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A TRANSGLUCOSYLASE IN *DAPHNE ODORA* CONVERTING DAPHNIN TO DAPHNETIN 8-GLUCOSIDE*

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Abstract-The hydrolysis of daphnin (daphnetin 7-glucoside) and the transglucosylation from this glucoside to daphnetin forming daphnetin 8-glucoside have been studied using an enzyme preparation from the flowers of ***Daphne odoru.*** Both the reactions seem to be controlled by the same enzyme having a high substrate specificity. Some other properties of this enzyme are described.

INTRODUCTION

IN A PREVIOUS paper, it was reported that a crude enzyme preparation from the flowers of ***Daphne odora*** can convert daphnin (daphnetin 7-glucoside) to daphnetin 8-glucoside according to the scheme shown in Fig. 1; daphnin is first hydrolysed by a hydrolytic enzyme to liberate daphnetin, and then the glucosyl residue of a second molecule of daphnin is transferred to its other hydroxyl group by a transglucosylase reaction.[1]

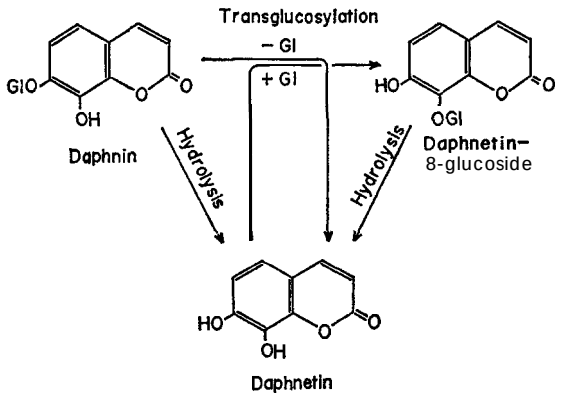

FIG. 1. SCHEME SHOWING THE CONVERSION OF DAPHNIN TO DAPHNETIN 8-GLUCOSIDE.

It has generally been accepted that a number of hydrolytic enzymes which hydrolyse glycosidic linkage can also transfer the glycosyl group to the compounds other than water and form new glycosides.[2] The two reactions in Fig. 1 may therefore be the double functions of the same enzyme.

* Part II in the series "Metabolism of Coumarins in ***Daphne odoru";*** for Part I see ***Phytochem. 8,*** 1211 (1969).

[1] M. SATÔ and M. HASEGAWA, ***Phytochem. 8, 1211 (1969).***

[2] W. Z. HASSID, in ***Metabolic Pathways*** (edited by D. M. GREENBERG), Vol. 1, p. 307, Academic Press, New York (1967).

RESULTS

Purification of the Enzyme

The stages of purifying an enzyme preparation are summarized in Table 1. The ratio of hydrolase to transglucosylase activities remained virtually unchanged at each stage, although the former activity tends to slightly increase as compared with the latter. It was therefore apparent that both activities are due to one protein. No separation of the two activities could be obtained by starch gel electrophoresis (Fig. 2) or by stepwise fractionation on DEAE cellulose column.

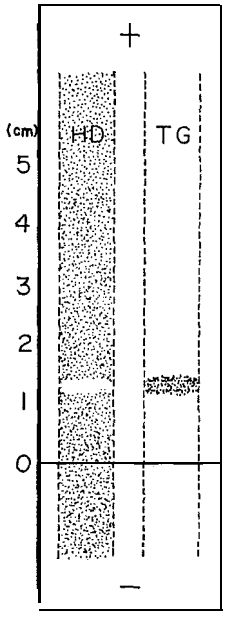

FIG. 2. A STARCH GEL ELECTROPHORESIS OF HYDROLASE (HD) AND TRANSGLUCOSYLASE (TG). DOTTED AREASHOWS THE FLUORESCENCE DUE TO D-8-G.

TABLE 1. PURIFICATION OF THE ENZYME

Fraction	Protein (mg)	Total activity (unit) HD	TG	Specific activity Ratio (unit/mg protein) HD/TG	ND	TG	Purification HD	TG	Recovery HD	TG
1	323	448	403	1.12	1.39	1·25	1·0	1·0	100	100
2	61.4	262	204	1·29	4.23	3.32	3·0	2·7	59	50
3	6.29	115	85.5	1.34	18.3	13.6	12·0	11·0	26	28
4	2.54	86.3	65.7	1.30	33.9	25.9	24.0	23·0	19	16
5	0.321	15.6	10·8	1.43	48.6	33.6	35·0	29.0	3.5	2·7

HD, hydrolysis; TG, transglucosylation.

