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PROPERTIES AND GENETIC CONTROL OF UDP-L-RHAMNOSE: ANTHOCYANIDIN 3-*O*-GLUCOSIDE, 6″-*O*-RHAMNOSYLTRANSFERASE FROM PETALS OF RED CAMPION, *SILENE DIOICA*

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Key Word Index—*Silene dioica*; Caryophyllaceae; anthocyanin biosynthesis; cyanidin glycosides; pelargonidin glycosides; glycosyltransferase; rhamnosyltransferase; UDP-rhamnose; genetics.

Abstract—In petals of *Silene dioica* plants the presence of a glycosyltransferase has been demonstrated, which catalyses the transfer of the rhamnosyl moiety of UDP-L-rhamnose to the glucose of cyanidin 3-*O*-glucoside. This enzyme can also use pelargonidin 3-*O*-glucoside as a substrate. The enzyme activity is controlled by a single dominant gene *N*; no rhamnosyltransferase activity is found in petals of *n/n* plants. The rhamnosyltransferase exhibits an optimum of pH 8.1 and is stimulated by the divalent metal ions Mg, Mn and Co. The biosynthetic pathway for the synthesis of cyanidin 3-rhamnosylglucoside-5-glucoside in petals of *S. dioica* is discussed.

INTRODUCTION

Genetic studies on flower colour in *Silene dioica* have demonstrated that several genes are involved in the anthocyanin biosynthesis [1]. The glycosylation of anthocyanins is governed by the genes *M* and *N*. Dominant alleles of both genes are required for the synthesis of cyanidin 3-rhamnosylglucoside-5-glucoside, the anthocyanin present in petals of wild populations. Gene *M* controls the glucosylation of the 5-position of anthocyanidin 3-rhamnosylglucosides, whereas gene *N* governs the attachment of rhamnose to the glucose at the 3-*O*-position. Previous biochemical and genetical studies [2] have demonstrated that gene *M* controls the formation of UDP-glucose: anthocyanidin 3-rhamnosylglucoside, 5-*O*-glucosyltransferase. Although no variant has been found of the gene, which controls the 3-*O*-glucosylation of cyanidin, the enzyme which catalyses this step (UDP-glucose: anthocyanidin 3-*O*-glucosyltransferase) has been demonstrated to be present in petals of *S. dioica* plants [3]. We here describe the identification and properties of the enzyme controlled by gene *N*.

RESULTS

Incubation of UDP-L-rhamnose, labelled uniformly in the rhamnose moiety, and cyanidin 3-*O*-glucoside with the supernatant of a crude petal homogenate of *m/m N/N Silene dioica* plants containing cyanidin 3-rhamnosylglucoside resulted in incorporation of radioactivity into cyanidin 3-rhamnosylglucoside. When trichloroacetic acid was added before incubation with the enzyme preparation, no incorporation was observed. Without the addition of cyanidin 3-*O*-glucoside to the supernatant of the crude homogenate, 20% of the synthesis occurred, indicating that the supernatant contains endogeneous cyanidin 3-*O*-glucoside. After removal of this endogeneous substrate by PVP-chromatography and gel filtration, no incorporation of radioactivity into cyanidin 3-rhamnosylglucoside was found when cyanidin 3-glucoside was omitted.

Partial hydrolysis according to Lynn and Luh [4] of the radioactive product demonstrated that only the rhamnosyl moiety was labelled. The radioactivity was only found in the remaining cyanidin 3-rhamnosylglucoside spot, but not in the cyanidin 3-glucoside and cyanidin spot. Of the sugars liberated, only rhamnose was labelled.

The product formation was found to be proportional to the amount of added enzyme and to time for periods up to 15 min for incubations at pH 7.5. The enzyme exhibits an optimum of pH 8.1 with half maximum velocities at pH 6.1 and 8.8. The skewness of the curve is probably due to the instability of anthocyanins at higher pHs. To circumvent this problem, all assays were performed at pH 7.5 at which still 90% of the activity is left.

The divalent metal ions Co, Ca, Mg, Mn and Zn were tested over the concentration range of 1–20 mM. One mM of Co and Mn did stimulate the reaction rate. With Mg a continuous rise in activity was found up to 10 mM; at this concentration there was a two-fold stimulation. EDTA (1 mM, pH 7.5) had no influence upon the reaction velocity. The enzyme was 55% inhibited by 1 mM $HgCl_2$. The molecular weight of the enzyme (approximately 45000 daltons) was determined by the method of Andrews [5] using a Sephadex G-150 column with cytochrome-*c*, chymotrypsinogen A, egg albumin, aldolase and catalase as standards. The rhamnosyltransferase could also use pelargonidin 3-*O*-glucoside as a substrate. Larger amounts of UDP-L-rhamnose will be needed to determine the K_m values for the various other substrates.

