

Are Spinach Chloroplasts Involved in Flavonoid O-Methylation?

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Intact spinach chloroplasts, which were isolated on a cushion of Percoll and purified on discontinuous sucrose gradients, were found to adsorb appreciable amounts of high specific activity, cytosolic O-methyltransferases. The latter activity was readily eliminated after washing the chloroplast pellet with 0.1 M phosphate in sucrose gradient buffer and finally with 0.1% nonidet. Furthermore, the OMT activity of spinach leaves was resolved by chromatography on DEAE-Sephadex CL-6B into caffeic acid (COMT) and flavonoid (FOMT) O-methyltransferase activities, and purified to 30- and 50-fold, respectively. The similarities between the FOMT/COMT activity ratios and the methylation patterns of intact chloroplasts and their supernatants, as well as those of purified leaf preparations, suggest non-specific binding of cytosolic OMTs to chloroplast envelopes.

It is concluded, therefore, that spinach chloroplasts are *not* involved in the methylation of phenylpropanoid or flavonoid compounds. These results call attention for the re-evaluation of the role of this organelle in the biosynthesis or accumulation of plant secondary metabolites.

Introduction

It is generally believed that flavonoid glucosides of medium polarity are found soluble in the cellular vacuole [1], whereas highly O-methylated lipophilic flavonoids may be secreted as farina on the plant surface [2]. During the last decade, several reports described the occurrence of flavonoids in the chloroplasts of several species [e.g. 3–7], which led to the assumption that the latter organelle may be the site of flavonoid synthesis and/or accumulation. A recent study [8], however, indicated that the high flavonoid content of isolated spinach chloroplasts was attributed to their contamination by vacuolar and extraplastidic contents during isolation, thus resulting in an artifact.

The intracellular localization of plant secondary metabolites and the enzymes involved in their biosynthesis remains a controversial subject. Whereas Charrière-Ladreix *et al.* [9] claimed the localization of a membrane-bound FOMT and a stromal COMT in spinach chloroplasts, Hrazdina *et al.* [10], on the other hand, could not detect any of the enzymes of flavonoid biosynthesis, including the OMT, in chloroplasts of four cultivars comprising spinach.

Abbreviations: COMT, caffeic acid O-methyltransferase; FOMT, flavonoid O-methyltransferase.

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Furthermore, Jonsson *et al.* [11] have recently demonstrated that the major part of anthocyanin-OMT activity in *Petunia* flowers was cytosolic. In view of these conflicting results and considering our interest in the methylation of flavonoid compounds [e.g. 12–16], an examination of the OMT system of spinach seemed appropriate.

We wish to confirm our previous observations [17] on the association of COMT and FOMT activities with purified, intact spinach chloroplasts. However, these enzyme activities were removed after successive washing with buffer. The similarity between both enzyme activities and those resolved from spinach leaf extracts indicates non-specific binding of cytosolic OMT to chloroplast membranes.

Materials and Methods

Plant material

Spinacia oleracea seeds were germinated in sand, then transferred in garden soil and maintained under 16-hr daily illumination (ca 250 Einstein/m²sec) at 21 °C and 15 °C at night. The plants were kept in the dark for 3–4 hr at 4 °C before chloroplast isolation in order to minimize contamination with starch grains.

Chemicals

S-Adenosyl-L-[¹⁴CH₃]methionine (60 mCi/mmol) was purchased from Amersham (Oakville, Ontario). Sephadex G-100, DEAE-Sephadex CL-6B and Per-



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coll were from Pharmacia Fine Chemicals (Uppsala, Sweden). Nonidet was obtained from Sigma Chemical Co. (St. Louis, MO). Most commercially unavailable flavonoid compounds were generous gifts from Dr. M. Jay (Lyon, France) and Prof. E. Wollenweber (Darmstadt, Germany).

Chloroplast isolation

Spinach leaves were cut into strips directly into ice-cold extraction buffer (1:3, w/v) as described by Wink and Hartmann [18] and homogenized in a Waring blender using seven, 1-sec strokes. The homogenate was filtered through a series of cheese cloth and nylon net layers. Chloroplasts were pelleted by layering the suspension on Percoll solution according to Mills and Joy [19] and centrifuged at $2,500 \times g$ for 2 min. About two ml of the chloroplast pellet was layered on to a discontinuous sucrose gradient (5 ml 57%, 6 ml 50%, 6 ml 43%, 6 ml 33% and 8 ml 20% sucrose w/v in 50 mM Tricine buffer, pH 8.0) and centrifuged in a Beckman SW-28 rotor at $18,000 \times g$ for 30 min. One-ml fractions were collected for the assay of OMT activity, protein and chlorophyll determinations.

