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6-Hydroxymellein synthetase as a multifunctional enzyme complex in elicitor-treated carrot root extract

Fumiya Kurosaki[1], Masataka Itoh[1], Mitsuhiro Yamada[2] and Arasuke Nishi[1]

[1]*Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, Sugitani, Toyama 930-01, Japan* and [2]*Department of Bioscience and Technology, Hokkaido Tokai University, Minamisawa, Minami-Ku, Sapporo 005, Japan*

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Synthetic activity of a polyketide compound 6-hydroxymellein was induced in elicitor-treated carrot root tissues. The activity was significantly inhibited by an antiserum raised against the acyl carrier protein (ACP) of fatty acid synthetase, suggesting that the enzyme(s) for 6-hydroxymellein synthesis require(s) a functional unit similar to ACP. However, the synthetic activity was not stimulated by the addition of ACP purified from *Escherichia coli* and was not lost even after fractionation by gel-filtration chromatography. The active fraction obtained by gel-filtration (136 kDa) was subjected to immunoblot analysis, and a 128 kDa polypeptide in the fraction was found to cross-react with anti-ACP serum. These observations suggest that the biosynthesis of 6-hydroxymellein in carrot cells is catalyzed by an enzyme consisting of a single peptide chain.

6-Hydroxymellein; Multifunctional enzyme complex; Phytoalexin; Polyketide; *Daucus carota* L.

1. INTRODUCTION

In higher plants and fungi, various secondary metabolites are synthesized by head-to-tail condensation of acetyl-CoA and malonyl-CoA [1]. Although enzymes involved in the synthesis of polyketide compounds are thought to resemble fatty acid synthetases (FASs), they are much more unstable than FASs and their biochemical nature is not well understood. Animal FASs have been shown to be a multicatalytic enzyme complex, whereas higher plants and bacterial FASs can be readily separated into individual catalytic units [2]. Dimroth et al. [3] reported that 6-methylsalicylate synthetase, an enzyme involved in polyketide synthesis from *Penicillium patulum*, was recovered as a single particle in sucrose density gradient centrifugation, but the possibility still exists that the catalytic units were non-specifically aggregated to each other under the conditions employed.

When challenged by pathogenic microbes, carrot cells produce 6-methoxymellein, an antimicrobial substance, as phytoalexin [4]. ^{13}C-NMR analysis [5] and feeding experiments with radiolabeled precursors [6] suggested that this compound is produced via the acetate-malonate pathway. In fact, we have reported [7,8] that in carrot extracts 6-hydroxymellein (Fig. 1 (1), the immediate precursor of 6-methoxymellein (2), can be synthesized from acetyl-CoA and malonyl-CoA in the presence of NADPH. Here we report evidence that 6-hydroxymellein synthetase consists of a single peptide chain containing multifunctional domains.

Correspondence address: F. Kurosaki, Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, Sugitani, Toyama 930-01, Japan

2. MATERIALS AND METHODS

2.1. *Chemicals*

6-Methoxymellein was isolated from infected carrot root tissues [4], and 6-hydroxymellein was prepared by demethylating the compound with boron tribromide as described previously [9]. 2-Chloroethylphosphonic acid (2-CEPA), acetyl-CoA, malonyl-CoA, NADPH were obtained from Sigma (St. Louis, MO), and [^{14}C]malonyl-CoA (sp. act 1.8 GBq/mmol) was from New England Nuclear (Boston, MA).

2.2. *Purification of acyl carrier protein (ACP)*

ACP from *Escherichia coli* was prepared according to the method of Rock and Cronan [10]. In brief, *E. coli* cells (130 g wet weight) were treated with 4 mg of lysozyme (Sigma) and homogenized with a Waring blendor in 100 mM Tris-HCl buffer containing 25 mM EDTA and 100 mM glycine (pH 8.0). Nucleic acids were removed by precipitation with isopropanol, and the proteins were absorbed to 15 ml of DEAE-Sephacel (Pharmacia). The resin was packed into a column, and successively washed with 10 mM PIPES buffer containing 250 mM LiCl (pH 6.1). ACP was then eluted with the PIPES buffer containing 500 mM LiCl. This preparation showed a single band in SDS-PAGE analysis (25.5 kDa) by the method of Laemmli [11]. Carrot ACP was partially purified in a similar manner. Carrot cells (150 g) were homogenized in the PIPES buffer, and the homogenates were centrifuged at 10 000 × *g* for 20 min. To the resultant supernatant was added ammonium sulfate to bring the concentration to 70% saturation, and the non-precipitated proteins were chromatographed on a DEAE-Sephacel column as described above.