Time Course of Daphnetin 8-Glucoside (D-8-G) Formation from Daphnin

This is shown in Fig. 3. The lag in the early reaction stage shows the time necessary for the accumulation of a sufficient amount of daphnetin (glucose acceptor). Addition of

daphnetin to the mixture at zero time eliminated the lag. As can be seen in Fig. 1, three reactions are involved in this system; (1) hydrolysis of daphnin, (2) transfer of glucose from daphnin to the liberated daphnetin, and (3) hydrolysis of D-S-G. D-8-G is formed almost linearly following the lag, but because of the decrease of daphnin (reactions 1 and 2) and also of D-8-G itself (reaction 3), it ceases to increase, gradually decreases and disappears after prolonged incubation.

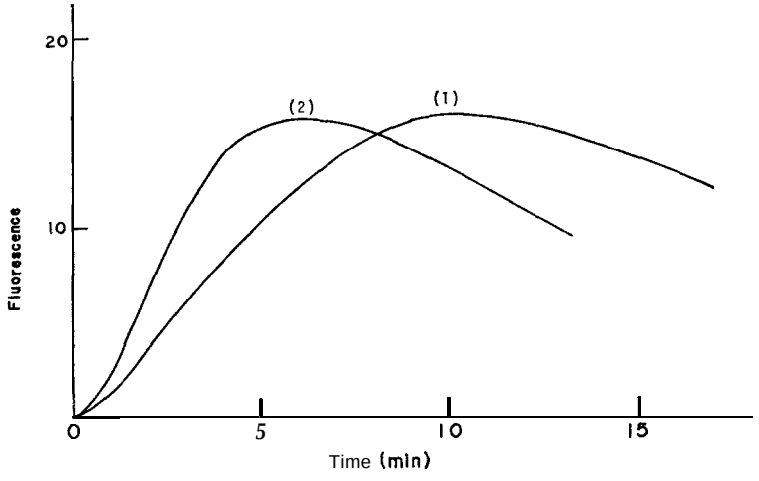

FIG. 3. TIME COURSE CURVES OF DAPHNETIN 8-GLUCOSIDE FORMATION WHEN DAPHNIN ALONE WAS INCUBATED WITH THE ENZYME.
Standard reaction system for estimation of hydrolase activity, from which daphnetin was omitted. Fluorescence intensity; arbitrary unit. In the solution (2), the amount of enzyme is twice as much as in (1).

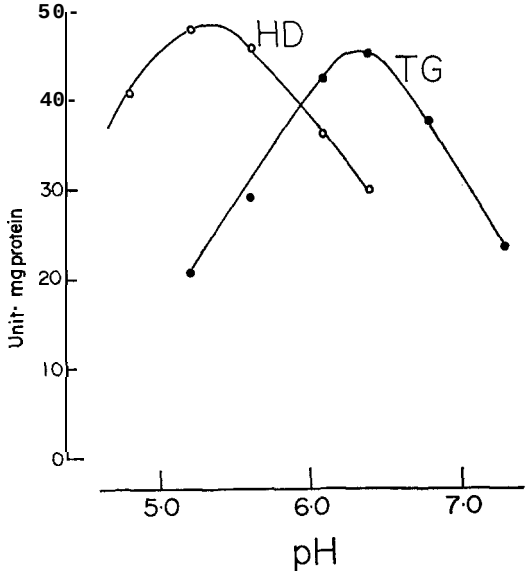

FIG. 4. THE pH OPTIMA OF HYDROLASE (HG) AND TRANSGLUCOSYLASE (TG).
At lower pH region, citrate instead phosphate buffer was used.

The pH Optima

The optimum pH of the hydrolysis is about 5·3, whilst that of the transglucosylation at 6·3 (Fig. 4).

Substrate Specificity

Of several β-glucosides tested as glucose donors to daphnetin, the enzyme could utilize only coumarin glucosides and p-glucosyloxycinnamic acid (Table 2). The enzyme did not produce daphnin when D-8-G was used as glucose donor to daphnetin. It showed a high substrate specificity also in hydrolytic reaction, exerting affinity only toward the same glucosides as in transglucosylation (Table 2).

TABLE 2. SUBSTRATE SPECIFICITY OF THE ENZYME

Glucoside	Activity (unit) Transglucosylation to daphnetin	Hydrolysis*
Daphnetin 8-glucoside		46.0
Daphnin	35.2	70.0
Cichoriin	+†	99.0
Esculin	+	62·0
Skimmin	+	50·0
p-Glucosyloxycinnamic acid	+	44.0
Arbutin	—‡	—
Salicin	—	—
Phlorizin	—	
Phenyl-β-glucoside	—	—
Isoquercitrin	—	—
Astragalin	—	—
Maltose	—	—
Cellobiose	—	—

* μmole glucose/mg protein/min.

† Activity could be detected by paper chromatography, but its estimation by fluorescence was impossible, because the glucoside itself or its aglucone fluoresces.

‡ No detectable activity.

