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PURIFICATION AND CHARACTERIZATION OF EMODINANTHRONE OXYGENASE FROM *ASPERGILLUS TERREUS*

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Key Word Index—*Aspergillus terreus*; biosynthesis; anthraquinone; emodinanthrone; emodin; oxygenase.

Abstract—Emodinanthrone oxygenase catalyses the fixation of molecular oxygen into emodinanthrone to give the anthraquinone emodin. The reaction proceeds without external electron donors. The enzyme activity was first detected in crude extracts of the fungus *Aspergillus terreus* IMI 16043 which produces the *seco*-anthraquinone, (+)-geodin. Emodinanthrone oxygenase from *A. terreus* was found to be membrane bound, could be solubilized by non-ionic detergents, and was activated by phospholipids. Although the instability and heterogeneity of the solubilized enzyme retarded its purification, emodinanthrone oxygenase was purified by chromatography on DEAE-cellulose, Hydroxyapatite, Q Sepharose, HPLC gel filtration, and Mono Q columns. The purified enzyme showed a single band with a M_r of 52000 on SDS–PAGE. The fact that the enzyme was inhibited by ferrous chelating reagents like *o*-phenanthroline in the presence of reducing reagents suggested that non-haem ferric iron is the catalytic cofactor of the enzyme. Based on these properties, a mechanism for the emodinanthrone oxygenase reaction is proposed.

INTRODUCTION

Polyketides are one of the most important groups of natural products in terms of biological functions and activities. A characteristic of compounds of polyketide origin is that oxygen atoms located at alternate carbons are derived from the carbonyl groups of acetate, malonate or other simple fatty acids. Emodin is a typical polyketide anthraquinone and the key intermediate in the biosynthesis of monomeric and dimeric anthraquinonoids [1], xanthones [2], and benzophenones [3, 4]. Of the five oxygen functional groups in emodin, all except the C-10 carbonyl oxygen are derived from the C_{16} β-polyketomethylene intermediate formed by condensation of one acetyl CoA molecule and seven malonyl CoA molecules. The first cyclization product is considered to be emodinanthrone, which is then oxidized to the anthraquinone emodin. Fixation of molecular oxygen into the anthraquinone moiety of averufin was observed by means of an *in vivo* $^{18}O_2$ feeding experiment and NMR analysis of the product [5]. The presence of emodinanthrone oxidizing activity to form emodin was first detected in our enzymological studies on (+)-geodin biosynthesis in *Aspergillus terreus* [6–8]. This anthrone oxidation is considered to be the common reaction in the biosynthesis of anthraquinonoids of polyketide origin [9]. In fact, all organisms tested which produce anthraquinonoids and related compounds showed significant anthrone oxygenase activities [7]. The derivation of the oxygen atom at C-10 of emodin from molecular oxygen was proved by an *in vitro* $^{18}O_2$ incubation experiment. The oxygenation reaction did not require any reducing reagents such as NADPH [7]. Thus, the enzyme was formally classified as an internal monooxygenase [10]. However, to date all the known enzymes of this type catalyse either the oxidative decarboxylation of substrates [11, 12] or hydroquinone epoxidation [13–15]. Emodinanthrone oxygenase, therefore, may be classified as a new type of internal monooxygenase.

(+)-Geodin, the first chlorinated compound isolated from fungi [16], possesses antibiotic activity [17], is a *seco*-anthraquinone and is biosynthesized from the anthraquinone emodin as shown in Fig. 1. We have purified the enzymes involved in its biosynthesis and characterized their molecular properties [6, 8, 18, 19]. To extend our enzymological studies further and to clarify the molecular properties of this new type monooxygenase which is involved in the formation of the quinone functionality of the anthraquinone emodin, the purification of emodinanthrone oxygenase from *A. terreus* was carried out.

