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TRANSGLUCOSYLASES IN *CICHORIUM INTYBUS* CONVERTING CICHORIIN TO ESCULIN

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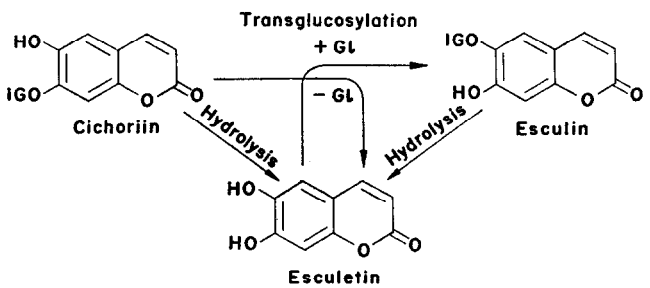
Key Word Index—*Cichorium intybus*; Compositae; *Daphne odora*; Thymelaeaceae; cichoriin; esculin; esculetin; transglucosylases; biosynthesis of dihydroxycoumarin glucosides.

Abstract—The heads of *Cichorium intybus* contain two enzymes concerned in the formation of esculin (esculetin 6-glucoside) from cichoriin (esculetin 7-glucoside). Both enzymes can catalyse two reactions, i.e. hydrolysis (HD) of cichoriin to give esculetin, and transglucosylation (TG) from this glucoside to the liberated aglucone forming esculin. One of them, designated enzyme A, is a high molecular weight protein with predominantly TG activity, and dissociates during isolation into the other enzyme having higher HD activity. Enzyme A shows high substrate specificity and different pH optima in HD and TG reactions, as in the case with the transglucosylase from *Daphne odora*.

INTRODUCTION

DURING the course of investigations on the metabolism of naturally-occurring dihydroxycoumarins[1-3] the authors found that *Daphne odora* contains a daphnetin monoglucoside, daphnetin 8-glucoside, in addition to daphnin which had already been reported.[4] The former glucoside is formed from the latter by a transglucosylase capable both of hydrolysing daphnin to daphnetin and of transferring the glucosyl residue of a second molecule of daphnin to the other hydroxyl group of the liberated aglucone.[1,2] The reverse reaction, i.e. the formation of daphnin from daphnetin 8-glucoside has not been observed (the equilibrium constant of the reaction would be low), and this was confirmed from feeding experiments.[3]



SCHEME 1. THE CONVERSION OF CICHORIIN INTO ESCULIN.

Cichorium intybus is known to contain esculetin, a dihydroxycoumarin with two adjacent hydroxyl groups similar to daphnetin, and its two monoglucosides, cichoriin and esculin, in the flowers from commercial stocks.[5] From the results with the *Daphne* enzyme which can catalyse the formation of esculin from cichoriin according to Scheme 1, but not

[1] M. SATÔ and M. HASEGAWA, *Phytochem.* **8**, 1211 (1969).
[2] M. SATÔ and M. HASEGAWA, *Phytochem.* **10**, 2367 (1971).
[3] M. SATÔ and M. HASEGAWA, *Phytochem.* **11**, 657 (1972).
[4] T. ASAI, *Acta Phytochim.* **5**, 9 (1930).
[5] J. B. HARBORNE, *Biochem. J.* **74**, 270 (1960).

vice versa, a transglucosylation of this type was expected in the tissues of *C. intybus*. The aim of the present study was to isolate and characterize the enzyme(s) concerned in the above reaction, and to compare its properties with those of *Daphne* transglucosylase.

RESULTS

Table 1 shows the hydrolase (HD) and transglucosylase (TG) activities and the ratio of HD/TG in nine fractions obtained by successive ammonium sulphate precipitation (Fraction II described in Experimental was used), suggesting that the Fraction II contains several enzymes with different HD/TG ratio.