2.3. *Enzyme preparation*

Induction of 6-hydroxymellein synthetic activity in carrot root disks with 2-CEPA was carried out as described previously [7]. 2-CEPA-treated carrot roots (5 disks) were frozen in liquid nitrogen and were ground in a mortar with a pestle. The disrupted tissues were further homogenized with a Waring blendor in 3 ml of 10 mM sodium phosphate buffer (pH 7.5) containing 2% mercaptoethanol (v/v) and

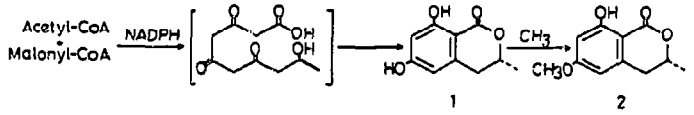

Fig. 1. Biosynthetic pathway of 6-methoxymellein. Condensation of 1 acetyl-CoA and 4 malonyl-CoA in the presence of NADPH results in a formation of 6-hydroxymellein (1), and following *O*-methylation leads it to 6-methoxymellein (2).

1 mM 1,10-phenanthroline in the presence of 0.5 g polyvinylpolypyrrolidone. The homogenates were filtered through double-layered gauze, and centrifuged at 10 000 × *g* for 20 min. The resultant supernatants were then treated with 0.2 g of AG 1-X8 resin (Bio-Rad) for 3 min in an ice bath, and the ion-exchange resin was removed by filtration through a glass fiber filter paper (Whatman GF/C). The filtrate was fractionated with ammonium sulfate, and the proteins precipitated with 20–40% saturation were redissolved in 5 ml of the homogenization buffer as the enzyme preparation of 6-hydroxymellein synthesis. For assay of FAS, proteins precipitated with 40–60% saturation of ammonium sulfate were used as the enzyme preparation.

2.4. *Enzyme assay*

6-Hydroxymellein synthetic activity was determined as described previously [8] with a small modification. The assay mixture contained 10 mM sodium phosphate (pH 7.5), 125 μl of enzyme preparation, 0.1 mM acetyl-CoA, 0.01 mM [^{14}C]malonyl-CoA (3.7 kBq), 1 mM NADPH and 1% (v/v) mercaptoethanol in a total volume of 250 μl. When the assay mixture contained sera, the buffer was replaced by the phosphate-buffered saline. The reaction was run for 20 min at 37°C, and stopped by the addition of 50 μl of 6 M HCl. Products were extracted with ethylacetate, and radioactivity incorporated into 6-hydroxymellein was determined after the separation by TLC as described previously in detail [8]. FAS activity was also determined radiochemically in a similar manner in which the assay mixture was idential to that for 6-hydroxymellein synthesis. Reaction mixtures were incubated for 1 h at 37°C, and fatty acids were extracted with hexane after converting them to methyl-ester with thionyl chloride [8].

2.5. *Gel-filtration and immunoblot analyses of the enzyme*

The enzyme preparation of 6-hydroxymellein synthesis obtained by ammonium sulfate precipitation (from 100 disks) was further purified by gel-filtration chromatography. The sample was applied onto a Toyopearl HW-55 F column (Tosoh, Tokyo, 2.0 × 80 cm), and was eluted with 10 mM sodium phosphate buffer (pH 7.5) containing 2% (v/v) mercaptoethanol. The active fraction obtained by gel-filtration was analyzed by immunoblotting probed with rabbit anti-spinach ACP serum which was prepared as described previously [12]. The sample was denatured by boiling for 1 min in the presence of 5% (v/v) mercaptoethanol and 2% (w/v) SDS, and was subjected to SDS-PAGE (5–20% gradient gel). Separated proteins were transferred to a nitrocellulose membrane in a semi-dry transfer cell (Bio-Rad, Transblot SD) according to the isntruction manual. After blocking with 3% dry fat milk in 20 mM Tris-HCl-buffered saline (pH 7.4), the membrane was incubated overnight with the antiserum diluted 100-fold with the buffered saline. Then it was washed and further incubated with peroxidase-labeled protein A (Seikagaku Kogyo, Tokyo) for 1 h. Cross-reacted proteins were monitored by an incubation with 4-chloro-1-naphthol.

Table I

Effect of the addition of *E. coli* ACP on FAS and 6-hydroxymellein synthetic activities in carrot

	Relative activity (%)
FAS	100
FAS + ACP	396
6-Hydroxymellein synthetic enzyme(s)	100
6-Hydroxymellein synthetic enzyme(s) + ACP	108

ACP was removed from the enzyme preparations by a fractionation with ammonium sulfate, and the effect of the addition of 10 μg ACP purified from *E. coli* on the respective enzymes was determined. Results were expressed as percentage to control without ACP.

