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CONVERSION OF DAPHNIN TO DAPHNETIN-8-GLUCOSIDE IN *DAPHNE ODORA*

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Abstract—Daphnetin-8-glucoside is found in *Daphne odora* and an enzyme preparation from the plant was found to be capable of converting daphnin (daphnetin-7-glucoside) to the 8-glucoside.

INTRODUCTION

DAPHNETIN (7,8-dihydroxycoumarin) and its 7-glucoside, daphnin, have been found in several species of the genous *Daphne*, for example, *D. alpina*,[1] *D. merezeum*[1] and *D. odora*.[2] Recently, daphnetin-8-glucoside has also been reported in *D. julia*.[3] However, the biogenesis of these substances has not been investigated, although many studies have been carried out on the metabolism of other natural coumarins.[4] The present paper deals with the detection of daphnetin-8-glucoside in *D. odora* and its enzymic formation from daphnin.

RESULTS

Detection of Daphnetin-8-Glucoside (D-8-G)

When an extract of *Daphne odora* flowers was examined by paper chromatography, three phenolic substances (*A*, *B* and *C*) were detected and subsequently isolated. Some of their properties are shown in Table 1. Substances *A* and *B* were readily identified as daphnetin and daphnin, respectively. Substance *C* rapidly lost its characteristic fluorescence when incubated with crude enzyme extract from *D. odora*, yielding a phenolic substance which was identical with daphnetin. Hydrolysis of *C* with emulsin showed it is a glucoside, glucose being detected on chromatograms with Bacon-Edelman's reagent, and the ratio of glucose and daphnetin was determined as 1:1. Thus the substance *C* must be daphnetin-8-glucoside (D-8-G): its identity with a synthetic sample[5–7] was confirmed by the criteria given in Table 1, by i.r. and fluorescence emission spectra and mixed m.p. test. The compound was detected in all the organs of this plant, being especially abundant in the flowers.

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TABLE 1. PROPERTIES OF COMPOUNDS *A*, *B* AND *C* FROM *Daphne odora*

	A (Daphnetin)	*B* (Daphnin)	*C* (8-Glucoside)
R_f values in			
5% acetic acid	0·45	0·72	0·76
n-Butanol:acetic acid:water (4:1:2)	0·77	0·43	0·49
Water-saturated *n*-butanol	0·74	0·21	0·34
Water	0·36	0·65	0·70
Colour with			
$FeCl_3$–$K_3Fe(CN)_6$	Blue	Blue	Blue
Alc. $FeCl_3$	Green	—	—
Phosphomolybdic acid	Yellow	—	—
Diazotized *p*-nitroaniline	Brown	Yellow	—
Fluorescence at			
365 nm $-NH_3$	Dull green	—	Bright blue
$+NH_3$	Yellow	—	Bright greenish blue
254 nm	Quenching	Quenching	—
Absorption spectrum			
λ_{max} (nm)	265·5, 330	258, 314	256·5,* 326
λ_{min} (nm)	241, 279	240, 272	268·5
M.p.	256°	223–224°	223–224°†

* Shoulder.
† See Experimental.

Enzymic Formation of D-8-G from Daphnin

When daphnin was incubated with a crude enzyme preparation from *Daphne*, the reaction mixture developed a bright greenish fluorescence within a few seconds. Paper chromatography showed two products which were identified as daphnetin and D-8-G. Thus the enzyme preparation can hydrolyse daphnin to daphnetin (D-8-G is also hydrolysed after a longer incubation), which can then act as a glucosyl acceptor. When the reaction was followed by determination of fluorescence intensity it was found that the D-8-G formation begins at once in the system supplied with daphnetin, but if daphnetin is not added, a clear lag phase was present before the reaction attains a maximum rate. This suggested that the formation of D-8-G is necessarily preceded by the liberation of daphnetin. It thus seemed that daphnin was partly hydrolysed to daphnetin, to which the glucosyl residue of daphnin was then transferred.

The incubation of daphnetin with esculin (esculetin-6-glucoside) with the crude enzyme preparation also gave D-8-G, so that esculin could serve as a glucosyl donor. D-8-G was also obtained from daphnetin and cichoriin (esculetin-7-glucoside). This reaction also gave esculin in addition to D-8-G, indicating the subsequent transfer of glucose to the liberated esculetin also can take place. The incubation of cichoriin alone with the enzyme gave rise to esculetin and esculin. However, daphnin and cichoriin were not produced from D-8-G and esculin, and it appeared that with this preparation glucose could not be transferred to the hydroxyl at seven of these coumarins. Umbelliferone (7-hydroxycoumarin) was not glucosylated in this system. Glucose itself, maltose and cellobiose or simple phenolic glucosides (phenyl-β-glucoside, arbutin and salicin) were all ineffective as glucose donors.