TABLE 1. DISTRIBUTION OF HYDROLASE AND TRANSGLUCOSYLASE ACTIVITIES AMONG FRACTIONS OBTAINED BY $(NH_4)_2SO_4$ PRECIPITATION

$(NH_4)_2SO_4$ saturation (%)	Protein* (mg)	Hydrolase (unit)	Transglucosylase (unit)	HD/TG†
0–20	4·05	5·06	4·72	1·08
20–25	1·35	3·55	2·29	1·55
25–30	1·99	3·89	2·73	1·42
30–35	1·50	3·51	2·40	1·46
35–40	1·19	4·28	4·32	0·99
40–45	0·41	2·82	2·73	1·03
45–50	0·38	3·27	4·01	0·82
50–55	0·66	6·62	8·41	0·79
55–60	1·09	5·69	5·01	1·13
Total	12·6	38·7	36·6	1·07

* From 1·5 g of the powder.
† HD; hydrolase, TG; transglucosylase activities.

In subsequent experiments, therefore, the enzyme was examined by thin-layer gel filtration (TLG) with Sephadex G150 (superfine). Within seconds of spraying a saturated cichoriin solution, bright blue fluorescence characteristic of esculin appeared at the places A, C and D (Fig. 1) (the proteins at positions A and C are designated enzymes A and C, respectively, but the designation of enzyme D would be unsuitable, because the gel at the origin (D) seems to contain more than one protein). In a 50% ethanolic extract of the gel at these places, esculin and esculetin were confirmed by PC, and this indicated that hydrolysis and transglucosylation yielded esculin from cichoriin according to Scheme 1, that is, the enzymes A and C can catalyse two different reactions. The fluorescence at C rapidly weakened, and only esculetin could be found by PC after several min, suggesting that active hydrolysis of esculin was effected by enzyme C having higher HD activity, i.e. higher HD/TG ratio. On standing for 20 min or more, several spots with weak blue fluorescence (proteins corresponding to these would be designated as enzyme B) could be observed in the area between A and C. After prolonged standing, the fluorescence due to esculin at the places A and D was replaced by that of esculetin, as in the case of C, and this indicates that esculin itself is also hydrolysed by enzymes at these places.

The extract from fresh heads contained no native enzymes other than A and C when applied immediately after preparation. This was also the case with the Fraction I applied just after preparation, but after 2 weeks storage at 2°, several proteins corresponding to B could be observed (Fig. 1). In the Fractions II and III, a trace of B and D was detected

immediately after preparation, and these proteins increased, especially in Fraction III on standing.

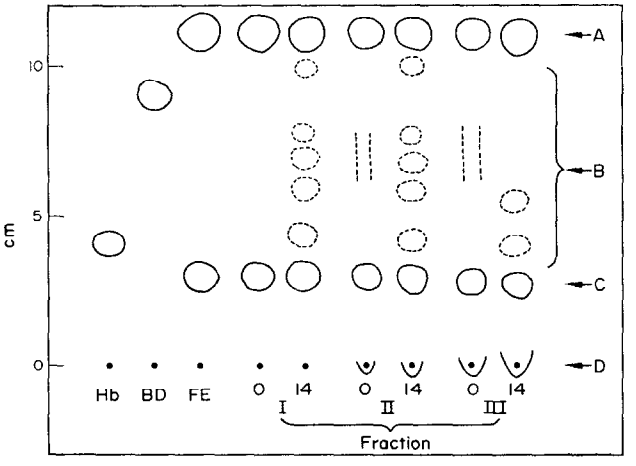


FIG. 1. THIN-LAYER GEL FILTRATION ON SEPHADEX G150 (SUPERFINE) PLATE OF THE EXTRACT OF FRESH HEADS (FE) AND THREE FRACTIONS (I, II AND III) OBTAINED FROM FROZEN MATERIALS. Sprayed with a saturated cichoriin solution: complete circle; fluoresced within several seconds, dotted circle; within 20–30 min after spraying. 0; applied immediately after preparation, 14; 14 days after preparation. BD; blue dextran (av. MW 2×10^6), Hb; horse hemoglobin (MW 68 000).

