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PARTIAL PURIFICATION AND CHARACTERIZATION OF 6-HYDROXYMELLEIN-*O*-METHYLTRANSFERASE FROM ELICITOR-TREATED CARROT CELLS

FUMIYA KUROSAKI

Laboratory of Cell Biology, Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, Sugitani, Toyama 930-01, Japan

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Key Word Index—*Daucus carota*; Umbelliferae; phytoalexin; biosynthesis; inducible enzyme; *O*-methyltransferase; product inhibition; reaction mechanism.

Abstract—6-Hydroxymellein-*O*-methyltransferase (6HMOMT), an inducible OMT involved in the biosynthesis of the carrot phytoalexin 6-methoxymellein (6MM), was partially purified with a 105-fold increase in the specific activity of the enzyme. The OMT showed a maximal activity within the pH range of 7.5–8.0, and its M_r was estimated to be 76000 while its pI was 5.7. The activity of 6HMOMT was appreciably inhibited in the presence of several divalent cations such as Cu^{2+}, Co^{2+}, Fe^{2+}, Mn^{2+}, and sulphydryl reagents. It was also inhibited by the addition of its reaction products, 6MM and *S*-adenosyl-L-homocysteine. 6MM was found to inhibit the enzymatic reaction with respect to the two co-substrates of 6HMOMT, 6HM and *S*-adenosyl-L-methionine, in a competitive manner. Competitive inhibition of the OMT was also observed for another product of the enzyme, *S*-adenosyl-L-homocysteine, as the function of these two substrates. These results suggest that the catalytic reaction of 6HMOMT proceeds by a sequential bireactant mechanism in which both the entry of the co-substrates into and the release of the co-products from the enzyme take place in random order.

INTRODUCTION

Higher plants produce phytoalexins upon invasion by pathogenic microorganisms as one of the active defense responses, and various chemicals of biotic and abiotic origin, called elicitors, are capable of inducing the biosynthetic activities received for the production of these compounds in plant cells [1]. Although numerous compounds have been reported as phytoalexins from a wide variety of plant sources [2], only little is known about the enzymes involved in the biosynthesis of this class of compounds. We showed that the carrot phytoalexin 6-methoxymellein (6MM, Fig. 1) is synthesized by two inducible enzymes, 6-hydroxymellein (6HM) synthase [3,4] and 6HM-*O*-methyltransferase (6HMOMT) [5]. 6HM synthase catalyzes the condensation of one acetyl-CoA and four malonyl-CoA in the presence of NADPH to form 6HM via a reduced ketomethylene chain as the putative intermediate [6]. It was demonstrated [5] that 6HM is the direct precursor of 6MM, and *O*-methylation catalysed by 6HMOMT with *S*-adenosyl-L-methionine (SAM) as the methyl donor leads to 6MM (Fig. 1). It was also shown [7] that the rate of accumulation of 6MM in elicitor-treated carrot root tissues was almost similar to that of 6HMOMT in the cell homogenates, and therefore, we concluded that the *O*-methyltransfer is the rate-limiting step and controls the overall rate of the biosynthesis of 6MM. It was demonstrated [8] that 6MM exhibits a considerable toxic effect on its host plant (carrot cells) as well as to invading microorganisms [9], whereas, the cytotoxicity of 6HM is appreciably lower [10]. These observations strongly suggested that the methyltransfer step in 6MM biosynthesis is a physiologically important process since it converts the less toxic precursor to the highly toxic final product as the rate-determining reaction. Therefore, it was reasonable to expect that certain direct mechanism(s) should be involved in the regulation of 6HMOMT activity in 6MM biosynthesis which strictly controls the cellular concentration of this compound to avoid the harmful effect to the host carrot cells caused by the overproduction. Recently, I have shown [11] that 6HMOMT activity is significantly inhibited in the presence of its reaction products, 6MM and *S*-adenosyl-L-homocysteine (SAH), *in vitro*, and therefore, it can be assumed that the product inhibition of the OMT activity is one of the important mechanisms controlling the enzyme activity in elicitor-treated carrot cells, *in vivo*. In order to understand the regulatory mechanisms of 6HMOMT activity in infected carrot cells in detail, in the present study, I attempted to purify the enzyme and characterize its catalytic processes. Special attention was focused on the mechanisms of the substrate entry and the product release in the reaction