Purification of leaf OMTs

Crude protein extracts of spinach leaves were prepared according to the methods previously described [14, 15] and the protein was fractionated with solid ammonium sulphate (35–70% salt saturation). The protein pellet was desalted on Sephadex G-100 using 25 mM potassium phosphate buffer, pH 7.6, containing 10% glycerol (buffer A) and 3-ml fractions were collected for the assay of OMT activity. The active fractions were further chromatographed on a DEAE-Sephacrose CL-6B column which had previously been equilibrated with buffer A. The column was washed with 2 volumes of the same buffer, followed by 2 volumes of 100 mM KCl in buffer A. The enzyme protein was eluted using a linear gradient of 100–300 mM KCl in buffer A, pH 7.6, and 3-ml fractions were collected for OMT assay.

OMT assay and identification of reaction products

The standard enzyme assay was the same as described in [14] using S-adenosyl-L-[$^{14}\text{CH}_3$]methionine as the methyl donor. The O-methylated products were extracted in a 1:1 (v/v) mixture of benzene-ethyl acetate and an aliquot of the organic phase was counted for radioactivity in a toluene-based scintilla-

tion fluid. The identity of reaction products was verified by co-chromatography with reference compounds on Polyamid-6 MN TLC plates using toluene-ethyl formate-butyl acetate-formic acid (25:50:23:2) as solvent system, then autoradiographed.

Determination of chlorophyll and chloroplast-related enzyme activities

Chlorophyll was determined as in [20]; NAD-dependent glutamate dehydrogenase and ribulose-1,5-bisphosphate carboxylase were determined as described in [21] and [22], respectively. Protein was determined by the method of Bradford [23] using the Bio-Rad protein reagent.

Results

Spinach leaf chloroplasts, isolated by centrifugation on a cushion of Percoll, exhibited a high degree of purity and intactness as was revealed by microscopic examination, photosynthetic activity [22] and the absence of NAD-dependent glutamate dehydrogenase activity [21]. Sucrose gradient centrifugation of isolated chloroplasts resulted in two distinct green bands; a lower band of intact, Type-I chloroplasts (ca 90%) and an upper band of broken chloroplasts with associated thylakoids (Fig. 1A).

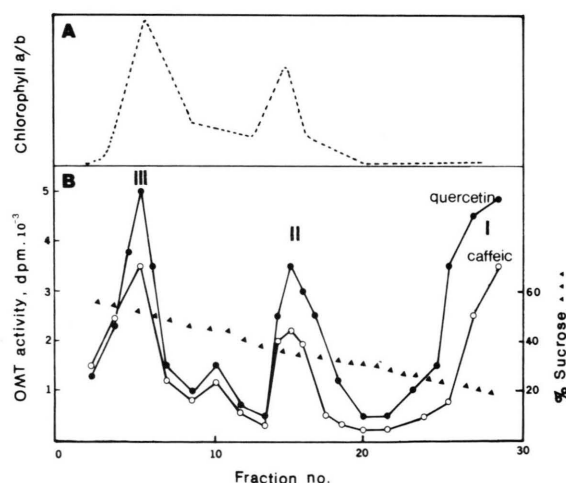


Fig. 1. A, Relative ratios of chlorophyll *a/b* of chloroplast preparations that were purified on discontinuous sucrose gradients. B, Relative enzyme activities of sucrose gradient fractions assayed with quercetin (FOMT) and caffeic acid (COMT) as described in the Methods section. Activity peaks I, II and III represent supernatant, broken and intact chloroplasts, respectively.

OMT activity of isolated chloroplasts

Examination of both COMT and FOMT activities of the sucrose gradients of isolated chloroplasts revealed three major activity peaks. One peak (I) was associated with the soluble fraction and the other two peaks (II and III) were associated with the chloroplast bands (Fig. 1B). It is interesting to note that the ratio of FOMT/COMT activity in the two chloroplast bands was similar to that of the soluble fraction.

In order to investigate whether the OMT activity in peak III was chloroplastic in origin or resulted from non-specific binding of cytosolic OMTs to chloroplast envelopes, an intact chloroplast pellet was prepared from two sucrose gradients. The latter was successively washed, three times, with 0.1 M phosphate in sucrose gradient buffer and finally with 0.1% nonidet (a mild non-ionic detergent), followed by centrifugation. When the supernatants S_1 - S_n were assayed for FOMT and COMT, almost 80% and 95% of total enzyme activities were recovered after the first two washings, respectively (Table I). This was true for the control assays in which no phenolic substrate was added to the assay mixture (results not shown). The fact that the nonidet extract (S_n) contained no appreciable enzyme activity (1.1% of the total) seems to indicate that the "chloroplastic" OMT activity may be the result of non-specific binding of cytosolic enzymes to chloroplast envelopes. Moreover, the similarities between the FOMT/COMT activity ratios (Fig. 1 and Table I) further support the above view.