The change of both activities in the three fractions were determined 2, 4 and 8 days after preparation. Generally speaking, the HD activity increased at first, then decreased, whilst the TG activity decreased slightly or did not change, then increased, that is, the increase of HD activity is accompanied by the decrease of TG, and vice versa. The total of both activities was however not sustained at the original level, but rather increased (if inactivation of the enzymes during storage is taken into consideration, this value should rather decrease).

These facts seemed to be best explained by assuming that molecules of protein A dissociate to give rise to fragments B, and the latter further dissociates to C having predominantly HD activity, and probably to the proteins at the place D where transglucosylation would predominate. This assumption was supported by an experiment where a two-dimensional TLG plate was stood overnight at 2° after running in the first direction and then run in the other with the same buffer. Enzyme A was sequentially converted into B, C and D in that order. On re-examining the results in Table 1 with the TLG technique, it was found that the main protein in the 0–20% (HD/TG ratio; *ca.* 1·1), 20–35% (*ca.* 1·5) and 35–60% (*ca.* 1·0) fractions of $(NH_4)_2SO_4$ precipitation is D, C and A, respectively, and this does not contradict the above assumption.

Enzyme A was obtained by fractionation on a Sephadex G150 column (Fig. 2 and Table 2), but no distinct elution pattern of proteins B and C could be observed, probably because of the weak activity in B and of contamination of endogenous esculin present in the fractions containing C.

Stability. Examination by TLG showed that enzyme A thus obtained gave protein B on standing several days, but to a less degree than in Fraction I. Heating at 60° for 20 min caused neither significant loss of activities (inactivation was less than 10%) nor distinct fragmentation.

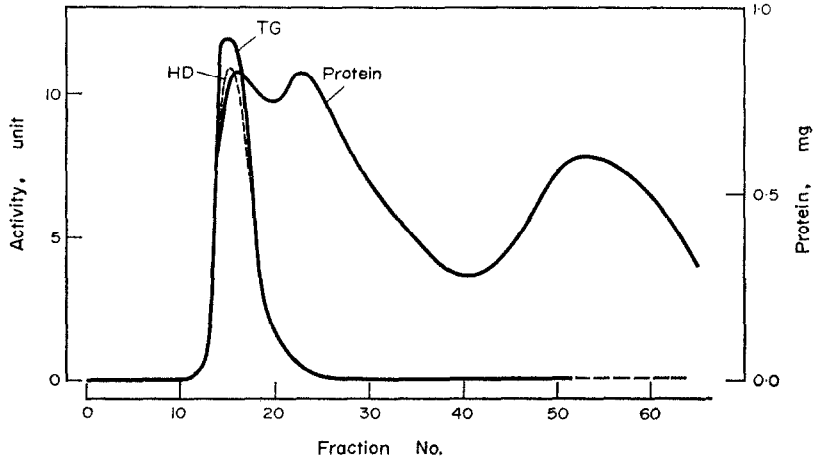


FIG. 2. FRACTIONATION OF THE PROTEINS IN FRACTION II ON SEPHADEX G150 COLUMN. Fractions on dotted line fluoresced due to endogenous esculin present in the Fraction II.

The pH optima. The optimum pH of hydrolysis was *ca.* 4·4 with half maximal activity at pH 3·8 and 5·8, whilst the pH optimum of transglucosylation was 6·1, with half maximal activity at pH 5·3 and 7·0.

TABLE 2. ISOLATION OF THE ENZYME A

Fraction	Protein (mg)	Total activity (unit) HD	TG	Ratio HD/TG	Specific activity (unit/mg protein) HD	TG	'Purification'* HD	TG	Recovery HD	TG
I	504	109	113	0·97	0·216	0·224	1·00	1·00	100	100
II	40·4	78·0	74·2	1·05	1·93	1·84	8·94	8·21	72	66
A	1·45	30·0	36·7	0·82	20·7	25·2	95·8	113	28	32

* The use of the term purification in this case is not suitable, because Fraction I contains the protein C besides A.