DISCUSSION

As shown above, *Daphne odora* contains two daphnetin glucosides: 7- and 8-glucosides, as in *D. julia*,[3] and its enzyme preparation can form 8-glucoside from 7-glucoside.

It has been known that the formation of phenolic glycosides is effected by the participation of glycosyl nucleotides,[8] but "low-energy glycosides" have also been shown to serve as glycosyl donors.[9,10] The glycosylation catalysed by *Daphne* enzyme is of the low-energy type. This reaction can use only certain coumarin glucosides as glucose sources and is unlikely to be effective for glucosylation of the 7-hydroxyl group of coumarins. It thus seemed that the 7-glucosylation of daphnetin (daphnin formation) in *D. odora* tissues is proceeded by a reaction different from the 8-glucosylation. The present data also suggest that, in the plants containing two esculetin glucosides, esculin and cichoriin,[11,12] the former may be obtained from the latter by a transfer reaction.

The *Daphne* enzyme was capable of catalysing two reactions, hydrolysis and trans-glucosylation. It has generally been accepted that a number of hydrolytic enzyme which normally hydrolyse the glycosidic linkage may also transfer glycosyl residue to compounds other than water and form new glycosyl derivatives.[8] But the present experiments have not shown whether the above two reactions are due to one enzyme or different enzymes.

EXPERIMENTAL

Isolation of the Substances A, B *and* C

100 g of the flowers were previously immersed in warm water (500 ml) at 40° (this procedure stimulated the enzyme(s) involved in the formation of the substance *C*), and then extracted twice in boiling water for 30 min. The combined extracts were reduced to *ca.* 50 ml and Et_2O-soluble substances were thoroughly extracted. The aq. phase was further brought to *ca.* 5 ml and the substance *B* (daphnin) separated was purified by three recrystallizations from EtOH. The residual solution was subjected to a large-scale paper chromatography (Whatman No. 1) in water-saturated *n*-BuOH. The greenish bands were eluted with EtOH and rechromatographed to remove trace amounts of daphnin. The substance *C* (daphnetin-8-glucoside) was crystallized three times from EtOH (*ca.* 30 mg); m.p. 223–224° (the previous authors recorded 216–217° (synthetic sample),[5–7] 191–193° (natural specimen);[4] crystals from alcohol with a trace amount of water melted at lower temperature). The substance *A* (daphnetin) was obtained by concentrating the ether extract and crystallizing from dilute EtOH.

Preparation of the Enzyme Solution

Flowers of this plant, previously frozen, were blended in cold acetone at −20° and treated with further acetone to give a finely ground powder, which was dried and stored at −20° until used. 1 g of the powder was suspended in 40 ml of 0·1 M phosphate buffer, pH 6·8, and the suspension stirred for 10 min in the cold. It was centrifuged at 15,000 *g* for 30 min, and the supernatant passed through Sephadex G-25 (M) column. To the almost colourless effluent (35 ml), 3 vol. of cold acetone were added, the protein collected by centrifugation and dissolved in a minimum volume of water. The solution was dialysed overnight against cold water and the dialysate (10 ml), in which no phenolic and fluorescent substances could be detected, was used as the enzyme solution.

Determination of Glucose/Daphnetin Ratio of the Substance C

5–10 mg of *C* in 1 ml of water were hydrolysed with emulsin and the daphnetin extracted with ethyl acetate and determined at 330 nm (molar extinction coefficient 12,800). Glucose was estimated in the aqueous phase by the method of Somogyi.[13]

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The Condition of the Reaction

The reaction mixture consisted of 2·5 ml of 0·04 M phosphate buffer, pH 5·9, 1 ml of 0·01 M glucoside solution, 0·1 ml of the enzyme solution (five times dilution of the original solution) and, in some reactions, 1 ml of 0·05 M coumarin solution (glucose acceptor) was added. The total volume was 4·6 ml and the reaction run at 20°. From time to time, 0·5 ml of the reaction mixture were transferred to 0·1 ml of 0·1 N HCl to stop the reaction, and the products examined by paper chromatography in 5% acetic acid. The products were identified by fluorescent colours with or without ammonia vapour, colour reactions to the reagents and chromatographic behaviours.

The fluorescence intensity (arbitrary unit) of daphnetin-8-glucoside produced in the reaction mixture was directly determined with a spectrophotometer using the 350 nm setting for the activation and measuring the output at 497 nm.

Chemicals

Daphnetin-8-glucoside[7] and umbelliferone[14] were synthesized, and cichoriin isolated from flowers of *Cichorium intybus*.[11] Esculin and esculetin were commercially obtained and purified before use.

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