3. RESULTS AND DISCUSSION

In higher plants and bacteria, ACP, a coenzyme of FAS, can be readily removed from other enzymes of FAS by ammonium sulfate fractionation. Fatty acid synthesis in carrot extracts thus depleted of ACP was markedly enhanced by the addition of 10 μg ACP purified from *E. coli*. Unlike FAS, however, 6-hydroxymellein synthetase activity of the extract was not influenced by ammonium sulfate fractionation and not stimulated by the addition of ACP (Table I). Nevertheless, 6-hydroxymellein synthetase activity was strongly inhibited by anti-spinach ACP serum but not by non-immune serum (Fig. 2). These findings suggest that the synthetase contains an ACP-like protein (or domain) that is tightly associated with the enzyme.

6-Hydroxymellein synthetic activity was further analyzed by gel-filtration chromatography on a Toyopearl HW-55 F column. The synthetic activity was not lost even after the chromatography, and a peak of the activity was found in the fraction of which the molecular mass was estimated to 136 kDa (Fig. 3). Proteins included in the fraction were denatured by boiling in the presence of SDS and mercaptoethanol, and were separated by SDS-PAGE (5–20% gradient gel). After transfer to a nitrocellulose membrane, they were probed with anti-spinach ACP serum. As shown in Fig. 4, the serum cross-reacted with a polypeptide (128 kDa) in

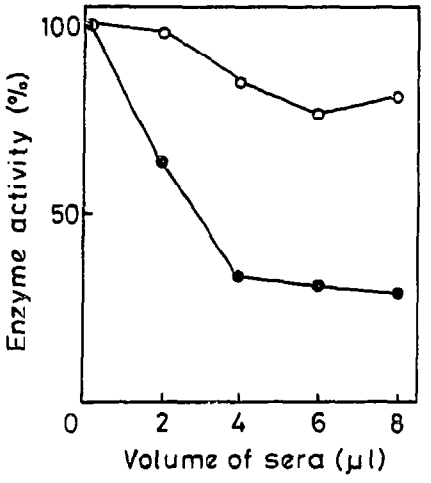

Fig. 2. Inhibition of biosynthetic activity of 6-hydroxymellein by anti-ACP serum. 6-Hydroxymellein synthetic activity was determined in the presence of various amounts of the antiserum (●), or non-immunized serum (○) in a total volume of 250 μl. Data were expressed as percentages to control in which sera were not included.

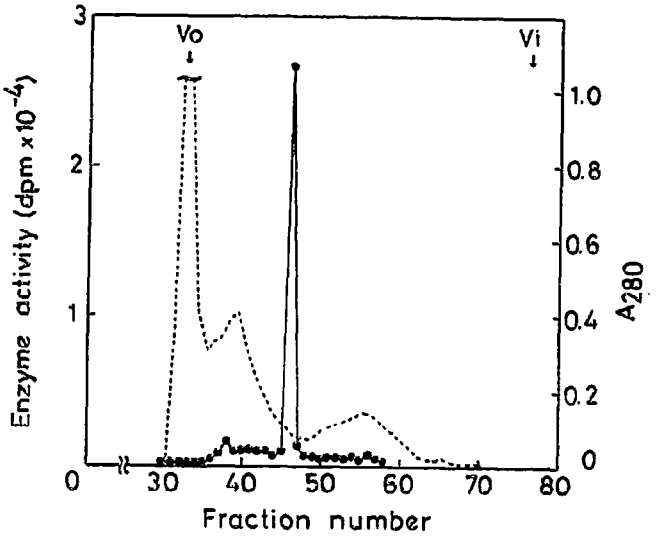

Fig. 3. Gel-filtration chromatography of 6-hydroxymellein synthetic activty on a Toyopearl HW-55 F column. The partially purified enzyme preparation was applied to the column and eluted with sodium phosphate buffer. Fractions of 2 ml were collected and assayed for synthetic activity (●). Proteins were monitored at 280 nm (--). Void (V_o) and inclusion (V_i) volumes were determined with Blue dextran and cyanocobalamin, and relative molecular mass of the active fraction was estimated with standard proteins (ferritin, 440 kDa; catalase, 232 kDa; aldolase, 158 kDa; bovine serum albumin, 66 kDa).