Using ordinary emulsin, hydrolysis and transglucosylation were studied with the same reaction system. All the glucosides were hydrolysed, but none could serve as glucose donor to daphnetin.

Inhibitors

Both activities were strongly inhibited by Hg^{2+} and δ-gluconolactone. Cu^{2+} showed a stronger inhibition on transglucosylation than on hydrolysis. Other heavy metal ions tested did not exhibit any significant inhibition (Table 3).

DISCUSSION

In accordance with earlier results,[2] the hydrolysis and transglucosylation catalysed by *Daphne* enzyme are again shown to be the functions of the same protein. Since both the activities are only observed toward the same glucosides and are similarily inhibited by Hg^{2+}

and δ-gluconolactone, it seems that the two reactions are proceeding at the same enzyme site. However, the pH optima are clearly different, and it is also probable that these reactions are effected on separate sites. That the rate of transglucosylation is lower than that of hydrolysis by Cu^{2+} may be due to copper complexing across the adjacent hydroxyls of daphnetin.

Like emulsin,[3,4] the hydrolytic activity of the enzyme is strongly inhibited by Hg^{2+} and δ-gluconolactone. However, the enzyme hydrolysed only coumarin glucosides and p-glucosyloxycinnamic acid, and this clearly sets it apart from emulsin. The transglucosylase activity of the enzyme also involves the same glucosides as in hydrolysis.

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

Substances	Concentration (M)	Relative activity Hydrolysis	Transglucosylation
$HgCl_2$	10^{-3}	0	4
$CuSO_4$	10^{-3}	77	17
$NiCl_2$	10^{-3}	89	83
$ZnCl_2$	10^{-3}	98	88
$CoCl_2$	10^{-3}	83	100
$BaCl_2$	10^{-3}	95	75
δ-Gluconolactone	10^{-3}	41	35
δ-Gluconolactone	10^{-2}	0	0
None (control)		100	100

Standard reaction mixtures were used (see Experimental).

While the formation of phenolic glycosides frequently requires the participation of glycosyl nucleotides,[2,5,6] these substances have also been obtained without any high energy sources being added to the system.[7-9] As has previously been suggested,[1] the glucosylation catalysed by *Daphne* enzyme is of the low energy type.

The role of the enzyme is to convert daphnin to D-8-G, and not vice versa. The authors have observed that, toward later stage of the flowering in *Daphne*, D-8-G is successively accumulated, while daphnin in turn decreases gradually, and that the label of radioactive precursors (cinnamic, p-coumaric and caffeic acids-2-^{14}C) administered to *Daphne* cuttings is incorporated into daphnin faster than into D-8-G.[10] The formation of D-8-G may therefore be preceded by that of daphnin, in metabolic sequence. This assumption is supported by the fact that D-8-G cannot be converted to daphnin in a cell free system. One of the factors controlling the accumulation of D-8-G toward later stage may be a shift of pH in cell sap. When the pH changes from acid to neutral where the activity of transglucosylation is stronger than that of hydrolysis (Fig. 4), the formation of D-8-G may predominate.

[3] S. HESTRIN, D. S. FEINGOLD and M. SCHRAMM, *Methods in Enzymology* (edited by S. P. COLOWICK and N. 0. KAPLAN), Vol. 1, p. 234, Academic Press, New York (1955).
[4] J. CONCHIE and G. A. LEWY, *Biochem. J. 65,389 (1957).*
[5] A. KLEINHOFS, F. A. HASKINS and H. J. GORZ, Phytochem. 6, 1313 (1967).
[6] G. A. BARBER and M. T. Y. CHANG, *Phytochem. 7, 35 (1968).*
[7] J. B. PRIDHAM, *Ann. Rev. Plant Physiol. 7, 35 (1965).*
[8] S. M. HOPKINSON and J. B. PRIDHAM, *Biochem. J.* 105, *655 (1967).*
[9] M. PŠENAK, P. KOVÁCS and A. JINDRA, *Phytochem. 8, 1665 (1969).*
[10] M. SATÔ and M. HASEGAWA, unpublished data.

EXPERIMENTAL

Material. The flowers (inflorescence) of ***Daphne odora*** picked in March and April were stored frozen until used.

Purification of the enzyme. Step 1. 10 g of the acetone powder of the flowers were suspended in 300 ml of 0·1 M phosphate buffer pH 6.4, the suspension stirred for 10 min in the cold and centrifuged at 15,000 ***g*** for 30 min. Three volumes of cold acetone at -20" were added to the supernatant, and after 30 min standing, the precipitate collected by centrifugation. It was taken in 50 ml of 0·01 M buffer, the viscous brownish suspension centrifuged to discard insoluble materials and the supernatant passed through Sephadex G-50 (fine) column (76 x 200 mm).