Inhibitors. Both activities were similarly inhibited by δ-gluconolactone at 10^{-3} and 10^{-2} M. Transglucosylation was completely inhibited by Hg^{2+} even at 10^{-3} M, but hydrolysis was inhibited by only 40% at this concentration. Other heavy metal ions tested did not exhibit any significant inhibition (Table 3).

Substrate specificity. All the coumarin glucosides tested served as substrates both of hydrolysis and of transglucosylation (as glucose donor to esculetin). The most efficient substance in both reactions was found to be cichoriin (Table 4). No activity was found with *p*-glucosyloxycinnamic acid, *o*-glucosyloxycinnamic acid, coniferin, arbutin, salicin, phenyl-β-glucoside, helicin, phloroacetophenone 4-glucoside, phlorizin, liquiritin, pinocembrin 5-glucoside, pinocembrin 7-glucoside, azalein or sulfurein.

TABLE 3. EFFECT OF HEAVY METAL IONS AND δ-GLUCONOLACTONE ON ENZYME ACTIVITY

Substance	Concn (M)	Relative activity Hydrolysis	Transglucosylation
None (control)		100	100
$HgCl_2$	10^{-3}	61	0
$HgCl_2$	10^{-2}	31	
$CuSO_4$	10^{-3}	91	88
$NiCl_2$	10^{-3}	89	91
$ZnCl_2$	10^{-3}	98	100
$CoCl_2$	10^{-3}	93	96
$BaCl_2$	10^{-3}	100	100
δ-Gluconolactone	10^{-3}	33	35
δ-Gluconolactone	10^{-2}	17	6

Some aglucones of coumarin glucosides were tested as glucose acceptors (Table 5) Regardless of glucose donors used, their glucosyl moiety is always introduced into 6-OH of esculetin and 8-OH of daphnetin to form esculin and daphnetin 8-glucoside, respectively. The hydroxyl group at the 7-position of these dihydroxycoumarins as well as of umbelliferone (7-OH coumarin) and scopoletin (6-OCH_3, 7-OH coumarin) was not glucosylated.

TABLE 4. SUBSTRATE SPECIFICITY OF THE ENZYME

Glucoside	Activity* Hydrolysis	Activity* Transglucosylation to esculetin	Glucoside	Activity* Hydrolysis	Activity* Transglucosylation to esculetin
Cichoriin	22·3	1·18	Tomenin‡	8·2	0·232
Esculin	8·2		Skimmin	14·8	†
Daphnin	15·4	0·348	Prunin	8·5	0·051
Daphnetin 8-glucoside	16·8	†			

* Unit/mg protein.
† Activity could be detected, but not determined by fluorimetry.
‡ 6,7-Dimethoxy, 5-glucosyloxycoumarin [M. HASEGAWA, *Bot. Mag. Tokyo* **82,** 458 (1969)].

DISCUSSION

From the results shown in Fig. 2 and Table 5, it can be concluded that the cell-free extract of *Cichorium* heads can form esculin from cichoriin according to Scheme 1. These reactions, however, do not seem to proceed actively in intact tissues, because esculin can be found only in a small amount.[3] A relatively large amount of esculin reported in the

cured materials of this plant[5] may be formed by breakdown of cellular structure during curing followed by mixing of the enzyme and the vacuolar substrate. A similar mechanism has been proposed concerning the flower-decolorization of this plant.[6]

TABLE 5. SPECIFICITY FOR GLUCOSE ACCEPTOR IN TRANSGLUCOSYLATION

Glucose acceptor	Position gluco-sylated	Glucoside formation* with glucose donors					Glucoside formed
		Esculin	Cichoriin	Daphnin	D-8-G†	Skimmin	
Esculetin	6-OH		+	+	+	+	Esculin
Esculetin	7-OH	−		−	−	−	Cichoriin
Daphnetin	7-OH	−	−		−	−	Daphnin
Daphnetin	8-OH	+	+	+		+	Daphnetin-8-glucoside
Umbelliferone	7-OH	−	−	−	−		Skimmin
Scopoletin	7-OH	−	−	−	−	−	Scopolin

* Glucoside can be formed (+) and not formed (−).
† Daphnetin-8-glucoside.