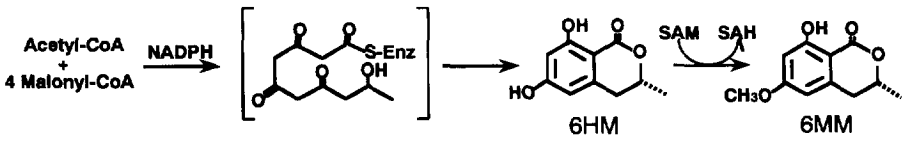

Fig. 1. Biosynthetic pathway for the formation of 6HM and 6MM.

Table 1. Partial purification of 6HMOMT from elicitor-treated carrot cell extracts

	Total proteins (mg)	Total activity (pkat)	Sp. activity (pkat/mg protein)	Recovery (%)	Purification (-fold)
Crude extracts	364	41.3	0.11	100	1
$(NH_4)_2SO_4$ ppt	129	29.9	0.23	72	2
DEAE-Sephacel	60	22.1	0.37	54	3
Sephadex G-100	3.3	4.6	1.39	11	13
Chromatofocusing	0.2	2.3	11.5	6	105

cycle of 6HMOMT by analysing the kinetic properties of the product-inhibition of the enzyme activity.

RESULTS

Partial purification of 6HMOMT

6HMOMT activity was induced in carrot root tissues by treatment of the root discs with 2-chloroethylphosphonic acid as the elicitor [3], and the enzyme in the cell extracts was partially purified by $(NH_4)_2SO_4$ precipitation, batch treatment with DEAE-Sephacel, and gel-filtration chromatography on a Sephadex G-100 column followed by chromatofocusing on a Polybuffer exchanger 94 column. The purification steps are summarized in Table 1. The specific activity of 6HMOMT was increased 105-fold, and maximal activity was observed within the pH range of 7.5–8.0 (data not shown). The M_r of 6HMOMT was estimated to be 76000 (Fig. 2a) by gel-filtration chromatography, and the pI was found to be 5.7 by chromatofocusing (Fig. 2b). 6HMOMT activity was unstable and was almost completely lost after the enzyme preparation was kept at 4° for seven days or frozen overnight. Addition of several protease inhibitors and/or polyalcohols did not show any significant effect on the rate of loss of the enzyme activity under these conditions.

Effect of divalent cations and sulphydryl reagents on 6HMOMT activity

The Effects of divalent cations and sulphydryl reagents on 6HMOMT activity was examined using partially purified enzyme preparation. Addition of 10 mM EDTA or EGTA did not inhibit the OMT activity suggesting that the methylation reaction has no requirement for cations (Table 2). By contrast, 6HMOMT activity was appreciably inhibited in the presence of several divalent cations such Cu^{2+}, Co^{2+}, Fe^{2+}, Mn^{2+}. It was reported [12–15] that some of the OMTs involved in berberine biosynthesis showed similar properties, and their activities were appreciably inhibited with these cations. It appeared that a SH group(s) is important for 6HMOMT activity since the activity was markedly inhibited in the presence of sulphydryl reagents.

Product inhibition of 6HMOMT activity

I showed previously [11] that 6HMOMT activity is appreciably inhibited in the presence of its reaction products. 6MM was found to inhibit the OMT activity as a function of 6HM concentration, and SAH also inhibits the activity with respect to SAM. *Ki* values of 6MM versus 6HM was estimated to be 37 μM while SAH versus SAM was 27 μM [11]. In the present experiments, the processes of this product inhibition of 6HMOMT have been characterized in detail employing the partially purified OMT, and it has been found that the enzyme activity is inhibited by 6MM as a function of not only 6HM but also SAM. The experimental results were analyzed by double reciprocal plot, and, where necessary, the method of least squares was employed. Primary reciprocal plots were determined at a series of concentrations of SAM (0.025–0.2 μM) and 6HM (1.25–10 μM) in the presence of 0–100 μM of 6MM, and this yielded families of lines which intersected on the vertical (1/V) axis, respectively. These results clearly indicated that the OMT activity was inhibited by 6MM in the competitive manner with respect to either 6HM or SAM. The *Ki* value of 6MM versus SAH was determined graphically from reciprocal plots of slopes against 6MM concentrations, and was estimated to be 47 μM. This figure is almost similar to that of 6MM versus 6HM as described above. 6HMOMT activity was also inhibited in the presence of SAH with respect to either 6HM or SAM in the competitive manner, and the *Ki* of SAH versus 6HM was determined to be 26 μM. These results suggest that the catalytic reaction of 6HMOMT proceeds by a sequential bi

