Pergamon

0031-9422(95)00004-6

Phytochemistry, Vol. 39, No. 3, pp. 515–519, 1995

0031-9422/95 $9.50 + 0.00

DIMER AS THE ACTIVE FORM OF A POLYKETIDE BIOSYNTHETIC ENZYME 6-HYDROXYMELLEIN SYNTHASE

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(*Received in revised form* 7 *December* 1994)

Key Word Index—*Daucus carota*; Umbelliferae; 6-hydroxymellein synthase; polyketide biosynthetic enzyme; multifunctional enzyme complex; dimeric structure.

Abstract—Activity of 6-hydroxymellein synthase, an inducible polyketide biosynthetic enzyme in carrot cell extracts, was appreciably inhibited in the presence of molar levels of NaCl or $(NH_4)_2SO_4$. However, the salt-induced inhibition of the synthase activity was reversible, and was almost fully restored after the salts were removed. Highly purified 6-hydroxymellein synthase showed essentially one band of M_r 128 000 as analysed by SDS–PAGE. However, in gel-filtration analysis, the synthase activity was recovered in fractions corresponding to M_r 285 000 when the enzyme was eluted with buffered saline. By contrast, the enzyme protein was eluted at the position of M_r 136 000 with loss of the activity under high-salt condition. Activity of the M_r 136 000 peptide was restored after desalting. The M_r of the re-activated enzyme was found to shift to 285 000. The results suggest that two polypeptides associate and form an active 6-hydroxymellein synthase.

INTRODUCTION

Many higher plants and fungi produce a variety of secondary metabolites by the head-to-tail condensation of acetyl-CoA and malonyl-CoA via a polyketomethylene chain as a putative intermediate [1]. Enzymes catalysing the biosynthesis of these polyketide compounds have been believed to share many common properties with fatty acid synthases (FASs). However, the biochemical nature of the polyketide synthetic enzymes has not been well understood because of the instability of these enzyme proteins [1,2]. Animal and yeast FASs are multifunctional complexes (type I) while higher plant and bacterial FASs can be readily separated into individual catalytic units (type II) [3]. Type I FASs are further divided into two subclasses, IA (dimeric structure consisting of two identical subunit) and IB (higher state of aggregation) [4].

It was shown that the carrot phytoalexin 6-methoxymellein (**2**) (Fig. 1), a very rare dihydroisocoumarin occurring in higher plants, is produced via the acetate-malonate pathway [5, 6]. Recently, we have demonstrated [7] that a reduced ketomethylene chain is an important intermediate in the biosynthesis of 6-methoxymellein, and 6-hydroxymellein (**1**) is a direct precursor of 6-methoxymellein. We have also shown that these processes are catalysed by two inducible enzymes, 6-hydroxymellein synthase [8] and 6-hydroxymellein-*O*-methyltransferase [9] (Fig. 1). Since the M_r of 6-hydroxymellein synthase estimated by gel-filtration chromatography was very similar to that shown by SDS–PAGE combined with immunoblotting (136 000 and 128 000, respectively) [10], we assumed that this enzyme should be a multifunctional complex which resembles type I FAS. However, in gel-filtration analysis, a minor peak of the synthase activity was reproducibly observed at the position corresponding to M_r 285 000 although its relative intensity varied in repeated experiments. These observations suggested the possibility that 6-hydroxymellein synthase might be composed of several subunits, as is type I FAS, *in vivo* and, therefore, in the present study, I attempted to elucidate the native structure of 6-hydroxymellein synthase. For this purpose, I examined changes in the activity and the M_r of 6-hydroxymellein synthase in the presence of various concentrations of salts, and obtained evidence that the dimeric form of the synthase is the only active species of the enzyme.

