Biosynthesis of Vitexin and Isovitexin: Enzymatic Synthesis of the C-Glucosylflavones Vitexin and Isovitexin with an Enzyme Preparation from *Fagopyrum esculentum* M. Seedlings

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Biosynthesis of Vitexin, *Fagopyrum esculentum* M., Flavonoid-C-glucosylation, 2-Hydroxyflavanones

A C-glucosyltransferase from *Fagopyrum esculentum* seedlings catalyzes the transfer of glucose from UDP-glucose or ADP-glucose to 2-hydroxyflavanones. In cell-free enzyme preparations it was shown that only 2-hydroxyflavanones, *e.g.* 2,4′,5,7-tetrahydroxyflavanone and 2,5,7-trihydroxyflavanone were appropriate substrates. Naringenin, naringenin-chalcone and the flavones apigenin and chrysin cannot act as glucosyl acceptors in C-glucosyl-flavonoid biosynthesis. This demonstrates that C-glucosylation occurs after oxidation of flavanones.

Introduction

C-glucosyl flavonoids are widespread in the plant kingdom with the most important representatives being vitexin and isovitexin [1]. The biosynthesis of the aglycon was subject of several investigations [2]. Nothing, however, is known about the mechanism of the C-glucosylation of these flavone compounds [3]. *In vivo* experiments demonstrated that flavanones might act as precursors for flavone-C-glucosides but not the flavones [4]. On the other hand the flavanone-C-glucosides are rarely found in plants. Oxidation of the flavonoids is not known to occur after glucosylation [5].

Britsch *et al.* [6] showed that flavanone oxidation might be dependent upon a dioxygenase but they could not isolate the postulated 2-hydroxyflavanone. The difficulty in isolation and characterization of the 2-hydroxyflavanones is due to their instability because of the easy intramolecular loss of water thereby forming the corresponding flavones. However, several examples of 2-hydroxylated flavanones or the tautomeric dibenzoylmethanes have been detected in plants [7], especially the more stable derivatives lacking hydroxylation in ring B.

Cotyledons of *Fagopyrum esculentum* are known to produce considerable amounts of vitexin and isovitexin [8]. The aim of the present work was to demonstrate at which stage during biogenesis of the aglycon C-glucosylation occurs. Enzyme extracts were prepared from this cotyledon material and incubated with several hypothetic glucosyldonors and acceptors such as naringenin-chalcone, naringenin, 2-hydroxynaringenin, apigenin and further with 2,5,7-trihydroxyflavanone and chrysin.

Material and Methods

Plant material

Fagopyrum esculentum M. seeds were grown on wet filter paper at room temperature and daylight. When the hypocotyl reached the length of 2–3 cm (after 5 days) the cotyledons were excised and frozen at −20 °C.

Enzyme preparation

All steps were carried out at 4 °C. 2 g of frozen cotyledons and 0.5 g PVP were homogenized with 2 ml 0.2 M Tris-HCl buffer pH 8.15, containing 10 mM DTE in a mortar and centrifuged at 40,000 × *g* for 40 min (= S40). The supernatant was fractionated by an ammoniumsulfate precipitation. The 30–80% pellet was redissolved in 0.1 M Tris-HCl buffer pH 8.15, containing 5 mM DTE (= ASP).

Abbreviations: PVP, polyvinylpyrrolidone; DTE, dithioerythritol; PC, paperchromatography; HPLC, high pressure liquid chromatography; TLC, thin layer chromatography; Tris, trishydroxymethylaminomethane.

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Aglycon preparation

Naringenin, apigenin and chrysin were purchased from Roth, Karlsruhe. Naringenin-chalcone was prepared as described by Moustafa and Wong [9].

2-Hydroxynaringenin and 2,5,7-trihydroxyflavanone were prepared by alkaline hydratation of apigenin according to the method of Hauteville and Chopin [10]. 20 mg of apigenin or chrysin were dissolved in 10 ml pyridine, containing 1% water. After addition of 2.5 g KOH (powdered under ether), the mixture was refluxed for 2 h. After neutralization and extraction with ethylacetate, the product was separated by PC [11] and HPLC. The product was further identified by UV spectroscopy and conversion into apigenin or chrysin after treatment with acetic acid (10% at 60 °C 30 min).