RESULTS

Solubilization of emodinanthrone oxygenase

When the mycelia of *A. terreus* were homogenized with 100 mM Tris–HCl buffer, pH 7.5, containing 15% (v/v) glycerol, the total activity of emodinanthrone oxygenase

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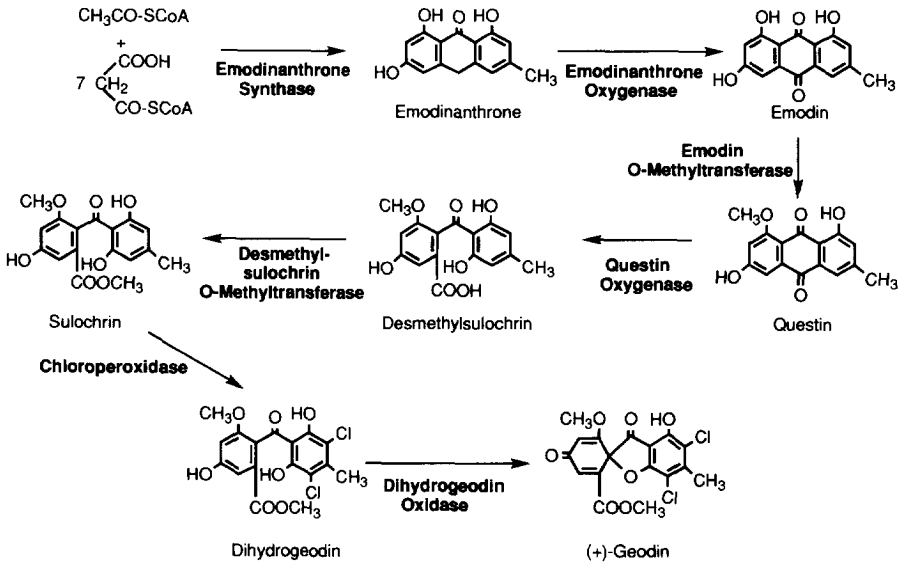


Fig. 1. Biosynthetic pathway for (+)-geodin in *Aspergillus terreus* IMI 16043.

in the crude extract was about three times higher than that obtained without glycerol in the extraction buffer. After ultracentrifugation at 100000 *g* for 60 min, more than 90% of emodinanthrone oxygenase activity was found in the microsomal fraction. Solubilization tests showed that emodinanthrone oxygenase could be solubilized efficiently by Lubrol PX or Triton X-100 each at 1% (v/v) concentration.

When the solubilized enzyme preparation with Lubrol PX was applied to a gel filtration column, a broad distribution of emodinanthrone oxygenase activity was observed from the void volume to later eluting low-M_r fractions. Also, isoelectric focusing of the enzyme preparations at different purification stages gave activity peaks with different p*I* values. These data indicated the complex or heterogeneous state of the solubilized enzyme. Although Lubrol PX solubilized the emodinanthrone oxygenase from *A. terreus* more efficiently than Triton X-100, Triton X-100 was used for the solubilization because the latter gave the relatively more homogeneous solubilized enzyme. For example, the enzyme activity showed a sharp peak around M_r 150000 on a gel filtration chromatography when Triton X-100 was used instead of Lubrol PX for solubilization.

Effect of lipids on emodinanthrone oxygenase

When the Lubrol PX solubilized enzyme preparation from *A. terreus* was applied to DEAE-cellulose column, the flow-through fraction could activate the emodinanthrone oxygenase which was eluted with 0.2 M NaCl. The activation effect of the flow-through fraction was not lost even if the fraction was boiled or digested with proteinase K, suggesting that the activator was a non-proteinaceous substance. As the sample loaded on to the DEAE-cellulose column was prepared from microsomes, lipids contained in the solubilized enzyme preparation