In contrast with the case of *D. odora*, in which only one enzyme concerning the glucoside formation could be isolated,[2] the heads of *C. intybus* contain two native enzymes, both of which can catalyse two reactions in Scheme 1 (Fig. 1). One of them, A, is characterized by its high MW and lower HD/TG ratio, and is converted, during isolation procedures and on standing, into the enzyme C having a higher HD/TG ratio with the formation of several fragments belonging to the proteins B. These fragments with MW between proteins A and C may be formed from A by successive release of a 'unit protein' being identical to C. It has been known that many protein molecules consist not of a single polypeptide chain, but form a complex made up from several polypeptide chains.[7–9]

The enzyme A has several properties common to those of *Daphne* transglucosylase.[2] Firstly, it shows a high substrate specificity (Tables 4 and 5); apart from prunin, it can hydrolyse only coumarin glucosides, and transfer the glucosyl moiety of these compounds always into 6-OH of esculetin to form esculin and into 8-OH of daphnetin to give rise to daphnetin 8-glucoside, and not into 7-OH of these dihydroxy- and other coumarins. The requirement of the *Cichorium* enzyme for the coumarin nucleus seems to be greater than that of the *Daphne* enzyme, because the former enzyme shows no affinity toward two glucosides of monohydroxycinnamic acids.

The second similarity of these enzymes is that in both of them the optimum pH of hydrolysis is acidic, while that of transglucosylation is neutral. The formation of esculin in intact tissues does not seem to occur at a high rate and this may be explained also by the lower pH value in the cell sap (less than *ca.* 4) where the transglucosylation becomes almost negligible.

The enzymes of both plants are similarly affected by δ-gluconolactone. The inhibitory effect of the heavy metal Hg^{2+} toward these two enzymes are however somewhat different. At a concentration of 10^{-3} M, both activities of the *Daphne* enzyme are completely inhibited, while the *Cichorium* enzyme undergoes a complete inhibition in transglucosylation

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[9] I. M. KLOTZ and D. W. DARNALL, *Science* **166,** 127 (1969).

but only 40% in hydrolysis (Table 3). The mode of action may therefore differ in these two reactions.

The transglucosylation catalysed by *Cichorium* enzyme as well as that by *Daphne* enzyme are of the so-called low energy type,[10–12] in which no high energy glycosyl nucleotides[13–16] are needed as glycosyl donors for the formation of phenolic glycosides.

EXPERIMENTAL

Materials. Heads (inflorescence) in bloom were collected in the morning on sunny days from June to September and stored frozen.

Isolation of protein fractions. The frozen materials were blended in cold acetone at −30° to give a powder, which was dried. The enzyme activity could be detected for 3 months.

Treatment 1. The powder (10 g) was suspended in 500 ml of a medium consisting of 0·05 M phosphate buffer pH 6·80, 10 g of Polyclar AT and 1·3 g ascorbic acid, and the suspension stirred for 10 min in the cold. It was centrifuged at 15 000 *g* for 30 min and the supernatant (Fraction I) was collected.

Treatment 2. To the supernatant, solid $(NH_4)_2SO_4$ was added to 60% saturation and the ppt. collected by centrifugation was taken up in 10 ml of 0·01 M phosphate buffer pH 6·80. The undissolved materials were removed by centrifugation at 15 000 g for 20 min. The supernatant was desalted with a Sephadex G25 column (2·5 × 20 cm).

Treatment 3. The proteins in the effluent (Fraction II) were adsorbed on a DEAE-cellulose column (1·6 × 7·5 cm), equilibrated with 0·01 M phosphate buffer pH 6·80 and eluted with 40 ml of 0·2 M phosphate buffer (pH 6·80)–0·5 M KCl solution (the extract; Fraction III).