Quantitative estimation of 2-hydroxyflavanone was carried out by peak integration after HPLC separation with naringenin as internal standard (molecular extinction coefficient: naringenin 4.23 at 288 nm [11]; 2,4′,5,7-tetrahydroxyflavanone 4.20 at 290 nm [14]).

Analytical methods

TLC was carried out on "Kieselgel 60 Merck" with the solvent system ethylacetate, methylethylketone, water, formic acid, acetic acid (50/30/10/7/3), vitexin rf 70, isovitexin rf 55, 2,5,7-trihydroxy-6(or 8)-C-glucosylflavanone rf 68, 6-C-glucosylchrysin rf 58, 8-C-glucosylchrysin rf 73.

HPLC was done on Lichrosorb RP 18, 7 μm column (250 × 4.6 mm). Solvent A: water, methanol, acetic acid (78/20/2). Solvent B: (18/80/2), linear gradient, 0 min 20% B, 18 min 60% B, 21 min 100% B, 29 min 100% B; flow rate 1 ml/min; retention times: vitexin 11 min, 2,5,7-trihydroxy-6(or 8)-C-glucosylflavanone 11.5 min, 8-C-glucosylchrysin 12.5 min, isovitexin 13 min, 2,4′,5,7-tetrahydroxyflavanone 17 min, 6-C-glucosylchrysin 19 min, naringenin 22 min, chalcone 24 min, 2,5,7-trihydroxyflavanone 25 min, apigenin 26 min, chrysin 29 min.

Isolation and identification of vitexin and isovitexin

The incubation mixture was acidified with 6% HCl (v/v) and extracted with 3 × 10 ml ethylacetate. The mixture was separated on TLC in the above solvent system. Vitexin and isovitexin were scraped out and separated by HPLC. In both cases, radioactivity corresponded with the two C-glucoside fractions. After treatment with 6% HCl at 100 °C for 60 min, radioactivity was not lost from the aglycon.

Isolation and identification of 2,5,7-trihydroxy-6-(or 8)-C-glucosylflavanone

The incubation mixture was extracted without acidification with 3 × 10 ml ethylacetate and separated on TLC. The radioactivity was scraped out and separated by HPLC.

2,6-Dichloroquinone-4-chlorimide reaction

TLC plates were dried after the separation, transfered for 10 min into an NH_3 saturated atmosphere and then sprayed with 0.5% 2,6-dichloroquinone-4-chlorimide solution in ethanol. For colour development they were transfered again into NH_3 atmosphere. Isovitexin and 6-C-glucosylchrysin appeared as blue spots, 2,5,7-trihydroxy-6(or 8)-C-glucosylflavanone as dark blue spot.

Enzyme assay

The incubation mixture contained 0.1 mM aglycon, 0.1 mM UDP-[^{14}C]glucose 4.625 kBq, 0.1 M Tris-HCl buffer pH 8.15, and enzyme preparation as indicated in the tables in a total volume of 0.33 ml. Incubation was carried out at 25 °C for 30 min.

Results

Specifity of the aglycon for O- and C-glucosylation

Naringenin and naringenin-chalcone

Incubation of naringenin and UDP-[^{14}C]glucose with the enzyme preparation S40 (see Material and Methods) resulted in the formation of one radioactive product which cochromatographed in TLC and HPLC with authentic prunin (naringenin-7-O-glucoside). The product could be hydrolyzed with 6% HCl at 100 °C within 45 min thereby forming naringenin and radioactive glucose.

Incubation with naringenin-chalcone instead of naringenin also produced prunin. The expected hemiphloin or isohemiphloin was not formed (Fig. 2).

