CHARACTERIZATION OF *O*-METHYLTRANSFERASE ACTIVITIES ASSOCIATED WITH SPINACH CHLOROPLAST FRACTIONS

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1. Introduction

During the last two decades the biosynthesis of flavonoids in plants has been studied extensively [1–5]. However, very little is known about the intracellular localization of the enzymes involved in the synthesis of flavonoids [6,7].

Here, we report, for the first time, the stepwise *O*-methylation of quercetin and quercetagetin by the chloroplast envelope. We report also the presence of a soluble *S*-adenosyl-L-methionine (SAM):caffeic acid *O*-methyltransferase in the chloroplast.

2. Materials and methods

2.1. *Preparation of intact and purified chloroplasts*

Chloroplasts were isolated from 1–2 kg spinach leaves obtained from local farms. Deveined leaves were homogenized for 2 s in a 4 liter Waring Blendor in the following medium: 330 mM mannitol, 50 mM tetrasodium pyrophosphate, 2 mM EDTA and 0.1% defatted BSA (pH 7.8). The brei was filtered through 8 layers of muslin (Ruby, Voiron) and intact chloroplasts (washed chloroplasts) were isolated as fast as possible according to [8]. Chloroplasts thus obtained were then purified by isopycnic centrifugation in a non-toxic silica sol (Percoll®, Pharmacia) gradient as in [9]. Final chlorophyll concentration of the chloroplast suspension was 7–8 mg/ml. The rate of ferricyanide-dependent O_2 evolution in the presence of 2 mM NH_4Cl before and after osmotic shock [10] indicated that 98–99.5% of the chloroplasts were intact.

2.2. *Isolation of chloroplast fractions*

Envelope, stroma (soluble fraction) and thylakoids were prepared from intact purified chloroplasts as in [9]. From 1 kg spinach leaves the yield of envelope membranes was 2–3 mg protein. Protein determination was done by the method in [11].

2.3. *Reaction mixture*

Caffeic acid and quercetin or quercetagetin were used as precursors to localize the *O*-methyltransferase reactions (fig.1) within the chloroplast.

The complete reaction mixture contained: 6.4 μM quercetin, 6.3 μM quercetagetin or 5.5 μM caffeic acid; 6.54 μM [*methyl*-^{14}C]SAM (116 600 dpm/nmol); 10 mM Tris–HCl (pH 8.2 for flavonol substrate, pH 7.6 for caffeic acid substrate) in a final volume of 1500 μl. The reaction was initiated by addition of known amounts of envelope membranes,

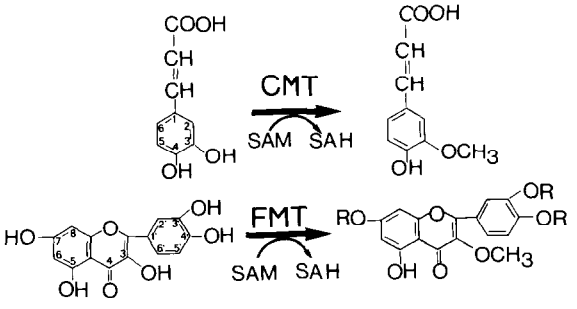

Fig.1. Reactions catalysed by the chloroplast *O*-methyltransferase. CMT = SAM:caffeic acid *O*-methyltransferase (caffeic acid → ferulic acid); FMT = SAM:quercetin *O*-methyltransferase (quercetin → 3-*O*-methyl quercetin derivatives; R = H or R = CH_3 (table 1); SAM = *S*-adenosyl-L-methionine; SAH = *S*-adenosyl-L-homocysteine.

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thylakoids or stroma. Aliquots (200 μl) were taken at different times for *O*-[^{14}C]methylated compound analysis and determination of the velocity of the reaction. The reaction was stopped by addition of 750 μl chloroform–methanol mixture (1:2, v/v). The proteins were eliminated according to [12]. The lower and upper phases were combined and evaporated to dryness in a stream of N_2. The residue, devoid of proteins, was dissolved in 100–200 μl methanol.