Table I. O-Methyltransferase activity associated with intact spinach chloroplast preparations^a.

Substrate (enzyme)	Enzyme activity (nmol/h/mg protein) ^b							
	S_1	P_1	S_2	P_2	S_3	P_3	S_n	P_n
Quercetin (FOMT)	198.7		83.6		28.2		9.25	
		141.8		59.1		16.3		6.37
Caffeic acid (COMT)	135.6		59.6		18.7		6.28	
		121.7		37.8		11.9		4.38

^a The intact chloroplast pellet (Fig. 1, peak III) was successively washed, three times, with 0.1 M phosphate in sucrose gradient buffer, pH 8, and finally with 0.1% nonidet to yield supernatants S_1 , S_2 , S_3 , S_n and pellets P_1 , P_2 , P_3 , and P_n . Enzyme activities of S_1 and P_1 are those of the original chloroplast pellet after the first washing; of S_2 and P_2 , after washing P_1 , etc.

^b Fractions were assayed for FOMT and COMT activities using quercetin and caffeic acid as substrates, respectively, as described in the Methods section.

Identification of the reaction products

The OMT reaction products of the fractions collected from sucrose gradients (Fig. 1B) and those recovered from washing the intact chloroplast pellet (Table I) were qualitatively similar. The O-methylated products were identified by co-chromatography with reference compounds on Polyamid-6 MN plates and autoradiography. Caffeic acid gave rise to ferulic acid. Quercetin was successively methylated to its 3-methyl and 3,7-dimethyl derivatives. The latter was further methylated to 3,7,4'-trimethylquercetin (Fig. 2). In contrast with a previous report [9], quercetagenin (6-OH-quercetin) was not accepted as substrate by any of the FOMT preparations tested. However, 3,7-dimethylquercetagenin was further methylated, both at the 6- and 4'-positions, and gave rise to 3,7,4'-trimethyl derivative as the major product and 3,6,7-trimethyl and 3,6,7,4'-tetramethyl analogs as minor products (Fig. 2). These results indicate the presence of a COMT and several OMT activities which were adsorbed to chloroplast envelopes and could be removed by washing with 0.1 M buffer.

The OMT system of spinach leaves

In view of the results reported above, it was considered necessary to investigate the OMT system of spinach leaves and compare their methylating activity with that of the chloroplast preparations. Both

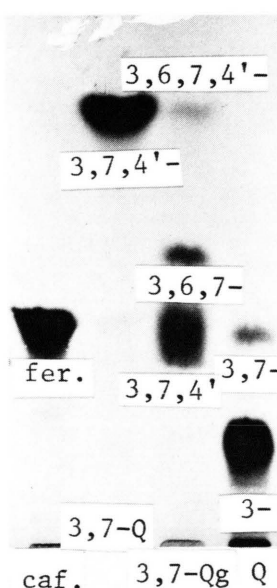


Fig. 2. Photograph of the autoradiographed reaction products of OMT activity in chloroplast pellets, supernatants or leaf enzyme preparations. Caf, caffeic acid; fer, ferulic acid; Q, quercetin; Qg, quercetagenin; numbers preceding substrates or products represent the methylated positions of the indicated substrates or products.

FOMT and COMT activities were purified by precipitation with ammonium sulfate and chromatography on Sephadex G-100 (profile not shown). Of the different ion exchangers used: DEAE-cellulose, DEAE-Sephacel, DEAE-Sephadex A-25 and DEAE-Sephacel CL-6B, the latter resulted in complete separation of both activities. This was achieved by washing the column first with 100 mM KCl, which eliminated 85% of the contaminating protein (profile not shown), followed by a linear gradient (100–300 mM) of KCl in 25 mM phosphate buffer, pH 7.6 (Fig. 3). This purification process resulted in an increased specific activity of 30- and 50-fold for COMT and FOMT activities, respectively, as compared with the crude extract (Table II). Identification of the reaction products revealed that the pat-

tern of methylation of both activity peaks (Fig. 3) was qualitatively similar to that described from the isolated chloroplast preparations (Fig. 2).

It is interesting to note that the OMT activity (nmol/h/mg protein) of the leaf Dowex extract (Table II) represented only 55–65% of that found in supernatants or pellets of chloroplast preparations (Table I). This was true whether the enzyme activity was calculated for FOMT and COMT. This result further indicates that, during the isolation of chloroplasts, an appreciable amount of high specific activity OMTs is being adsorbed on their envelopes.

Discussion

The spinach leaf OMT system was resolved, for the first time, into separate COMT and FOMT activities. The latter catalyzed the successive O-methylation of quercetin in 3-methyl, 3,7-dimethyl and 3,7,4'-trimethyl derivatives. Moreover, 3,7-dimethylquercetagenin was further methylated at the 4'- and 6-positions, thus suggesting the presence of several FOMTs in spinach leaves. Similar results were recently reported for *Chrysosplenium americanum* [14, 24] and apple cell suspension culture [16].

