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SHORT REPORTS

POSITION SPECIFICITY OF AN *o*-DIHYDROXYCOUMARIN GLUCOSYLTRANSFERASE FROM TOBACCO CELL SUSPENSION CULTURE

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Key Word Index—*Nicotiana tabacum*; Solanaceae; *o*-dihydroxycoumarins; esculetin; daphnetin; glucosyltransferase; position specificity; tobacco cell culture.

Abstract—A partially purified UDP-glucose: *o*-dihydroxycoumarin glucosyltransferase from tobacco cell culture required an intact coumarin ring system with *o*-dihydroxy groups for highest activity. The enzyme exhibited strict position specificity towards the 7-OH group of both esculetin and daphnetin with the formation of cichoriin and daphnin, respectively. The apparent K_m values were 111 and 95 μM, while the V_{max} for esculetin was 26.4 pkat/mg protein.

INTRODUCTION

Glucosyltransferases are believed to possess a broad specificity [1]; however, some enzymes have been reported recently to exhibit high affinity towards certain phenolic substrates such as lignin precursors [2, 3], flavones or flavonols [4–6] and anthocyanidins [7, 8]. Although only a few coumarin glucosides are known to occur in plants [9], little information is available on the enzymes involved in their formation. Earlier isotopic evidence has indicated that scopolin (scopoletin-7-*O*-glucoside) and fabiatrin (scopoletin-7-*O*-primeveroside) are readily formed from [^{14}C]-scopoletin administered to tobacco seedlings [10]. On the other hand, esculin (esculetin-6-*O*-glucoside) and daphnetin-8-*O*-glucoside appear to be formed by intramolecular *trans*-glucosylation of cichoriin (esculetin-7-*O*-glucoside) and daphnin (daphnetin-7-*O*-glucoside), respectively [11, 12]. Recently, we described the partial purification and some properties of a glucosyltransferase which exhibited a high affinity towards the *o*-dihydroxycoumarins, esculetin and daphnetin [13]. We wish to report here on the position specificity of this enzyme for the 7-OH group, with the formation of cichoriin and daphnin respectively.

RESULTS AND DISCUSSION

The enzyme extract used in this study was partially purified by $(NH_4)_2SO_4$ precipitation and chromatography on DEAE-Sephadex A-50 which resulted in an 80-fold increase in specific activity [13]. Table 1 lists a number of coumarin derivatives that were tested for their relative glucose acceptor ability. The results indicate that this enzyme exhibits highest affinity for the two *o*-dihydroxycoumarins esculetin and daphnetin. The reaction products were rigorously identified as the 7-*O*-glucosides, cichoriin and daphnin, respectively by cochromatography with reference compounds in a number of solvents, autoradiography and hydrolysis with β-glucosidase followed by identification of the aglucone and [^{14}C]-glucose.

Table 1. Position specificity of tobacco glucosyltransferase against some phenylpropanoid substrates*

Substrate†	Relative activity‡
Daphnetin (7,8-diOH-coumarin)	100
Esculetin (6,7-diOH-coumarin)	95
Caffeic acid (3,4-diOH-cinnamic)	50
Hydrangetin (7-OH-8-OMe-coumarin)	30
Scopoletin (6-OMe-7-OH-coumarin)	25
Umbelliferone (7-OH-coumarin)	25

* The standard assay was used as described in the Experimental, at saturated concn (0.2–0.3 mM) of substrate.

† Some activity (10–15%) was observed with protocatechuic, vanillic and syringic acids; slight activity (*ca* 5%) with ferulic, 5-OH-ferulic and sinapic acids; no activity with isoscopoletin, isoferulic, isovanillic, *p*-coumaric, *p*-hydroxybenzoic acids, luteolin, chrysoeriol, quercetin or isorhamnetin.

‡ Relative to daphnetin = 100; activity in daphnin amounted to 1 240 000 dpm/mg protein.

Opening of the lactone ring of the *o*-dihydroxycoumarin, as in caffeic acid, or removal of one OH group, as in umbelliferone, resulted in a 50% decrease in enzyme activity; whereas methylation of one OH group, as in scopoletin or hydrangetin, brought about a 70–75% decrease in activity. The combination of both structural changes, as in ferulic, 5-OH-ferulic or sinapic acids, resulted in almost complete loss of activity. Furthermore, this enzyme seems to exhibit strict position specificity towards the OH group *para* to the side chain of the phenolic ring since neither esculin nor daphnetin-8-*O*-glucoside appeared among the reaction products when their aglucones were used as substrates. In addition, the enzyme did not react with isoscopoletin, isoferulic or isovanillic acids, nor did it accept coumarin glucosides with a free 7-OH group such as esculin or daphnetin-8-*O*-glucoside. There was some activity observed (10–15%)

with protocatechuic, vanillic and syringic acids, but none with the monohydroxy compounds, *p*-coumaric or *p*-hydroxybenzoic acids, nor with any of the flavonoid substrates tested—luteolin, chrysoeriol, quercetin or isorhamnetin.

We propose, therefore, the systematic name UDP-glucose: *o*-dihydroxycoumarin 7-*O*-glucosyltransferase. However, due to the limited extent of enzyme purification, it is possible that two enzymes/isoenzymes exist in tobacco cell culture which may be involved in the separate glucosylation of esculetin and daphnetin.

EXPERIMENTAL

Enzyme source. Tobacco cell culture glucosyltransferase was isolated and partially purified by $(NH_4)_2SO_4$ precipitation (60–80% satn) and chromatography on Sephadex G-25 and DEAE-Sephadex A-50 as previously described [13]. The enzyme preparation used in specificity studies had a sp. act. of 26.4 pkat/mg protein which represented an 80-fold increase in activity as compared with the crude extract.

Enzyme assays. The standard assay was similar to that described in ref. [13] using UDP-[U-^{14}C]-glucose as the glucose donor.

Identification of reaction products. After acidification and addition of $(NH_4)_2SO_4$ (40% final concn) the products were extracted with EtOAc then cochromatographed with reference compounds on Whatman No. 3 paper or cellulose–Si gel G (1:1, w/w) TLC plates using *n*-BuOH–HOAc–H_2O (4:1:2); 2% HOAc; *n*-BuOH–NH_4OH–EtOH–C_6H_6 (5:3:1:1) or *iso*-PrOH–NH_4OH–H_2O (8:1:1) or a combination of these for 2D chromatography, then autoradiographed against X-ray films. The spots corresponding to cichoriin and daphnin were cut out and the activity determined in a liquid scintillation counter. Products from several assays were pooled and subjected to hydrolysis with β-glucosidase and their aglucones and [^{14}C]-glucose were identified by the common chromatographic methods.

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