2.4. *Identification of reaction products*

[*methyl*-^{14}C]Ethers of cinnamic acids were chromatographed on silica gel-precoated thin-layer chromatography (TLC) plates containing fluorescent indicator (Merck, 60 F 254) using benzene, acetic acid (80:10, v/v) as solvent system. [*methyl*-^{14}C]Ethers of flavonols were chromatographed on polyamide-precoated thin-layer chromatography plates (Merck, polyamide 11 F 254) using petroleum ether (boiling point 40–65°C), benzene, methanol, methylethylketone (40:60:20:20. by vol.) as solvent system. The radioactivity was localized by autoradiography (NS-2T, Eastman Kodak). Correlation of R_F values on thin-layer chromatograms with standards, when available, and spectral chacteristics before and after treatment with $AlCl_3$, $AlCl_3$/HCl, CH_3COONa, CH_3COONa/H_3Bo_3 and CH_3ONa provided the basis for the identification of [*methyl*-^{14}C]ethers of cinnamic acids or of flavonols. Using an Aminco DW_2 UV spectrophotometer, as little as 1 nmol flavonoids could be analyzed in 3 ml methanol.

3. Results

3.1. *Characterization of SAM:caffeic acid* O-*methyltransferase activity associated with chloroplast soluble fraction*

Fig.2 indicates that the soluble fraction from spinach chloroplasts was able to catalyse the methylation of caffeic acid from [*methyl*-^{14}C]SAM. In our conditions, a linear rate of [*methyl*-^{14}C]SAM incorporation was sustained for at least 30 min. The reaction products were separated by TLC (section 2) and they were identified by co-chromatography with reference compounds. The *O*-methylation catalysed by the stroma yielded 70% ferulic acid and 30% 3,4-dimethyl caffeic acid after 60 min incubation. Consequently the *O*-methyltransferase (SAM:caffeic acid *O*-methyltransferase) involved catalysed the *O*-methylation of

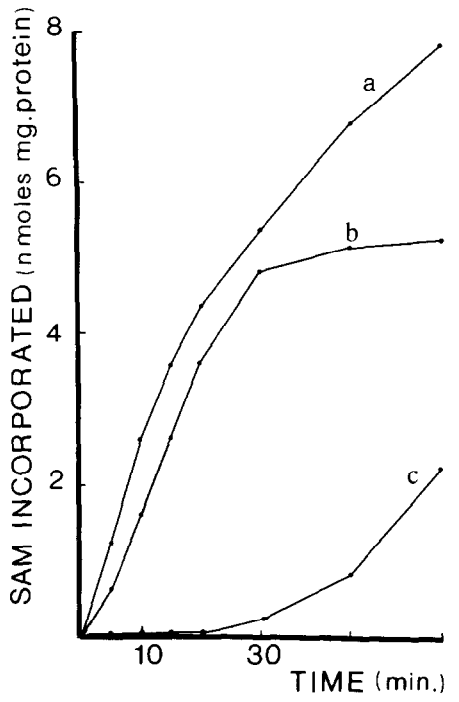

Fig.2. Time course of incorporation of *methyl*-^{14}C from [*methyl*-^{14}C]SAM by the chloroplast soluble fraction into caffeic acid derivatives. The incubation mixture, containing 735 μg soluble fraction protein, was as described in section 2. (a) Total incorporation with caffeic acid as substrate; (b) incorporation into ferulic acid; (c) incorporation into 3,4-dimethyl caffeic acid.

caffeic acid predominantly at the *meta*-position. Fig.2 also shows that the initial product was ferulic acid which was then slowly *O*-methylated to yield the corresponding dimethyl derivative (3,4-dimethyl caffeic acid).

3.2. *Characterization of SAM:flavonol* O-*methyltransferase activity associated with chloroplast envelope membranes*

Fig.3 clearly shows that quercetin was readily *O*-methylated by the envelope membranes. The same holds true for quercetagetin as substrate (fig.3). In our conditions, a linear rate of [*methyl*-^{14}C]SAM incorporation was sustained for at least 10 min.

The *O*-methylation of quercetin catalysed by the envelope membranes yielded several compounds after 60 min (table 1). The availability of a number of reference compounds made it possible to demonstrate the presence of 3-monomethyl, 3,3′-, 3,7-dimethyl,

