeoyl-CoA were a gift from W. Heller (Neuherberg, F.R.G.). The anthocyanins used as substrates were isolated from flower extracts. Pelargonidin 3-*O*-(6"-malonyl)glucoside was isolated from line 06 [5] of *Callistephus chinensis* and identified by TLC in BAW (*n*-butanol–acetic acid–$H_2O$, 6:1:2) [3]. All anthocyanins were purified by descending PC in BAW and in 30% acetic acid. Their purity was checked by HPLC [7].

*Enzyme preparation.* All steps were performed at 4°. 1.0 g petals were homogenized in a prechilled mortar with 1.0 g quartz, 0.5 g PVP (Serva, FRG) and 8 ml 0.1 M KPi buffer pH = 7.0 containing 5 mM 2-mercapto ethanol. The homogenate was centrifugated for 15 min at 10000 *g* and the supernatant for 10 min once more. The crude extract was freed from low $M_r$ substances by passing it through a Sephadex G-50 (fine) column (bed volume 1 ml).

*Enzyme assay.* The standard reaction mixture (100 μl) contained 20 μl enzyme extract (4 μg protein), 20 μl malonyl-CoA (75 nmol) 50 nmol dried pelargonidin-3-glucoside and 60 μl 0.05 M KPi buffer pH 7.0. The incubation was performed at 30° for 10 min. The reaction was started by adding malonyl-CoA and stopped by the addition of 50 μl $CHCl_3$–MeOH = 2:1 (0.5% HCl) resulting in a Folch partition [18]. In the upper phase of the Folch partition, the anthocyanins were concentrated.

*Determination of the pH-optimum.* The total volume of 100 μl assay consisted of 50 nmol pelargonidin-3-glucoside, 5 μl

enzyme (1 μg protein), 5 μl malonyl-CoA (75 nmol) and 90μl 0.1 M Kpi buffer between pH 5.5 and 8.5. Incubation was performed as described.

*Analytical methods.* For analysis of the reaction mixture, both HPLC and TLC were used. For HPLC analysis the same conditions were used as described before [7]. The anthocyanins present in the reaction mixture were compared with appropriate references and identified by their $R_f$-value. Furthermore, the single fractions of the HPLC-separation (Frac-100, Pharmacia) were put on TLC and also compared to appropriate references and to the reaction mixture charged without HPLC-separation on TLC. TLC was performed on cellulose plates (Schleicher & Schüll, F.R.G.) in BAW and 30% HOAc. Protein was determined by the method of ref. [19].

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