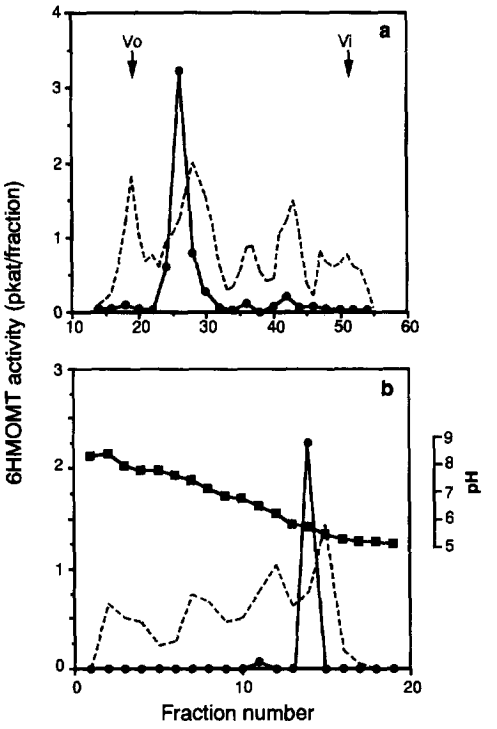

Fig. 2. Elution profiles on gel-filtration chromatography (a) and chromatofocusing (b) of 6HMOMT. (a) 6HMOMT partially purified by $(NH_4)_2SO_4$ fractionation and batch treatment with DEAE-Sephacel, was applied onto a Sephadex G-100 column. The OMT was eluted with Na-phosphate buffer as the eluting solvent, and the enzyme activity in the fractions (2 ml each) was determined (●). Proteins were monitored by measuring the absorbance at 280 nm (dashed line). Void (Vo) and inclusion (Vi) volumes were determined by blue dextran and vitamin B_{12}, respectively, and the M_r of 6HMOMT was estimated with standard proteins (Bio-Rad, thyroglobulin 670 kDa; gamma globulin 158 kDa; ovalbumin 44 kDa; myoglobin 17 kDa). (b) Active fractions obtained by gel-filtration chromatography were equilibrated with 25 mM Tris–HCl buffer (pH 8.3) by dialysis, and applied onto a Polybuffer exchanger 94 column which had been previously equilibrated with the same buffer. The column was eluted with the mixture of Polybuffer 96 and Polybuffer 74 of which pH was adjusted at 5.0 to make the pH gradient (■). Proteins were monitored at 280 nm (dashed line), and 6HMOMT activity (●) in the fractions (5.5 ml each) was determined.

bi mechanism, and both the entry of the two subtrates, 6HM and SAM, into and the release of the two products, 6MM and SAH, from the enzyme protein take place in random order (Fig. 3).

DISCUSSION

6HMOMT, an inducible OMT involved in the biosynthesis of carrot phytoalexin 6MM, was partially purified, and the mode of product inhibition of the enzyme was characterized in detail to understand the regulatory mechanism of the activity, *in vivo*. It is well known that

Table 2. Effect of various reagents on 6HMOMT activity

Reagents	Concentration (mM)	Relative activity (% of control)
EDTA	10	98
EGTA	10	103
Mg^{2+}	1	101
	10	108
Ca^{2+}	1	96
	10	89
Cu^{2+}	1	52
	10	12
Co^{2+}	1	39
	10	10
Fe^{2+}	1	84
	10	11
Mn^{2+}	1	63
	10	13
Hg^{2+}	10	8
p-Chloromercuribenzoate	10	11
Iodoacetoamide	10	22

Divalent cations were added to the assay mixture as their chloride salts, respectively.