2-Hydroxynaringenin and 2,5,7-trihydroxyflavanone

Incubation of 2-hydroxynaringenin and UDP-[^{14}C]glucose with the enzyme preparation (S40) produced two radioactive compounds which were shown

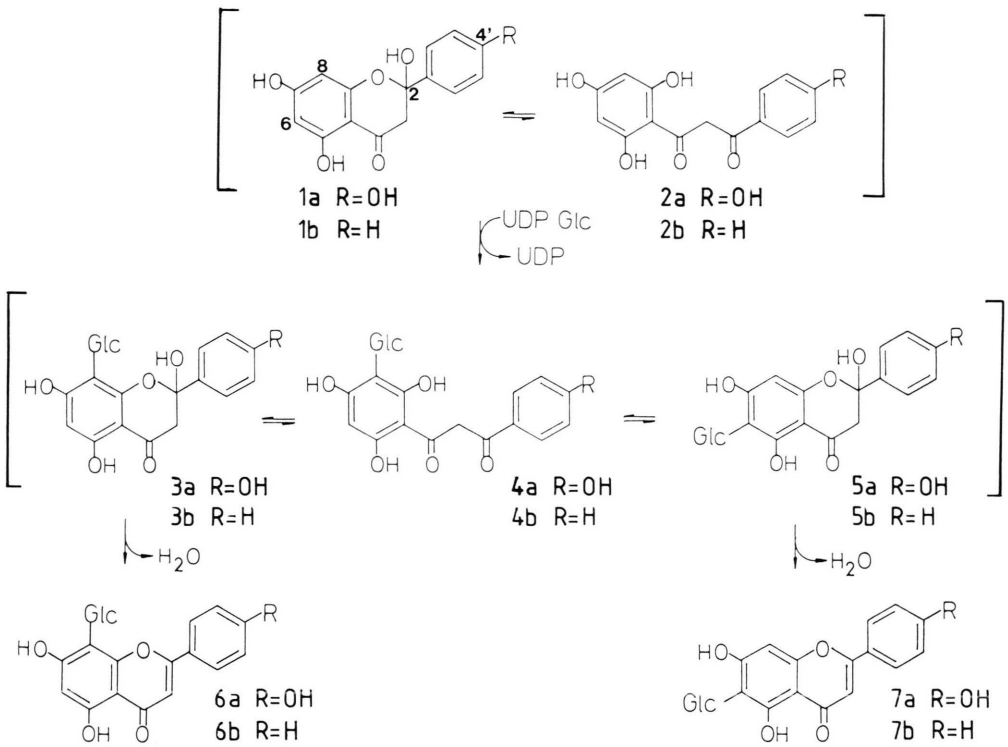

Fig. 1. Proposed reaction sequence for C-glucosylation of flavonoids. **1a**, 2-hydroxynaringenin; **1b**, 2,5,7-trihydroxyflavanone; **2**, dibenzoylmethane; **3**, **4**, **5**, three possible forms of C-glucosylated 2-hydroxyflavanones, being in rapid equilibrium; **6a**, vitexin; **6b**, 8-C-glucosylchrysin; **7a**, isovitexin; **7b**, 6-C-glucosylchrysin.

Table I. Aglycon specifity for O- and C-glucosylation.

Enzyme source	Aglycon (0.1 mM)	[^{14}C]-Products formed *in vitro* (Bq) per mg of protein
S40	naringenin-chalcone	prunin (4)
S40	naringenin	prunin (5)
S40	2-hydroxynaringenin	vitexin (48), isovitexin (53)
S40	apigenin	none
ASP	2-hydroxynaringenin	vitexin (129), isovitexin (147)
ASP	apigenin	none
ASP	2,5,7-trihydroxy-flavanone	"2,5,7-trihydroxy-6(or 8)-C-glucosylflavanone" (170)
ASP	chrysin	none

Glucosyl donor was in all assays UDP-[^{14}C]glucose, 0.1 mM 4.625 kBq. S40: 40,000 × *g* supernatant of cotyledon homogenate containing 6 mg protein in a total volume of 0.33 ml. ASP: redissolved ammonium sulfate precipitate containing 2.5 mg protein in a total volume of 0.33 ml.