Step 2. To the effluent (Fraction 1), being free from D-8-G, $(NH_4)_2SO_4$ was added to 30 % saturation and after 1 hr the precipitate was discarded. The supernatant was further made up to 60% saturation, the protein collected by centrifugation and dissolved in *ca.* 10 ml of 0·01 M phosphate buffer pH 6·4. The solution was dialysed overnight against the same buffer.

Step 3. The dialysate (Fraction 2) was aoolied on a DEAE cellulose column (10 x 60 mm) which had been equilibrated with 0·01 M sodium phosphate buffer pH 6.4. The discharged'column was successively washed with 10 ml of 0·01 M and 50 ml of 0·08 M buffer, and the enzyme then extracted with 50 ml of 0·12 M buffer (Fraction 3).

Step 4. The protein collected between 35-55 % saturation of $(NH_4)_2SO_4$ was dissolved in water (10 ml). The solution was dialysed against cold water and the precipitated protein was taken in 0·05 buffer (Fraction 4).

Step 5. The above solution was further applied on a DEAE cellulose column (equilibrated with 0·05 M buffer) and a fraction was obtained bv extraction with 0·11 M buffer following washing with 0·09 M buffer (Fraction 5).

The estimation of the enzyme activity. The hydrolase and transglucosylase activities were principally estimated from decrease and increase, respectively, of the fluorescence intensity of D-8-G. It was determined with a spectrophotometer using the 350nmsetting for activation and measuring the output of 497 nm.[1] Linear relationship to the intensity can be observed only with the D-8-G concentration less than 10^{-5} M and D-8-G up to 10^{-7} M can be estimated. Because the fluorescence intensity depends upon pH, the results in Fig. 2 were obtained from a pH-intensity relation curve at D-8-G concentration of 5×10^{-6} M.

Hydrolase. Mixtures consisting of 1 m-mole of buffer (pH 6·0), 0·05 μmoles of D-8-G and enzyme in a total volume of 10 ml were incubated at room temp., and the decrease of fluorescence at 493 nm was recorded every 30 sec.

Transglucosylase. The reaction system is composed of 1 m-mole of buffer (pH 6·0), 1 μmole of daphnetin (glucose acceptor) and 2 μmoles of daphnin in a final volume of 10 ml. The reaction was proceeded as above and the increase of fluorescence was estimated.

One enzyme unit is defined as the amount of enzyme which transformed 1 μmole of D-8-G for 1 min under the qualified conditions.

Starch gel electrophoresis. Starch gel electrophoresis (Fig. 2) was conducted in 0·01 M phosphate buffer pH 6.4, using 10 V/cm at 1·3 mA and running for 3·5 hr at 5". For detecting the hydrolase band on the block, a paper strip sprayed with 10^{-3} M D-8-G solution was placed for several minutes on the one side (HD in Fig. 2) and the area where the fluorescence disappeared was located. The transglucosylase band was detected by placing another strip sprayed with a saturated daphnin solution on the other side of the block (TG in Fig. 2) and by observing the appearance of fluorescence.

Substrate specificity. Hydrolase. In place of 0·05 μmoles of D-S-G in standard reaction mixture, 20 μmoles each of glucoside were used. The reaction was run for 10 min and stopped by adding 1 ml of 0·1 M 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 to a definite volume. The amount of glucose was determined by the method of Somoghi.[11]

Transglucosylase. Daphnin in standard reaction solution was replaced, if necessary, by 2 μmoles each of glucoside and the formation of D-8-G was examined by fluorometry and paper chromatography.

Measurement of protein. This was carried out by the method of Lowry ***et al.***[12]

Chemicals. Cichoriin was isolated from the flowers of ***Cichorium intybus***[13] and esculin commercially obtained. Skimmin[14] andp-glucosyloxycinnamic acid[15] were synthesized according to the literature. D-8-G was readily obtained in a large amount from mature ***Daphne*** flowers in which only a small amount of daphnin is present. After the evaporation of 80% ethanol extract of the flowers, the residue was extracted with hot ethanol. Ethanol was replaced by water, daphnetin removed by extraction with ethyl acetate, and the aqueous phase concentrated to a moderate volume. Crude crystals, obtained by standing overnight, were recrystallized from hot water, m.p. 223-224".

[11] M. SOMOGHI, ***J. Biol. Chem.*** 195, 19 (1952).
[12] 0. H. LOWRY, N. J. ROSENBROLJGH, A. L. FARR and R. J. RANDALL, ***J. Biol. Chem.*** 193, 265 ***(1951).***
[13] J. B. HARBORNE, ***Biochem. J. 74, 270*** (1959).
[14] E. SPÄTH and 0. HEUFELD, ***Rec. Trav. Chim. 57, 535*** (1938).
[15] D. J. AUSTIN and M. B. MEYERS, ***Phytochem. 4, 245 (1965).***