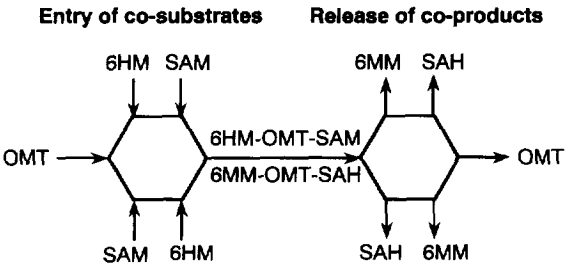

Fig. 3. Schematic presentation of the reaction process of 6HMOMT. The catalytic reaction of 6HMOMT is likely to proceed by a sequential bireactant mechanism in which both the entry of the co-substrates into and the release of the co-products from the enzyme take place in random order.

SAH, a common product of many OMTs which utilize SAM as the methyl donor, is usually a potent inhibitor of these enzymes. In the 6HMOMT reaction, however, I have previously reported [11] that another product of this OMT, 6MM, exhibits a marked inhibitory activity as well as SAH. This observation suggests that 6HMOMT activity is regulated rather specifically in response to an increase in the cellular concentrations of its reaction products in carrot cells since, in addition to SAH, the 'specific' product of the OMT, 6MM, is capable of inhibiting the enzyme activity [11]. In the present study, it has been demonstrated that 6MM inhibits 6HMOMT activity with respect to not only 6HM but also SAM in a competitive manner. This cross competitive inhibition was also found in SAH as the function of these co-substrates of 6HMOMT. These observations indicate that the reaction catalyzed by 6HMOMT proceeds by a sequential bireactant mechanism in which both the entry of the co-substrates to form the enzyme-substrate

complexes and the release of the co-products to generate free enzyme take place in the random order (Fig. 3). This result also implies that 6MM and SAH are able to associate with only the free OMT to exhibit their inhibitory activities, and that they cannot work as the inhibitors after the OMT forms the enzyme-substrate complexes. Therefore, if 6HMOMT activity is controlled by its specific product 6MM, *in vivo*, this compound should efficiently block the entry of the both co-substrates, 6HM and SAM, prior to the formation of the primary enzyme-substrate complexes, OMT-6HM and OMT-SAM. As described above, *Ki* values of 6MM are almost comparable with respect to the co-substrates, SAM and 6HM (47 and 37 μM, respectively), suggesting that 6MM appreciably prevents the binding of the both co-substrates to 6HMOMT protein and inhibits the formation of the enzyme-substrate complexes. SAH also showed comparable *Ki* values toward these substrates (27 μM for SAM and 26 μM for 6HM, respectively), and it is assumed that, as is in 6MM, this compound is able to inhibit the entry of the two co-substrates with a similar efficiency. These observations, therefore, strongly suggest that both of the two products of 6HMOMT are capable of inhibiting the formation of 6HMOMT-substrate complexes within the range of physiological concentrations, *in vivo*. The results obtained in the present study appear to support my previous idea that 6HMOMT activity is strictly and specifically controlled by its own product 6MM, as well as SAH, to maintain the cytoplasmic concentration of 6MM at the appropriate levels to avoid damage to the host carrot cells themselves possibly caused by the overproduction of this toxic secondary metabolite.

EXPERIMENTAL

Chemicals. 6MM was isolated from fungi-infected carrot roots [9], and 6HM was synthesized by demethylating 6MM with BBr_3 in anhydrous CH_2Cl_2 as reported previously in detail [8]. Polyvinylpolypyrrolidone, EDTA, EGTA, p-chloromercuribenzoate and iodoacetoamide were purchased from Wako Pure Chemicals, while 2-chloroethylphosphonic acid, SAM and SAH were from Sigma. [*Me*^{14}C] SAM (sp. act 2.1 GBq mmol^{-1}) was obtained from New England Nuclear. All other chemicals were of reagent grade.

