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SHORT COMMUNICATION

THE ENZYMIC GLYCOSYLATION OF QUERCETIN TO QUERCITRIN

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Abstract—An enzyme preparation from leaves of *Leucaena glauca* is shown to catalyze the transfer of the ^{14}C-labeled L-rhamnosyl moiety of uridine 5′-diphosphate L-rhamnose to quercetin to form the widely distributed flavonol glycoside, quercitrin. The reaction requires quercetin and is stimulated by manganous ion. In this system, thymidine 5′-diphosphate L-rhamnose is a considerably less effective donor of L-rhamnosyl groups than is the uridine derivative.

INTRODUCTION

THE L-RHAMNOSYL nucleotide uridine 5′-diphosphate L-rhamnose is formed by enzymes of various higher plants and of the green alga, *Chlorella pyrenoidosa*.[1,2] It has also been isolated from a species of golden brown algae, *Ochromonas malhamensis*.[3] Transfer of L-rhamnose from this nucleotide to the D-glucosyl moiety of quercetin-3-glucoside is catalyzed by an enzyme preparation from leaves of *Phaseolus aureus*.[4] The common flavonol glycoside rutin is the product of that reaction.

Evidence will be presented in this report that an enzyme from *Leucaena glauca*, a species of the Mimosa family, brings about the transfer of L-rhamnose from UDP-L-rhamnose directly to the 3-hydroxyl of quercetin to form another flavonol glycoside, quercitrin.

RESULTS

Properties of the Glycosylating System

That fraction of the soluble leaf proteins of *Leucaena glauca* precipitating between 45 and 60 per cent ammonium sulfate saturation contains most of the enzyme catalyzing the transfer of L-rhamnose to quercetin. Attempts to purify the enzyme further on a column of Sephadex G-150 were not successful.

The rhamnosylation of quercetin was proportional to time and to enzyme concentration when the mixtures were incubated at 25°. With the low concentration of enzyme used in the routine assay, however, the reaction ended after about 20 min when only 50 per cent or less of the substrate had been utilized. This is due presumably to inactivation of the enzyme and

[1] G. A. BARBER, *Arch. Biochem. Biophys.* **103**, 276 (1963).
[2] G. A. BARBER and M. T. Y. CHANG, *Arch. Biochem. Biophys.* **118**, 659 (1967).
[3] H. KAUSS, *Biochem. Biophys. Res. Commun.* **18**, 170 (1965).
[4] G. A. BARBER, *Biochemistry* **1**, 463 (1962).

not to equilibrium considerations since at higher enzyme concentrations the reaction went to completion. A pH optimum was reached at about pH 7 and the rate of reaction was essentially zero at pH 5 and 9. These results are shown in Table 1.

There was an absolute requirement for quercetin with an optimal concentration about equal to the concentration of UDP-L-rhamnose present in the mixture (Table 2). Neither the rate nor the extent of the reaction appeared to be influenced by further increases in the concentration of quercetin.

Several divalent cations (Mg^{2+}, Mn^{2+}, Co^{2+}, and Ca^{2+}, as their chlorides) were tested for their effect on the reaction. Of these substances only $MnCl_2$, at a concentration of $1{\cdot}7 \times 10^{-3}$ M, stimulated the rate of quercitrin formation (Table 3). Concentrations higher than

TABLE 1. EFFECT OF pH ON THE L-RHAMNOSYLATION OF QUERCETIN

pH	% of total radioactivity incorporated into quercitrin
5·1	0
5·9	11
6·2	13
6·5	16
6·8	17
7·1	17
7·9	13
8·8	4

The reaction mixture contained 20 μl of enzyme (about 2·5 mg protein/ml) previously adjusted to the appropriate pH with sodium-potassium phosphate buffer, 0·1 M (0·1 M sodium acetate, pH5, and 0·1 M glycylglycine, pH 8·9, were added in addition to the phosphate buffer at the lower and upper pH respectively), 5 μl $MnCl_2$ (0·01 M), 2 μl UDP-L-rhamnose-^{14}C (0·012 μc or $1{\cdot}9 \times 10^{-4}$ μmole) and 1 μl quercetin suspension (1 per cent in water). The mixtures were incubated for 5 min at 24° and assayed for the formation of quercitrin as described in the text. Incubation under these conditions provides a measure of the reaction rate since L-rhamnosylation of quercetin is proceeding linearly in this portion of the time curve.