Thin-layer gel filtration. Samples were applied to a thin-layer gel plate of Sephadex G150 (superfine, thickness; 1 mm), and developed with 0·01 M phosphate buffer pH 6·80 for 5–6 hr (the angle of inclination of the plate; *ca.* 20°). The extract of fresh heads was prepared by crushing 2 g of fresh materials in 5 ml of the above medium with a pestle and mortar and by centrifuging the brei at 15 000 *g* for 20 min.

The estimation of enzyme activity. The hydrolase and transglucosylase activities were principally estimated from the decrease and increase, respectively, of the fluorescence intensity of esculin. Activation was at 350 nm and the output measured at 495 nm. Linear relationship to the intensity can be observed only with the esculin concentration less than 10^{-5} M, and esculin down to 10^{-7} M can be estimated. Because the fluorescence intensity depends upon pH, the results for the pH optima were corrected from a pH-intensity curve at esculin concentration of 5×10^{-6} M.

Hydrolase. The reaction system is composed of 2 ml McIlvaine's phosphate-citrate buffer pH 4·40, 0·05 μmol of esculin and enzyme solution in a total vol. of 5 ml, incubated at room temp. and the decrease of fluorescence was recorded every 30 sec.

Transglucosylase. Mixture consisting of 2 ml of McIlvaine's phosphate-citrate buffer pH 6·10, 0·05 μmol of esculetin, 0·5 μmol of cichoriin (final concentration is 10^{-4} M; cichoriin solution more than 10^{-3} M lowers the fluorescence intensity of esculin formed), and enzyme in a final vol. of 5 ml, was incubated as above and the increase of fluorescence due to esculin was measured. Although esculetin, which is to be formed from esculin and cichoriin by hydrolytic reaction also fluoresces, its fluorescence intensity is negligible at pH 4·40 and less than 1/20 at pH 6·80 as compated with an equal amount of esculin. One enzyme unit is defined as the amount of enzyme which transforms 10^{-2} μmol of esculin or other product under the standard conditions.

Isolation of the enzyme A. Fraction II was applied on Sephadex G150 (superfine) column (2·5 × 30 cm) which had been equilibrated with 0·01 M phosphate buffer pH 6·80 and the proteins were fractionated with the same buffer by upward flow. Every 3·0 ml was collected at a velocity of 5 ml per hr. The fractions from Nos. 14 to 16 were combined and used as the solution of the enzyme A.

Substrate specificity. The fluorimetric method shown above was inadequate for this purpose. *Hydrolysis.* In place of 0·05 μmol of esculin in the standard reaction solution, 10 μmol of each glucoside were used. The reaction was stopped after 10 min by adding 1 ml of 0·1 N HCl. A part of the solution was chromatographed on Whatman No. 1 filter paper with *n*-BuOH–HOAc–H_2O (4:1:2) and glucose was eluted, which was concentrated and the amount of glucose was determined by the method of Somoghi.[17] *Transglucosylation to*

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esculetin. 0·5 μmol of esculin and 0·05 μmol of esculetin in the standard reaction solution were replaced by 10 μmol of each of glucoside and 5 μmol of esculetin, and esculin formed for 10 min was isolated, if possible, by PC and determined by fluorimetry. *Examination of glucose acceptors.* The reaction systems in Table 5 are composed of 10 μmol of glucoside, 5 μmol of acceptor and enzyme in a total vol. of 5 ml, and the reaction products were determined by PC.

Estimation of protein. This was carried out by the method of Lowry *et al.*[18]

Chemicals. *o*-Glucosyloxycinnamic acid was synthesized from helicin and malonic acid according to the literature.[19]

Acknowledgement—The authors wish to express their thanks to Mr. A. Kamiya of Applied Microbiological Institute of Tokyo University for synthesizing a specimen of *o*-glucosyloxycinnamic acid.

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[19] B. HELFERICH and H. LUTZMANN, *Ann. Chem. Liebigs* **537,** 11 (1939).