Induction and partial purification of 6HMOMT from carrot root tissues. Carrot roots were purchased from a local market, and induction of 6HMOMT activity was carried out according to the method described previously [3]. The root tissues (500 g) were surface-sterilized with EtOH, and sliced into pieces 2 mm thick. These were placed in a Petri dish (21 cm diameter), and 20 ml of 2-chloroethylphosphonic acid soln (10 mg/ml in 0.1 M Na-citrate buffer) was dropped onto the surfaces. After incubation at 26° for 4 days, the root slices were harvested, frozen in liquid N_2, and then homogenized in a mortar with a pestle. They were further homogenized with a Waring Blender in 400 ml of 40 mM Na-Pi buffer (pH 7.5) containing 4 mM cysteine, 0.2% (v/v) mercaptoethanol in the presence of 40 g polyvinylpolypyrrolidone. The homogenate was passed through a double layered gauze, and centrifuged at 10 000 *g* for 15 min (crude extract). To the resultant supernatant was added crystalline $(NH_4)_2SO_4$, and proteins precipitated with 40–70% saturation of $(NH_4)_2SO_4$ collected by centrifugation (10 000 *g* for 15 min). They were redissolved in, and dialysed against, the homogenization buffer. The soln was then mixed with DEAE-Sephacel (Pharmacia, 40 ml packed vol) which had been previously equilibrated with the same buffer. The suspension was stirred at 4° for 20 min, and the non-adsorbed proteins removed by centrifugation (1000 *g* for 5 min). The adsorbed proteins were recovered by stirring the suspension in Na-Pi buffer containing 0.8 M NaCl followed by centrifugation (twice). The vol of the soln was reduced to *ca.* 3 ml with an ultrafiltration cell (Amicon, YM-10 membrane), and the sample applied onto a column of Sephadex G-100 (Pharmacia, 2.0 × 75 cm). The column was eluted with 20 mM Na-Pi buffer (pH 7.5, 0.2% mercaptoethanol), and frs (2 ml each) containing 6HMOMT activity were combined and dialysed against 25 mM Tris–HCl buffer (pH 8.3). The sample was then applied onto a 0.7 × 22 cm column of Polybuffer exchanger 94 (Pharmacia) equilibrated with Tris–HCl buffer, and was developed with a mixture of Polybuffer 96 and Polybuffer 74 (Pharmacia, 4.5 ml and 10.5 ml, respectively, in a total vol. of 150 ml) the pH of which was set to 5.0 according to the instruction manual. Frs (5.5 ml each) were collected, and their enzyme activity and pH value were determined. The active fraction was passed through a Sephadex G-75 column (Pharmacia, 0.8 × 60 cm) with 40 mM Na-Pi buffer (pH 7.5, containing 0.2% mercaptoethanol) as the eluting solvent to remove the Polybuffer mixture. The sample thus purified was concentrated in an Amicon cell, and served as the 6HMOMT preparation. Protein concentrations of the samples were determined according to the method of Bradford [16].

Assay of 6HMOMT activity. The assay of 6HMOMT activity was carried out according to the method described in ref. [5] with some modifications. The standard assay mixture contained enzyme protein (*ca* 1 pkat/assay), 10 μM 6HM, 2 mM cysteine and 1 μM [*Me*–^{14}C] SAM (3.7 kBq) in 20 mM Na-Pi buffer (pH 7.5) containing 0.1% mercaptoethanol in a total vol. of 250 μl. In some experiments, the assay was carried out in the presence of 0–100 μM of 6MM or 0–40 μM of SAH with various concentrations of the substrates (0.025–0.2 μM of SAM plus 10 μM of 6HM or 1 μM of SAM plus 1.25–10 μM of 6HM). After 1 hr incubation at 37°, the reaction was terminated by the addition of 50 μl 6 M HCl. The product was extracted with 200 μl hexane by blending, a 100 μl-aliquot was removed, mixed with 3.5 ml of a commercial scintillation cocktail (Amersham, ACS II), and the mixture assayed for radio activity.

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