The presence of the dominant allele of gene *N* is necessary for production of the enzyme that catalyses the transfer of the rhamnosyl moiety of UDP-L-rhamnose to the 6-position of the glucose bound at the 3-position of the anthocyanidin skeleton. No rhamnosyltransferase activity was found in petals of *n/n* plants. From this it can be concluded that the anthocyanidin 3-*O*-glucoside, 6″-*O*-rhamnosyltransferase exhibits the properties expected of a rhamnosyltransferase controlled by gene *N*.

EXPERIMENTAL

Plant material. The genotypes *C/C A/A P/P N/N ac/ac* with cyanidin 3-rhamnosylglucoside in the petals and *C/C A/A P/P n/n ac/ac* which contained cyanidin 3-glucoside were grown in the greenhouse of our experimental garden. The petals were collected in tubes placed in crushed ice and stored at −17°.

Preparation of substrates. UDP-[U-^{14}C]-glucose, 293 Ci/mol (Radiochemical Centre, Amersham, England) was converted to UDP-[U-^{14}C]-rhamnose using a protein preparation of *Nicotianum tabacum* var. Xanthi according to the method of ref. [6] except that the electrophoris step was omitted. Ten individual incubations were combined. After precipitation of the protein by adjusting the pH at pH 4–5 and centrifugation, the supernatant was concd under red. pres. and purified by PC on Whatman I in EtOH–1 M ammonium acetate (7:3). In this solvent system UDP-L-rhamnose runs slightly ahead of the UDP-D-glucose. For further purification the eluted UDP-L-rhamnose was re-chromatographed several times. In total 90 nmol were synthesized.

Cyanidin 3-glucoside was isolated from petals of appropriate genotypes of *S. dioica.* Pelargonidin 3-glucoside was isolated from strawberries [7]. The anthocyanins were purified by PC on Whatman III in BuOH–HOAc–H_2O (4:1:5, upper phase) and 1% HCl, eluted with 70% MeOH, 0.01% HCl and concd to dryness under red. pres., re-dissolved in a small vol. of MeOH i.e. 0.01% HCl, dried over $CaCl_2$ and precipitated in a large vol. of Et_2O. The precipitate was dried over silica. The anthocyanins used as substrate in the assay were dissolved in 2-methoxyethanol (EGME) H_2O. The final concns of the anthocyanins were determined by spectrophotometry (cyanidin 3-*O*-glucoside, EtOH, 0.01% HCl: 538 nm (ε 14000); pelargonidin 3-*O*-glucoside; 515 nm (ε 13000) [8, 9].

Enzyme preparation. 5 g of petals were homogenized at (0–4°) with an all glass Potter Elvehjem homogenizer in 5 ml 20 mM β-mercaptoethanol, 5% soluble PVP, 1% Triton X-100, 50 mM K–Na phosphate buffer (pH 7.5) and centrifuged for 15 min at 38000 *g*. The enzyme was purified from endogeneous substrate by filtration over polyclar AT (PVP) and Sephadex G-50 columns, which had been equilibrated before use with a 4 mM β-mercaptoethanol, 0.02% Triton X-100, 10 mM K–Na phosphate buffer (pH 7.5). This buffer was also used for the elution of the enzyme.

Enzyme assay. The standard reaction mixture consisted of 50 μl enzyme, 10 μl cyanidin 3-*O*-glucoside (14.6 mM) and 10 μl UDP-[U-^{14}C]-rhamnose (40 μM; S.A. 293 Ci-mol). The reaction mixture was incubated for 15 min at 30°, stopped by addition of trichloroacetic acid in MeOH, applied quantitatively, together with carrier cyanidin 3-rhamnosylglucoside, as a spot on Whatman III paper and developed two-dimensionally in BuOH–HOAc–H_2O (4:1:5, upper phase) and 1% HCl. After drying, the cyanidin 3-rhamnosylglucoside spot was cut out, placed in a scintillation vial with toluene liquifluor and counted in a liquid scintillation spectrometer (counting efficiency 80%).

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