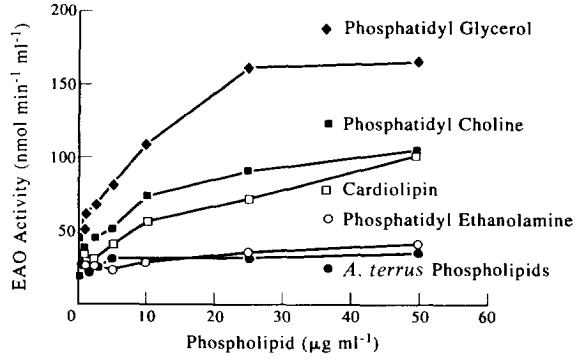


Fig. 2. Activation effects of phospholipids on emodinanthrone oxygenase. Emodinanthrone oxygenase activities in the presence of various phospholipids were measured and plotted. ◆, phosphatidyl glycerol; ▲, phosphatidyl choline; △, cardiolipin; ○, phosphatidyl ethanolamine; ●, *A. terreus* crude phospholipid extracts.

were assumed to be possible activation factors. Indeed, lipid extracts of the flow-through fraction prepared by the method of Floch *et al.* [20] activated emodinanthrone oxygenase, and crude phospholipid extracts obtained from the mycelia of *A. terreus* by the Bligh–Dyer method [21] had an apparent activation effect on the enzyme (data not shown). To confirm the effect of phospholipids, the ability of commercially available phospholipids to activate the enzyme was examined. As shown in Fig. 2, all phospholipids tested exhibited some activation effect. Among these, phosphatidyl glycerol was found to be the strongest activator. Hence, we concluded that lipids are the activation factors in the flow-through fraction from DEAE-cellulose column. Therefore, phosphatidyl choline was added when the *A. terreus* emodinanthrone oxygenase was assayed.

Table 1. Purification of emodinanthrone oxygenase from *A. terreus*

Purification step	Protein (mg)	Activity (nmol min^{-1})	Specific activity (nmol min^{-1} mg^{-1})	Recovery (%)
1% Triton X-100	26.8	40264	1504	100
DEAE-cellulose	16.5	16780	1018	41.7
1st Hydroxyapatite	10.4	13642	1317	33.9
Q Sepharose	9.07	2549	281	6.33
2nd Hydroxyapatite	2.11	1583	751	3.93
TSK G-3000 SW	1.13	376	334	0.93
Mono Q HR 5/5	0.052	117	2254	0.29

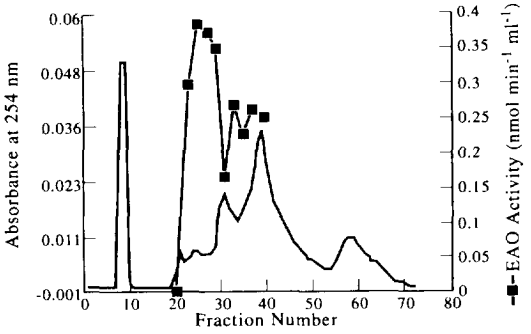


Fig. 3. Mono Q FPLC of emodinanthrone oxygenase from *A. terreus*. —, Absorbance at 254 nm; ■, emodinanthrone oxygenase activity (nmol/min^{-1} ml^{-1}).

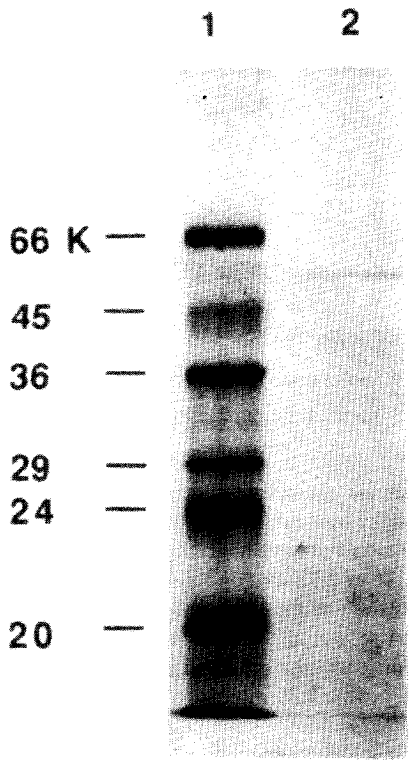


Fig. 4. SDS-PAGE analysis of the purified emodinanthrone oxygenase from *A. terreus*. Right lane, purified emodinanthrone oxygenase from *A. terreus*; left lane, M_r marker proteins. The gel was stained with silver reagent.