RESULTS

We reported previously [10] that antibody raised against spinach acyl carrier protein cross-reacted with a M_r 128 000 polypeptide of partially purified 6-hydroxymellein synthase. To elucidate the polypeptide component(s) of 6-hydroxymellein synthase active in the production of the compound, the enzyme was highly purified and subjected to an SDS–PAGE (Fig. 2). Essentially one protein band of 128 000 was detected, although several scant bands, probably contaminant proteins, were also observed. It was revealed that the M_r 128 000 peptide comprised 83% of the total proteins as analysed on a

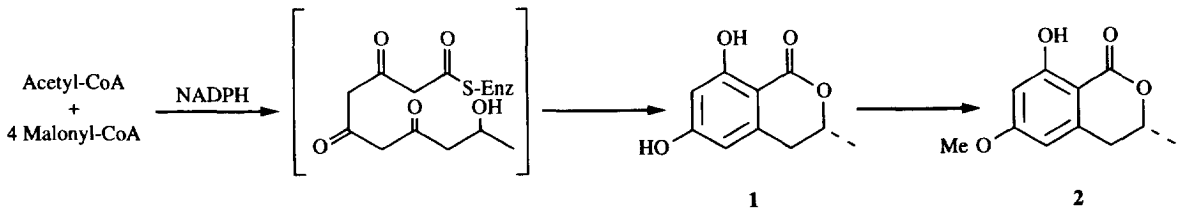

Fig 1. Biosynthesis of 6-methoxymellein. 6-Hydroxymellein synthase catalyses the condensation of acetyl-CoA and malonyl-CoA in the presence of NADPH, and produces 6-hydroxymellein (1) via a reduced ketomethylene chain as an intermediate. *O*-Methylation by 6-hydroxymellein-*O*-methyltransferase leads to 6-methoxymellein (2).

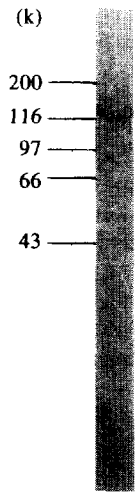

Fig. 2. SDS–PAGE of the polypeptide components of 6-hydroxymellein synthase. Highly purified 6-hydroxymellein synthase was denatured, subjected to SDS–PAGE, and the M_r of the main band was estimated using standard proteins (myosin, 200 000; *Escherichia coli* galactosidase, 116 000; rabbit muscle phosphorylase, 97 000; bovine serum albumin, 66 000; ovalbumin, 43 000).

densitometer. This result strongly suggests that the active form of 6-hydroxymellein synthase consists of a single polypeptide or is organized by several identical subunits of M_r 128 000.

In type I FASs, it was shown [11] that the association of the polypeptides to form the active FAS is highly sensitive to ionic strength of the buffer *in vitro*. Therefore, I examined the possible effect of salt concentration on the activity of 6-hydroxymellein synthase. The synthase activity was markedly affected by NaCl concentration (Fig. 3a). It appeared that the enzyme activity was not inhibited in the presence of less than 0.5 M of NaCl, although a marked decrease in activity was observed with 0.7 M NaCl. The enzymatic reaction was almost completely inhibited with 1–2 M NaCl. This NaCl-induced inhibition of 6-hydroxymellein synthase was found to be reversible, and its activity was recovered essentially without loss after the sample was desalted by dialysis against buffered saline or NaCl-depleted buffer. Similar results were obtained when NaCl was replaced by $(NH_4)_2SO_4$ (Fig. 3b), and the synthase activity was significantly inhibited with high concentrations of $(NH_4)_2SO_4$ in a reversible manner.

In the next experiments, I examined the possible change of M_r of 6-hydroxymellein synthase in the presence or absence of high concentrations of NaCl. However, an attempt to detect possible oligomeric structure(s) of native 6-hydroxymellein synthase with the use of cross-linking reagents was unsuccessful. Several aggregates of the synthase with a variety of molecular masses were observed in immunoblot analysis [10] probably because multiple cross-linking sites are included in the enzyme structure and non-specifically bound products were formed even in the diluted solution of the enzyme proteins. Therefore, I employed gel-filtration chromatography to examine the change in molecular mass of 6-hydroxymellein synthase under various conditions. The synthase preparation was equilibrated by dialysis with Na-phosphate buffered saline containing mercaptoethanol, and subjected to gel-filtration chromatography using buffered saline as the eluting solvent. After fractionation, only one activity peak was observed, and its M_r was estimated to be 285 000 (Fig. 4a). This M_r did not change even when the synthase was equilibrated and eluted with the phosphate buffer free of NaCl and/or mercaptoethanol (data not shown). In case the synthase preparation was eluted with and assayed in NaCl-containing buffer (2M), the enzyme activity could not be detected (Fig. 4b). However, the activity peak was observed at a position corresponding to M_r 136 000 after the collected fractions were desalted by dialysis against Na-phosphate buffered saline. In order to determine the M_r of this re-activated synthase, the desalted sample recovered in the 136 000 fractions was applied to the column and eluted with the buffered saline (Fig. 4c). Its activity was found to shift to a position corresponding to M_r 285 000. These results strongly suggested that the dimeric form of 6-hydroxymellein synthase is an active species, and the salt-induced inhibition of the synthase observed above was caused by dissociation of the active dimers to inactive monomeric peptides under conditions of high ionic strength. To confirm this further, in the next experiments, I equilibrated and eluted the synthase preparation with 0.7 M NaCl-containing buffer with which it was shown that 6-hydroxymellein synthase activity is partially inactivated (Fig. 3a). Collected fractions were dialysed against the buffered saline prior to the enzyme assay to detect either the active dimers or the inactive monomeric peptides.