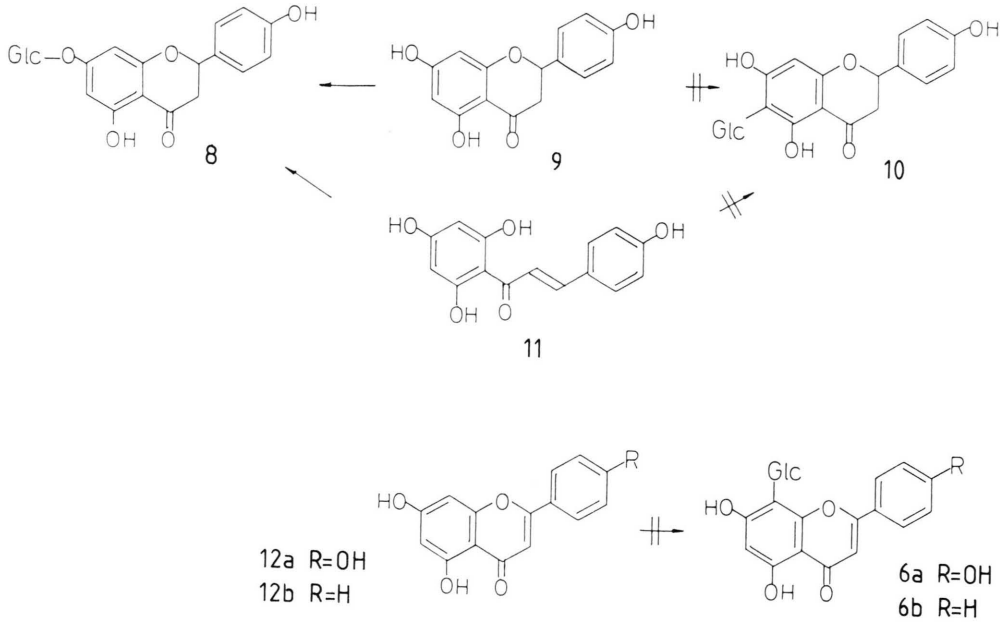

Fig. 2. Reaction sequence of flavonoid aglycons as shown with enzyme preparations from *Fagopyrum esculentum* cotyledons and UDP-[^{14}C]glucose. **8**, Prunin; **9**, naringenin; **10**, hemiphloin; **11**, naringenin-chalcone; **12a**, apigenin; **12b**, chrysin.

to be identical after TLC and HPLC with authentic vitexin and isovitexin. The products could not be hydrolyzed with 6% HCl at 100 °C within 3 h but a Wessely-Moser isomerization was observed [11].

In a parallel experiment apigenin and UDP-[^{14}C]-glucose were incubated with the S40 preparation, but no radioactive glucoside was formed. Therefore it can be excluded, that C-glucosylation takes place after dehydration of 2-hydroxynaringenin.

When the experiments were carried out with the enzyme preparation ASP (see Material and Methods) the formation of the same products could be demonstrated.

2-Hydroxyflavanones without hydroxylation in ring B are considered to be more stable than 2-hydroxynaringenin [12]. 2,5,7-Trihydroxyflavanone was prepared by alkaline hydratation of chrysin. After incubation of 2,5,7-trihydroxyflavanone and UDP-[^{14}C]glucose with the enzyme preparation ASP only one radioactive product (Fig. 1, **3b**, **4b**, **5b**) could be demonstrated by TLC (Fig. 3) and HPLC. The UV spectrum was similar to 2,5,7-trihydroxyflavanone. Reaction with 2,6-dichloroquinone-4-chlorimide showed a *para*-nonsubstituted phenolic group.

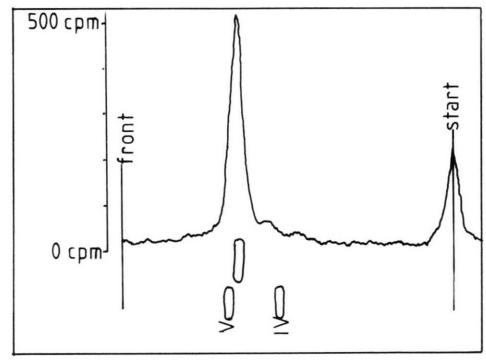

Fig. 3. TLC of the ethylacetate extract obtained from an enzymatic incubation with 2,5,7-trihydroxyflavanone, UDP-[^{14}C]glucose and ASP. The radioactive fraction is 2,5,7-trihydroxy-6(or 8)-C-glucosylflavanone (Fig. 1, **3b**, **4b**, **5b**).