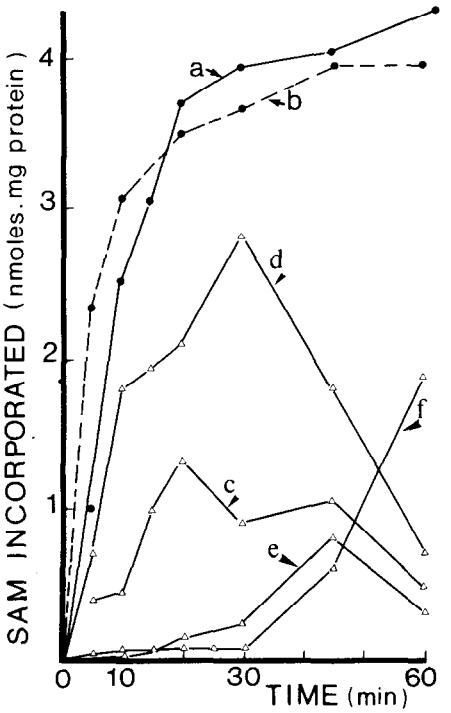

Fig.3. Time course of incorporation of *methyl*-^{14}C from [*methyl*-^{14}C]SAM by the chloroplast envelope membranes into quercetin (a) and quercetagetin (b) derivatives. The incubation mixture, containing 675 μg envelope membrane protein, was as in section 2. (a) Total incorporation with quercetin as substrate; (b) total incorporation with quercetagetin as substrate; (c) incorporation into monomethylether quercetin derivative (3-methyl quercetin); (d) incorporation into dimethylether quercetin derivatives; (e) incorporation into trimethylether quercetin derivatives; (f) incorporation into tetramethylether quercetin derivatives.

3,7,3'-, 3,3', 4'-, 3,7,4'-trimethyl and 3,7,3'4'-tetramethyl ethers of quercetin among the reaction products.

In addition, fig.3 indicates the stepwise *O*-methylation of quercetin to mono-, di-, tri- and tetramethyl derivatives. This result suggests that a coordinated sequence of *O*-methylation steps occurred on the envelope membranes. This result also suggests that the envelope membranes exhibited the highest rate of *O*-methylation for position 3.

In marked contrast to the soluble fraction, the envelope membranes were unable to catalyse the *O*-methylation of caffeic acid. Finally, washed thylakoids were unable to catalyse the introduction of methyl groups from [*methyl*-^{14}C]SAM either into caffeic acid or flavonols.

4. Discussion

These results demonstrate that spinach chloroplasts contain at least two *O*-methyltransferase. The first *O*-methyltransferase catalysed the methylation, predominantly at the *meta*-position, of caffeic acid. This soluble enzyme is localized either in the stroma or in the compartment between the two envelope membranes. The second *O*-methyltransferase catalysed the stepwise *O*-methylation of quercetin and quercetagetin, but not of caffeic acid, at the *meta*- and/or *para*-positions of rings A and B. Furthermore, this enzyme (which was bound to envelope membranes) operated first on the 3-hydroxyl of flavonols. The problem of whether separate enzymes or only one enzyme actu-

Table 1
Chromatographic and spectrophotometric data for *O*-methylated derivatives synthesized from quercetin by chloroplast envelope membranes

Quercetin derivatives	R_F	λ_{max} (nm)		
		Methanol	$AlCl_3$	$AlCl_3$/HCl
Methyl-3	0.25	255 362	278 435	476 365
Dimethyl-3,3'	0.60	256 356	267 364 404	264 276 359
Dimethyl-3,7	0.72	270 365	278 437	278 368
Trimethyl-3,7,3'	0.76	255 355	268 370 408	268 370
Trimethyl-3,3',4'	0.82	253 353	267 360 408	265 359
Trimethyl-3,7,4'	0.89	356 353	270 366 402	268 360
Tetramethyl-3,7,3',4'	0.96	250 270 352	277 359 405	270 359

Flavonols were chromatographed on polyamide-precoated thin-layer chromatography plates using petroleum ether, benzene, methanol, methylethylketone as solvent system (section 2)

ally catalysed the reactions at the *meta*- and *para*-positions has not been resolved.

Consequently, these results demonstrate for the first time that the plastid envelope is directly involved in flavonoid biosynthesis. As a matter of fact, the SAM:flavonol *O*-methyltransferase represent a central enzyme of this metabolic process. Our preliminary experiments also demonstrate that the first enzyme (= flavonone synthase) responsible for the formation of the flavonoid skeleton is soluble and localized in the chloroplasts. We can therefore conclude, in contrast with [7], that several sequences of the flavonoid pathway are present in plastids.

The reason for this discrepancy is that the chloroplast is in general a delicate structure and it is very difficult to avoid large scale rupture of the two membranes of the envelope during chloroplast isolation. As pointed out in [13], many methods which are currently used yield preparations containing an appreciable proportion of broken chloroplasts and may lead to ambiguous and possibly misleading results.

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