TABLE 2. EFFECT OF QUERCETIN CONCENTRATION ON THE INCORPORATION OF L-RHAMNOSE-^{14}C INTO QUERCITRIN

Quercetin concentration (M)	% of total radioactivity incorporated into quercitrin
0	0
1×10^{-5}	6
5×10^{-5}	25
1×10^{-4}	41
1×10^{-3}	56
1×10^{-2}	52

The reaction mixtures each contained 20 μl of enzyme in 0·025 M sodium–potassium phosphate buffer, pH 7·0 (about 2·5 mg protein/ml), 5 μl $MnCl_2$ (0·01 M), 2 μl UDP-L-rhamnose-^{14}C (0·012 μc) and 1 μl quercetin in ethanol at the appropriate concentration. The mixtures were incubated at 37° for 10 min and were assayed for the formation of quercitrin as described in the text.

TABLE 3. STIMULATION OF L-RHAMNOSE TRANSFER BY $MnCl_2$

Concentration of $MnCl_2$ (M)	% of total radioactivity incorporated into quercitrin
0	17
8×10^{-4}	17
$1{\cdot}7 \times 10^{-3}$	23
4×10^{-3}	21
8×10^{-3}	18
$1{\cdot}5 \times 10^{-2}$	10

The reaction mixtures were constituted as described in Table 2 except that 1 μl quercetin suspension (1 per cent in water) was used and $MnCl_2$ was added in the indicated concentrations. Mixtures were incubated for 5 min at 24° and were assayed for the formation of quercitrin as described in the text.

4×10^{-3} M were inhibitory. ATP (4×10^{-3} M and 8×10^{-3} M) did not stimulate the reaction rate.

dTDP-L-rhamnose was the first L-rhamnosyl nucleotide described[5] and has been shown to be involved extensively in the metabolism of L-rhamnose by bacteria.[6] From the data in Fig. 1, however, it is evident that UDP-L-rhamnose is a considerably more effective donor of

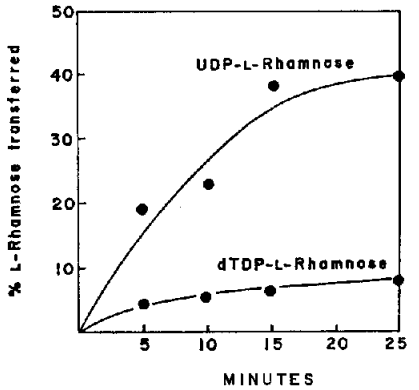

FIG. 1. COMPARISON OF THE RATES OF L-RHAMNOSYL TRANSFER FROM TWO NUCLEOTIDE DERIVATIVES. The reaction mixtures each contained 100 μl enzyme (about 2·5 mg protein/ml), 5μl quercetin suspension (1 per cent in water), 25 μl $MnCl_2$ (0·01 M) and in one 0·06 μc UDP-L-rhamnose-^{14}C ($9{\cdot}5 \times 10^{-4}$ μmole) and in the other 0·06 μc dTDP-L-rhamnose-^{14}C (16×10^{-4} μmole). The mixtures were incubated at 25° and at the indicated times a 25 μl aliquot was removed from each and assayed for quercitrin formation as described in the text.