The OMT activity data obtained with intact spinach chloroplasts (Fig. 1 and Table I) clearly indicate that the latter are *not* involved in the methylation of either phenylpropanoid or flavonoid compounds. Both COMT and FOMT activities associated with the purified chloroplast pellets were readily eluted with 0.1 M phosphate, and the residual activity (ca. 1.1%) with 0.1% nonidet. The similarities between FOMT/COMT ratios, as well as the qualitative pattern of O-methylation, in both intact chloroplast and leaf preparations further support the above view

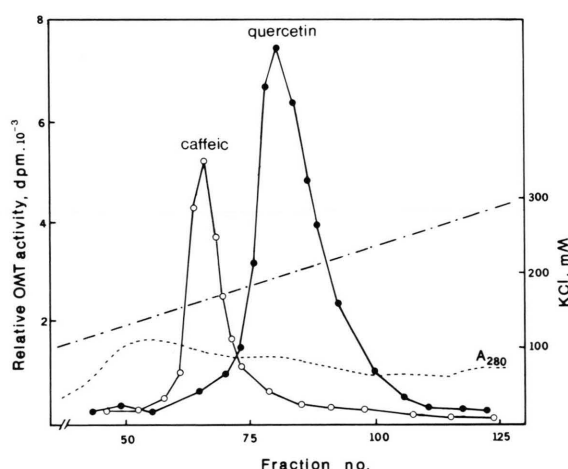


Fig. 3. Elution profile of OMT activity of spinach leaves chromatographed on DEAE-Sephacel CL-6B column. Fractions were assayed against caffeic acid and quercetin, as described in the Methods section.

Table II. Purification of spinach leaf O-methyltransferases^a.

Fraction	Total protein [mg]	Substrate ^b	Specific activity [nkat/mg]	Total activity [nkat]	Recovery [%]	Purification (-fold)
Crude extract ^c	72	Q	0.084	6.05	100	—
		C	0.062	4.46	100	—
(NH ₄) ₂ SO ₄ (35–70% satn) ^d	16.8	Q	0.29	4.87	80.5	3.5
		C	0.21	3.53	70.1	3.5
Sephadex G-100	6.7	Q	0.58	3.75	62	6.9
		C	0.37	2.48	41	5.9
DEAE-Sephacel CL-6B	0.42	Q	4.2	1.76	29.2	50
	0.49	C	1.9	0.93	20.8	30.6

^a Twenty g of leaves were used in the purification process as described in the Methods section.

^b Caffeic acid (C) and quercetin (Q) were used as substrates for COMT and FOMT activities, respectively.

^c After treatment with Dowex 1X2.

^d After desalting on Sephadex G-25.

and suggest non-specific binding of cytosolic OMTs to chloroplast membranes.

Our results are in contrast with a recent study [9] which claimed that stepwise O-methylation of both quercetin and quercetagenin by spinach chloroplast membranes and of caffeic acid, by chloroplast stroma. Apart from the lack of sufficient evidence for the identity of reaction products, the latter report [9] was based on enzyme assays containing 675 µg and 735 µg protein which converted 4 nmol of quercetin and 8 nmol of caffeic acid to their respective methyl derivatives. This represented 0.05% and 0.2% of the respective FOMT and COMT activities that were partially purified from leaf preparations (Table II). Although spinach leaves are considered to be a poor source of methylated flavonoids [4], they exhibited a fairly high FOMT activity (ca 4.2 nkat/mg protein, Table II). It is not unexpected, therefore that non-specific adsorption of small amounts of cytosolic OMT to organelle membranes may take place during ultracentrifugation; as was recently reported with the anthocyanin OMT of *Petunia hybrida* flowers [11]. The contamination of chloroplasts by cytoplasmic or other enzymes [25, 26] and vacuolar contents [7, 8] is well known. A very recent study [27] showed that, until chloroplasts were highly purified from isolated plant protoplasts, spermidine synthase was consid-

ered a chloroplastic enzyme. These results, together with the present study, signal attention as to the re-evaluation of previous reports [3–7, 9] on the implication of chloroplasts in the synthesis/accumulation of flavonoid compounds.

Current studies in our laboratory (unpublished results) indicate that none of the flavonol-specific OMTs [24] nor the flavonol-ring B glucosyltransferase [28] could be localized in the chloroplasts of *C. americanum*, a rich source of highly methylated flavonol glucosides [29]. These results are concordant with the recent finding [30] that chloroplasts lack two enzymes necessary for S-adenosyl-L-methionine synthesis, methionine synthase and methionine adenosyltransferase. This makes the chloroplast even more unlikely a site for the methylation of phenylpropanoid or flavonoid compounds.

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