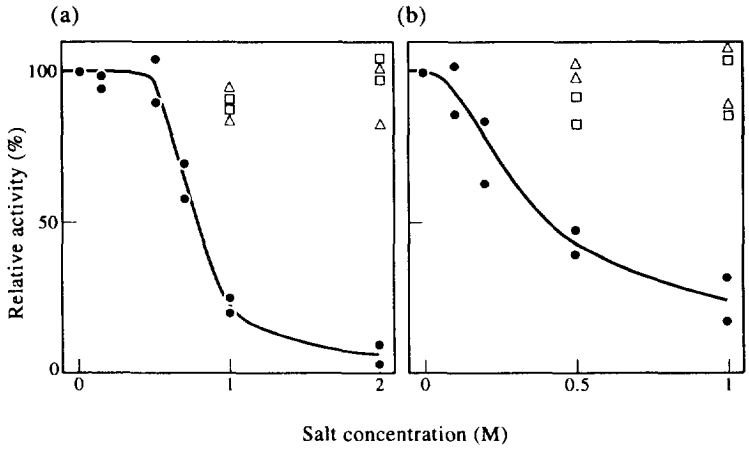

Fig. 3. Effect of NaCl (a) or $(NH_4)_2SO_4$ (b) on 6-hydroxymellein synthase activity. A 6-hydroxymellein synthase preparation was equilibrated with Na-Pi buffer containing various concentrations of NaCl or $(NH_4)_2SO_4$, and the activity was assayed in the buffers containing the same concentrations of the salts, respectively (●). Results obtained from two independent experiments were expressed as percentages to controls which were treated with and assayed in the buffer free from these salts [1.3. and 0.9 pkat in (a), and 1.5 and 1.1 pkat in (b), respectively]. In some experiments, the synthase activity of the salt-treated samples was determined after being dialysed against the buffered saline (△) or the salt-free buffer (□).

Two peaks of the synthase activity (285 000 and 136 000) were observed in Fig. 4d indicating that both monomeric and dimeric forms of the enzyme were included in the 0.7 M NaCl-treated sample. This observation is consistent with the assumption that there is an equilibrium between the active dimer and inactive monomer of 6-hydroxymellein synthase *in vitro*, and ionic strength is an important factor to determine the ratio of these two forms of the synthase.

DISCUSSION

In the present study, it is strongly suggested that the dimeric structure of 6-hydroxymellein synthase is an active form of the enzyme, and, as far as tested, higher aggregated states of the synthase could not be detected (Fig. 4). The monomer of the enzyme was shown to be inactive, and therefore, it is very likely that the dimer is the only active species of 6-hydroxymellein synthase. Since highly purified 6-hydroxymellein synthase showed essentially one band in SDS–PAGE (Fig. 2), it is assumed that the active synthase is a homodimer and resembles type IA FAS reported to occur in various animal cells [4, 11]. However, the possibility cannot be ruled out that the dimeric structure of the synthase consists of two polypeptides of which the M_rs are very similar but not identical.