L-rhamnose to quercetin in this system than is the thymidine derivative. Similar results were obtained in studies of the enzymic rhamnosylation of quercetin-3-glucoside to rutin.[4]

Characterization of ^{14}C-labeled Quercitrin

The product of the enzymic reaction was characterized as quercitrin by the following criteria: (1) Upon chromatography in solvent systems I and II the movement of the radioactive

[5] R. OKAZAKI, *Biochim. Biophys. Res. Commun.* **1**, 34 (1959).
[6] L. GLASER, *Physiol. Rev.* **43**, 215 (1963).

compound coincided with that of authentic quercitrin; (2) When the ^{14}C-labeled compound was mixed with authentic quercitrin and cocrystallized, there was essentially no decrease in specific radioactivity after three crystallizations (Table 4); (3) Hydrolysis (0·2 N HCl, 30 min

TABLE 4. COCRYSTALLIZATION OF ^{14}C-LABELED QUERCITRIN WITH AUTHENTIC QUERCITRIN

Quercitrin	Specific radioactivity (cpm/mg quercitrin)
Mixture before crystallization	435
Crystallized once	407
Crystallized twice	461
Crystallized three times	392

^{14}C-labeled quercitrin isolated from various reaction mixtures was mixed with 15 mg authentic quercitrin and dissolved in a known volume of methanol. The radioactivity of an aliquot was determined. Quercitrin was crystallized by reducing the volume of the methanol solution to less than 0·1 ml, adding 0·4 to 0·7 ml cold water, and allowing the dilute methanol solution to stand in the refrigerator for 15 or more hours.

at 100°) of the material previously purified by paper chromatography yielded only ^{14}C-labeled rhamnose as indicated by the movement of the ^{14}C-labeled sugar in solvent I and by its electrophoretic mobility in sodium borate buffer (0·05 M, pH9).

DISCUSSION

The data presented in this paper further support the hypothesis that UDP-L-rhamnose is the principal donor of L-rhamnosyl units to various non-carbohydrate aglycones of higher plants. Preliminary work in this laboratory also suggests that that nucleotide derivative is used by enzymes of the developing flax seed to form portions of a heteropolysaccharide which is normally found in that species[7] and contains about 25 per cent L-rhamnose.

Recently an interesting observation has been made in regard to the metabolism of another ubiquitous 6-deoxyhexose, L-fucose.[8] Thus, in certain animal tissues there exist enzymes that phosphorylate free L-fucose to L-fucose l-phosphate and then catalyze its reaction with GTP to form GDP-L-fucose. Heretofore, it had been thought that this nucleotide could be synthesized only by the reduction and epimerization of GDP-D-mannose. The existence of such enzymes would seem to provide the cell with a mechanism for recovering 6-deoxyhexose released by the breakdown of various compounds within the organism and, perhaps more important to the animal, it would provide the means for incorporating dietary 6-deoxyhexose into various glycoproteins, mucopolysaccharides, etc.

So far no such phenomenon has been reported to occur in plants where large quantities of rhamnosides often accumulate in the vacuoles of cells. On the contrary, in one experiment when ^{14}C-labeled rhamnose was administered to buckwheat seedlings, little if any of the radioactivity appeared in rutin which that species synthesizes at a considerable rate.[9] If this

[7] R. L. WHISTLER and C. L. SMART, *Polysaccharide Chemistry*, p. 327, Academic Press, New York (1953).
[8] H. ISHIHARA, D. J. MASSARO and E. C. HEATH, *Federation Proc.* **25**, 1895 (1966).
[9] J. E. WATKIN and A. C. NEISH, *Phytochemistry* **1**, 52 (1961).

implies that plants lack enzymes able to effect the conversion of free L-rhamnose to the appropriate nucleotide derivative, a biological role for glycosides such as rutin and quercitrin as endogenous sources of L-rhamnose seems unlikely. It is even possible that these deoxysugars, as well as the flavonoids to which they are usually linked, are merely end products of irreversible metabolic processes and hence are without functional significance in plants.