Treatment with 10% acetic acid at 60 °C for 15 min formed two substances (Fig. 4), which could not be hydrolyzed with 6% HCl at 100 °C for 3 h. Therefore an O-glucosylation was excluded. UV spectra of the HPLC purified products demonstrated the presence of chrysin derivatives. Reaction with

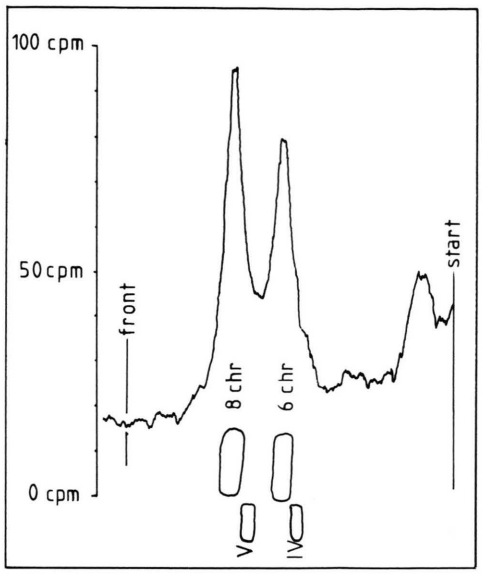

Fig. 4. Separation of the products obtained after acid treatment (with 10% acetic acid, 60 °C, 15 min) of the radioactive fraction from Fig. 3. 6chr, 6-C-glucosylchrysin; 8chr, 8-C-glucosylchrysin.

2,6-dichloroquinone-4-chlorimide showed that only one product had a *para*-nonsubstituted phenolic group. From these data it is concluded that the formed products are 6-C-glucosylchrysin and 8-C-glucosylchrysin. They were both treated with 6% HCl at 100 °C for 3 h. Thereby a Wessely-Moser isomerization was observed according to the pair of vitexin and isovitexin.

Specifity of the glucosyl donor

In an other series of experiments 2-hydroxynaringenin and the enzyme preparation ASP were incubated with further hypothetic glucosyl donors. The substrate specifity of the glucosyltransferase for the glucosyl donors apparently is not very high. Both, UDP-[^{14}C]glucose and ADP-[^{14}C]glucose but not GDP-[^{14}C]glucose or glucose-1-phosphat are effective glucosyl donors (Table II).

Discussion

The above results strongly suggest that the 2-hydroxylation of flavanones is an essential prerequisite for C-glucosylation in positions 6 or 8 of the aromatic ring A. Thereby the pyran ring C of flavanone is converted into a cyclic hemiketal. Hemiketals are known to be rather unstable. Studies of Hauteville *et al.* [13, 14] demonstrated that 2-hydroxyflavanones preferentially exist in the cyclic form. In addition Chopin *et al.* [12] could show that 6(or 8)-formyl-2,5,7-trihydroxy-8(or 6)-methylflavanone from *Unona lawii* exists as an equilibrium mixture of isomers, which cannot be separated. These observations confirm our findings that the Wessely-Moser isomers of 2,5,7-trihydroxy-6(or 8)-C-glucosylflavanones (Fig. 1, **3b**, **5b**) are in a rapidly formed state of equilibrium and cannot be separated from each other (Fig. 3).

Which aglycon structure, *i.e.* flavanone or dibenzoylmethane, is accepted preferentially from the enzyme is still unknown (Fig. 1, **1** or **2**). Because naringenin or chalcone cannot act as C-glucosyl

Table II. Specifity of the glucosyl donor for the formation of C-glucosides vitexin and isovitexin.

[^{14}C]-Glucosyl donor	(Bq)	[^{14}C]-Reaction products formed per mg of protein	
		Vitexin (Bq)	Isovitexin (Bq)
UDG-glucose	4625	129	147
ADP-glucose	4625	84	106
GDP-glucose	4625	3	4
Glc-1-P	37000	none	none
UDP-glucose[a]	4625	none	none

The incubation mixture contained: 0.1 mM glucosyl donor as indicated above, 0.1 mM 2-hydroxynaringenin, enzyme (ASP, 2.5 mg) in a total volume of 0.1 M Tris-HCl buffer pH 8.15 of 0.33 ml.
[a] Control experiment, containing heat inactivated enzyme.

acceptor it is suggested that the enzyme accepts the dibenzoylmethane isomer. Further experiments with inhibitors, *e.g.* dibenzoylmethanes to characterize the purified enzyme are in progress.

Acknowledgement

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