We reported previously [10] that the activity of partially purified 6-hydroxymellein synthase was mainly found in the fractions of 136 000 in gel-filtration analysis even though the chromatography was carried out with low ionic strength-buffer (10 mM Na-Pi, pH 7.5) as an eluting solvent. This apparent discrepancy may be explained as follows. In the previous experiment, the enzyme preparation precipitated with 40% saturation of $(NH_4)_2SO_4$ (approximately 2.3 M) was redissolved in a small volume of the buffer, and directly applied onto the column without desalting. Therefore, the synthase dissociated to monomers in this solution of high-salt concentration, and the majority of the enzyme molecules eluted through the gel-matrix without formation of a dimer. The enzyme eluted at the position of M_r 136 000, then associated to form the active dimer in the fraction tubes or the assay mixtures.

In our previous study, it was demonstrated [6] that the activity of 6-hydroxymellein synthase did not change in the presence of various divalent cations or chelating reagent. As described above, addition of mercaptoethanol also did not influence the M_r of the synthase. These results suggest that maintenance of the enzyme structure active in 6-hydroxymellein synthesis depends neither on cations nor on the formation of disulphide bond. The fact that 6-hydroxymellein synthase dissociates to monomers in the buffers of high salt concentrations suggests that electrostatic interaction plays an important role in the association of the two polypeptides. By contrast, it was shown [12] that type IA FASs of chicken and rat livers dissociated and lost their activity in the buffer of low ionic strength. These observations clearly indicate that the physicochemical force which drives the formation of the dimeric structure as an active form of 6-hydroxymellein synthase is different from that in type IA FASs.

EXPERIMENTAL

Chemicals. 6-Methoxymellein was isolated from fungus-infected carrot root tissues [13], and 6-hydroxymellein was prepared by demethylating the compound with BBr_3 in anhydrous methylene chloride as reported previously [14]. 2-Chloroethylphosphonic acid, acetyl-CoA,

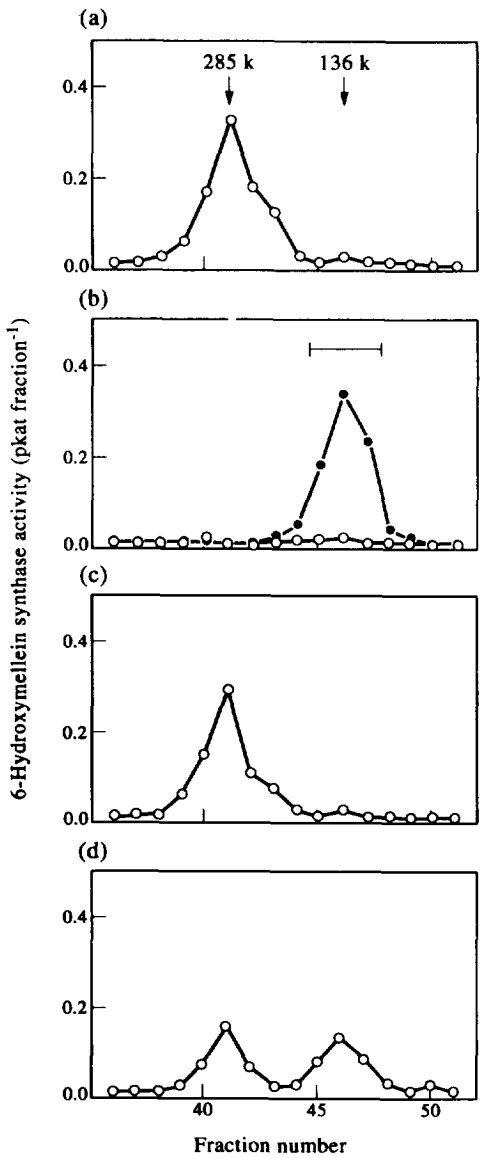

Fig. 4. Analyses of M_r of 6-hydroxymellein synthase by gel-filtration chromatography on a Toyopearl HW-55 column under various conditions. (a) Elution profile of 6-hydroxymellein synthase which was equilibrated and eluted with Na-Pi buffered saline. (b) 6-Hydroxymellein synthase was eluted with Pi buffer containing 2 M NaCl, and the enzyme activity in the collected fractions was assayed under high salt condition (○) or after being dialysed against the buffered saline (●). (c) Fractions including the synthase proteins in (b) (indicated with a bar) were desalted and eluted with the buffered saline. (d) 6-Hydroxymellein synthase was treated and eluted with 0.7 M NaCl-containing buffer, and the enzyme activity in the fractions was determined after dialysis against the saline. M_rs of the synthase were estimated with standard proteins (ferritin 44 000; catalase, 232 000; aldolase, 158 000; bovine serum albumin, 66 000), and indicated in the panel (a) with arrows and numbers.

malonyl-CoA, NADPH were purchased from Sigma, and 1,10-phenanthroline and polyvinylpolypyrrolidone were from Wako Pure Chemicals. [2-^{14}C]Malonyl-CoA (sp.act.2.2 GBq mmol^{-1}) was from New England Nuclear. All other chemicals were reagent grade.