EXPERIMENTAL

Plant Material

Young green leaflets were picked immediately before use from variously aged plants of *Leucaena glauca*. This species is known locally as "koa-haole" and is widespread on this island.

Preparation of Enzyme

The larger petioles were removed from the leaflets and discarded. The leaf tissue was washed several times with distilled water and chilled in an ice bath. All subsequent operations were conducted in the cold. The washed leaves were ground in a mortar with sea sand and 1½ times their weight of a buffer at pH7 consisting of 0·05 M sodium-potassium phosphate, 0·02 M mercaptoethanol and 5 per cent soluble polyvinvylpyrrolidone. The homogenate was squeezed through 4 layers of cheesecloth, and particulate material was removed from it by centrifugation at 20,000 X *g* for 20 min. Proteins in the supernatant solution were then fractionally precipitated with a saturated solution of ammonium sulfate, pH7. (Most of the polyvinylpyrrolidone becomes insoluble between 25–35 per cent ammonium sulfate saturation.) The material that precipitated between 45–60 per cent saturation was suspended in a minimal volume of 0·025 M sodium-potassium phosphate 0·01 M mercaptoethanol buffer, pH 7·0 and dialyzed for about three hr against 1 l. of that buffer. Phenolic substances and other low molecular weight compounds were then removed from the mixture by passing it through a 1 × 35 cm column of Sephadex G-25. Protein was eluted from the column with the dilute buffer and precipitated by adding two volumes of saturated ammonium sulfate solution to the eluate. It was suspended in a minimal volume of the dilute buffer and dialyzed overnight against two 1 l. volumes of the same buffer.

In a typical preparation, 80 g of leaves treated as described yielded 2 ml of the dialyzed solution containing about 25 mg protein/ml. The enzyme could be stored at −10° for at least several weeks without loss of activity.

Chromatography and Electrophoresis

Chromatographic separations were carried out on Schleicher and Schuell paper, No. 389 Blue Label. The following solvent systems were employed: I, propanol-ethyl acetate–water, 7:1:2; II, *n*-butanol–acetic acid–water, 52:13:35. Electrophoresis was performed in an apparatus modeled after the one described by Crestfield and Allen[10] on Schleicher and Schuell paper, No. 389 Orange Label.

Assay of Enzymic Activity

Unless otherwise noted reactions were carried out in capillary tubes in a total volume of 28 μl. The standard reaction mixture was composed of 20 μl of enzyme solution (about 2·5 mg protein/ml), 2 μl UDP-L-rhamnose labeled uniformly with ^{14}C in the L-rhamnosyl moiety (0·012 μc or $1{\cdot}9 \times 10^{-14}$ μmole), 5 μl $MnCl_2$ (0·01 M) and 1 μl quercetin suspension (1 per cent in water). After incubation the mixture was subjected to electrophoresis on paper in 0·1 M ammonium formate buffer, pH 3·7, at about 25 V/cm for 1 hr. ^{14}C-labeled quercitrin was eluted from the origin of the electrophoretogram with methanol, and unreacted UDP-L-rhamnose-^{14}C and traces of L-rhamnose-^{14}C 1-phosphate were eluted with water from their respective areas of the paper. The eluates were dried on aluminium planchets and their radioactivities determined with a conventional Geiger–Müller counter and scaler. That percentage of the total radioactivity recovered in the methanol eluate of quercitrin was taken as a measure of the extent to which the reaction had proceeded.

Other Materials

Uridine 5′-diphosphate L-rhamnose and thymidine 5′-diphosphate L-rhamnose labeled uniformly with ^{14}C in the L-rhamnosyl moieties (62 and 37 uc/umole respectively) were synthesized enzymically as described previously.[1,4] All other compounds were obtained from commercial sources.

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[10] A. M. CRESTFIELD and F. W. ALLEN, *Anal. Chem.* **27**, 422 (1955).