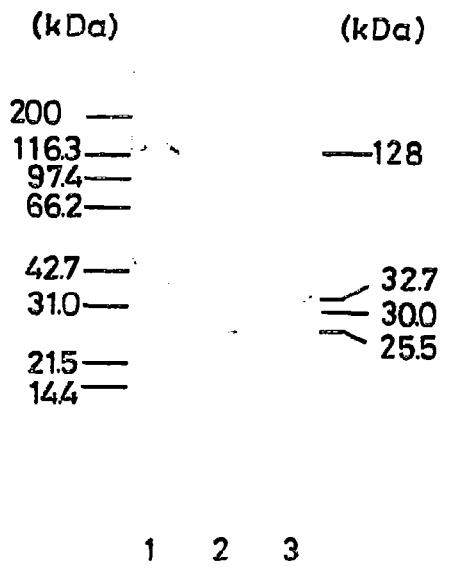

Fig. 4. Immunoblot analysis of 6-hydroxymellein synthetic activity. The activity fraction obtained by gel-filtration chromatography (136 kDa) was subjected to SDS-PAGE (5-20% gradient gel), and after transfer to a nitrocellulose membrane, proteins were probed with anti-ACP serum (lane 1). ACPs purified from *E. coli* (lane 2) and carrot (lane 3) were analyzed similarly. Molecular masses were estimated with standard proteins (myosin, 200 kDa; *E. coli* galactosidase, 116.3 kDa; rabbit muscle phosphorylase, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 42.7 kDa; bovine carbonic anhydrase, 31.0 kDa; soybean trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa).

the fraction. Coxon et al. [13] reported that 95% of ether extractable phenolics in elicitor-treated carrot roots was 6-methoxymellein, suggesting that polyketide synthetic enzymes other than 6-hydroxymellein synthetase would give, if any, very weak signals in the immunoblotting. Immunoreacted signals were also observed at the position of 25.5 kDa in purified *E. coli* ACP, and 32.7 and 30.0 kDa in partially purified carrot ACP. Two isoforms of ACP have been reported in spinach [14], castor bean and soybean [15].

Molecular mass of 6-hydroxymellein synthetic enzyme estimated by gel-filtration chromatography was quite similar to that of the peptide detected by the immunoblot analysis (136 and 128 kDa, respectively) indicating that the ACP-like protein in the enzyme was not dissociated in these treatments. These results strongly suggested that a functional structure corresponding to ACP in 6-hydroxymellein synthetic system binds covalently to the enzyme, and catalytic unit for biosynthesis of the compound consists of a single polypeptide chain with multifunctional domains. Since FAS of higher plants is known to be made up of separable catalytic units, present results indicate that two differently organized enzyme systems are included in carrot cells even though they catalyze many similar reactions such as acyl-CoA condensation and NADPH-dependent keto-reduction. Further characterization of 6-hydroxymellein synthetase is in progress in our laboratory.

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REFERENCES

[1] Leistner, E. (1981) in: The Biochemistry of Plants (Stumpf, P.K. and Conn. E. eds.) vol. 7, pp. 403-423, Academic Press, New York.
[2] Wakil, S.J., Stoop, J.K. and Joshi, V.C. (1983) Annu. Rev. Biochem. 52, 537-579.
[3] Dimroth, P., Walter, H. and Lynen, F. (1970) Eur. J. Biochem. 13, 98-110.
[4] Kurosaki, F. and Nishi, A. (1983) Phytochemistry 22, 669-672.
[5] Stoessl, A. and Stothers, J.B. (1978) Can. J. Bot. 56, 2589-2593.
[6] Condon, P. and Kuć, J. (1962) Phytopathology 52, 182-183.
[7] Kurosaki, F. and Nishi, A. (1988) FEBS Lett. 227, 183-186.
[8] Kurosaki, F., Kizawa, Y. and Nishi, A. (1989) Eur. J. Biochem. 185, 85-89.
[9] Kurosaki, F., Matsui, K. and Nishi, A. (1984) Physiol. Plant Pathol. 25, 313-322.
[10] Rock, C.O. and Cronan, J.E. (1980) Anal. Biochem. 102, 362-364.
[11] Laemmli, U.K. (1970) Nature 227, 680-685.
[12] Kato, M., Ozeki, Y., Suzuki, T., Yamada, T. and Yamada, M. (1989) Plant Cell Physiol. 30, 1129-1132.
[13] Coxon, D.T., Curtis, R.F., Price, K.R. and Levett, G. (1973) Phytochemistry 12, 1881-1885.
[14] Kuo, T. and Ohlrogge, J.B. (1984) Arch. Biochem. Biophys. 234, 290-296.
[15] Ohlrogge, J.B. and Kuo, T. (1985) J. Biol. Chem. 260, 8032-8037.