Methylation of 3′,4′ Di-OH C-Glycosyl-flavones in *Silene*

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In green parts of Silene plants of the genotype gl^R two methylated flavonoids were found: isoscoparin and isoscoparin 2″-O-rhamnoside. An enzyme has been demonstrated to catalyze the transfer of the methyl moiety of S-adenosyl methionine to iso-orientin and iso-orientin 2″-O-rhamnoside. Maximal activity takes place at pH 8.0–8.2. Of the metal ions Mn^{2+}, Mg^{2+}, Ca^{2+}, Co^{2+}, Zn^{2+} and Hg^{2+}, only Co^{2+} stimulated the reaction at conc. > 2 mM. For the methylation of isoorientin the K_m values were 4×10^{-6} M for S-adenosyl methionine and 0.32×10^{-3} M for iso-orientin. When isoorientin 2″-O-rhamnoside was used as substrate the K_m values were 5×10^{-6} M for S-adenosylmethionine and 7×10^{-6} M for iso-orientin 2″-O-rhamnoside.

Introduction

Contrary to the petals of *Silene*, in which isovitexin is the only basic flavone present, the green parts show a greater flavonoid variation. Besides glycosides of isovitexin, iso-orientin glycosides have been demonstrated as well [1].

It appeared that the glycosylation pattern of isovitexin and iso-orientin is the same, *i.e.* the same glycosylation genes govern the glycosylation of isovitexin and iso-orientin [2–4].

The presence of iso-orientin derivatives is strongly influenced by the light regime. High light levels (above 30 000 lux) induce the hydroxylation of isovitexin to iso-orientin.

Besides the rise in iso-orientin concentration, high light levels also induce the formation of some previously unknown compounds. The structure elucidation and biosynthesis of these compounds will be described in this paper.

Results and Discussion

In the green parts of *Silene alba* plants, with isovitexin-2″-O-rhamnoside in the petals, besides isovitexin-2″-O-rhamnoside (R_f: BuOH-HOAc-H_2O, 4:1:5, upper phase: 0,43, 1% HCL: 0.52), and iso-orientin 2″-O-rhamnoside (R_f: BuOH-HOAc-H_2O, 4:1:5, upper phase: 0.33, 1% HCL: 0.40), an unknown compound X is present as well (R_f: BuOH-HOAc-H_2O, 4:1:5, upper phase: 0.37, 1% HCL: 0.44). At low light levels (12 000 lux) iso-orientin 2″-O-rhamnoside and the unknown compound are hardly detectable. The unknown compound was isolated by paper chromatography on Whatman III in BuOH-HOAc-H_2O 4:1:5, upper phase and 1% HCL subsequently. Acid hydrolysis liberated rhamnose and a mixture of two flavones A_1 (R_f: BuOH-HOAc-H_2O 4:1:5, upper phase: 0.30, 1% HCL: 0.02) and A_2 (R_f: BuOH-HOAc-H_2O 4:1:5, upper phase: 0.52, 1% HCL: 0.09). Hydrolysis of each of the two purified "aglycones" gave again a mixture of these "aglyglycones". This suggests that the unknown basic compound has a C-glycosyl flavone skeleton which on acid hydrolysis gives two isomers. There were no differences in the spectra of A_1, A_2 and the unknown compound, which suggests that all phenolic hydroxyl groups are free, and the rhamnose of our unknown compound is released from the C-C bound glucose. The spectra of A_1, A_2 and X correspond with the spectra of scoparin [5]: MeOH: 253 sh, 270, 345; NaOMe: 277, 330, 409; $AlCl_3$: 258 sh, 276, 300, 359, 387 sh; $AlCl_3$ + HCL: 260 sh, 277, 300, 355, 384 sh; NaOAc: 277, 318, 398; NaOAc + H_3BO_3: 273, 343. The R_f values of compound A_1 correspond with scoparin. From this it can be concluded that compound A_2 is isoscoparin and that compound X is probably isoscoparin O″-rhamnoside. This structure is further confirmed by synthetic studies.

Incubation of an enzyme preparation, freed from phenolic compounds by PVP and G-10 chromatography, with iso-orientin and S-adenosyl methionine gave a product which co-chromatographed with compound A_2. Maximal synthesis took place at pH 8.0–8.2. Of the divalent metals ions tested, Mg^{2+}, Mn^{2+}, Ca^{2+}, Co^{2+}, Zn^{2+} and Hg^{2+}, only Co^{2+} stimulated the reaction at conc. > 2 mM (150%). The true K_m values for iso-orientin and S-adenosylmethionine were respectively 0.32×10^{-3} M and 4×10^{-6} M. When iso-orientin-O″-rhamnoside was used as substrate, product X was formed. The true K_m values for iso-orientin O″-rhamnoside and S-adenosyl methionine were respectively 7×10^{-6} M and 5×10^{-6} M. The 46 times higher affinity for iso-orientin O″-rhamnoside than for iso-orientin suggests that not iso-orientin but its O″-rhamnoside is *in vivo* a substrate for the enzyme.

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It has been shown before that in isovitexin the rhamnosylation, governed by gene gl^R, takes place at the 2″ OH of the C-C bound glucose [6]. Genetic studies (unpublished) showed that the rhamnosylation of iso-orientin and iso-scoparin is also governed by gene gl^R. It therefore is probable that in iso-orientin and isoscoparin the rhamnose is also bound to the 2″ hydroxyl group of the C-C bound glucose. So the unknown compound is isoscoparin 2″-O-rhamnoside.

Experimental

Plant material. Silene alba was grown in the open in the experimental garden of the Department of Population and Evolutionary Biology, University of Utrecht. For all experiments fresh material was used.

Chemicals. S-[Methyl ^{14}C]adenosyl-L-methionine, S. A. 57.6 Ci/mol, was supplied by New England Nuclear. Iso-orientin and iso-orientin 2″-O-rhamnoside were isolated from *Silene* plants of the appropriate genotype.

Enzyme preparation. 1 g leaves was homogenized in 3 ml 100 mM phosphate, 20 mM β-mercaptoethanol, 2.5% PVP, 0.01% triton X-100, pH 8.0, and centrifuged for 20 min at 38 000 × *g*.

The supernatant was purified by PVP and G-10 chromatography. For all experiments the G-10 eluate was used.

Enzyme assay. The standard reaction mixture consisted of 2 µl iso-orientin 1.5% in EGME, (or 2 µl iso-orientin O″-rhamnoside), 6 µl S-adenosyl methionine (57.6 Ci/mol, 0.347 mM), and 25 µl protein fraction. The reaction mixture was incubated for 1 h at 30 °C. The reaction was stopped by the addition of 50 µl trichloro acetic acid 15%. The reaction product was isolated by 2-dimensional chromatography (BuOH/HOAc/H_2O 4:1:5, upper phase, and 1% HCl). The spots A_2 and X were cut out and counted in a toluene based mixture in a liquid scintillation spectrometer.

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