Induction and partial purification of 6-hydroxymellein synthase in carrot root tissues. Carrot roots were purchased from a local market, and induction of 6-hydroxymellein synthase activity in the root disks was carried out using 2-chloroethylphosphonic acid as the elicitor as described in ref [15]. The elicitor-treated carrot root (5 disks) was frozen in liquid N_2 and ground in a mortar with a pestle. They were further homogenized with a Waring blender in 5 ml of 20 mM Na-Pi buffer (pH 7.5) containing 0.2% mercaptoethanol and 1 mM 1, 10-phenanthroline in the presence of 0.5 g polyvinylpolypyrrolidone. The homogenates were passed through double layered gauze, and were centrifuged at 10000 *g* for 20 min. The resultant supernatants were fractionated with $(NH_4)_2SO_4$ and proteins precipitated with 20–40% satn served as the enzyme prepn. Since 6-hydroxymellein synthase is very unstable as reported previously [8], the enzyme was freshly induced and prepared for each set of the experiments. In some experiments, 6-hydroxymellein synthase was highly purified following methods described previously [8]. Purified synthase was heat denatured in the presence of 5% mercaptoethanol and 2% SDS, and subjected to SDS-PAGE (5–20% gradient gel) according to the method of ref. [16]. Separated proteins were stained with Coomassie Brilliant Blue, and intensities of the bands were quantified on a densitometer (Shimadzu, CS-910).

Assay of 6-hydroxymellein synthase. Activity of 6-hydroxymellein synthase was determined as reported previously [5] with some modifications [8]. The assay mixture consisted of 10 mM Na-Pi (pH 7.5), 50 μM acetyl-CoA, 5 μM of [^{14}C]malonyl-CoA (1.85 kBq), 1 mM NADPH, 0.1% of mercaptoethanol and 100 μl of the enzyme soln (*ca* 1 pkat/assay) in total vol. of 0.2 ml. If necessary, various concns of NaCl or $(NH_4)_2SO_4$ were included in the assay mixture. The reaction was run for 30 min at 37°, and was terminated by the addition of 50 μl of 6 M HCl. Products were extracted with 200 μl of EtOAc by blending and were separated by silica gel TLC [7]. Silica gels corresponding to the position of authentic co-chromatographed 6-hydroxymellein were scraped off, and the radioactivities were determined after mixing the gels with a commercial scintillation cocktail (Amersham, ACS II).

Salt-treatment and gel-filtration chromatography of 6-hydroxymellein synthase. Partially purified 6-hydroxymellein synthase pptd with 20–40% satn of $(NH_4)_2SO_4$ was dissolved in 10 ml of 20 mM Na-Pi buffer (pH 7.5, 0.2% mercaptoethanol), and 1 ml-aliquots were transferred into cellophase tubes. They were allowed to equilibrate by dialysis (2l each, total 3 h with one change) with Na-Pi buffer containing various concs of NaCl or $(NH_4)_2SO_4$. After the equilibration, the sample vols were adjusted to 1.5 ml with the respective buffers, and 6-hydroxymellein synthase activity was determined in the presence of the same concs of the salts, respectively.

M_r of 6-hydroxymellein synthase was determined by gel-filtration chromatography under various conditions.

The enzyme solns (*ca* 1 pkat/experiment as determined under the saline condition), which were equilibrated with the Na-Pi buffer containing various concns of NaCl, were applied onto a Toyopearl HW-55 column (Tosoh, 2 × 80 cm), and the column was eluted with the respective NaCl-containing buffers at a flow rate of 11 ml h^{-1}. The fractions (2 ml each) collected were, if necessary, dialysed against the Na-Pi buffered saline, and the enzyme activity was determined.

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