Purification of emodinanthrone oxygenase from A. terreus

Protocols for the purification of emodinanthrone oxygenase from *A. terreus* were developed and are described in detail in the Experimental. A typical purification is presented in Table 1. Two main problems encountered in the course of the enzyme purification were the instability and heterogeneity of the solubilized enzyme. Although emodinanthrone oxygenase activity appeared to be stable in the crude preparations, it became unstable after solubilization and was easily lost on chromatography. Also, a relatively broad distribution of enzyme activity was observed on most chromatographic procedures. As shown in Table 1, the specific activity decreased at each purification step, except for the Mono Q step, owing to loss of enzyme activity. Removal of the enzyme associated phospholipids, which seemed to be required for both activation and stabilization, by chromatographic procedures might be a reason for these phenomena.

Although emodinanthrone oxygenase activity was resolved into three peaks on Mono Q column chromatography (Fig. 3), possibly due to the heterogeneous state of the enzyme preparation, the main peak (fractions 22–28) gave a single band with M_r 52000 on SDS-PAGE (Fig. 4).

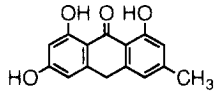
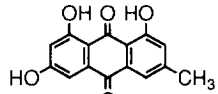
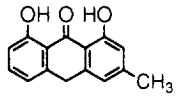
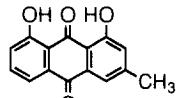
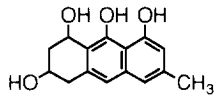
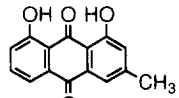
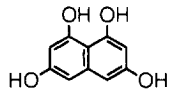
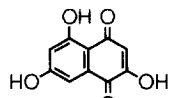
Substrate specificity

The substrate specificity of the emodinanthrone oxygenase was examined using chrysophanol anthrone (chrysarobin), germichrysone, 1,3,6,8-tetrahydroxynaphthalene and scytalone as substrates. Interestingly, chrysophanol anthrone was oxidized much more efficiently than emodinanthrone. But, germichrysone, a dihydro- equivalent of emodinanthrone, did not react at all. Also, naphthalene derivatives could not act as substrates for the enzymes (Table 2).

Emodinanthrone oxygenase cofactor requirement

Three kinds of cofactors, i.e. flavins, haems or metal ions, are required by oxygenases. It was important to find

Table 2. Substrate specificity of emodinanthrone oxygenase from *Aspergillus terreus*

Substrate	Product	Relative activity (%)
 emodinanthrone	 emodin	100
 chrysophanolanthrone	 chrysophanol	211
 germichrysone	 chrysophanol	0
 1,3,6,8-tetrahydroxynaphthalene	 flaviolin	0

out the cofactor requirement of emodinanthrone oxygenase in order to establish the reaction mechanism.

Flavin or haem groups have characteristic absorption maxima at 380 and 450 nm or 350 to 650 nm, respectively [10]. However, the final enzyme preparation from *A. terreus* showed no absorption above 300 nm. Flavin cofactor removal by the modified procedure of Manstein and Pai [22] caused no inhibition on the enzyme. Also, haemoprotein inhibitors such as metyrapone and SKF 525A had no effect on emodinanthrone oxygenase activity (Table 3). These facts suggested that emodinanthrone oxygenase does not contain haem or flavin prosthetic groups.

The addition of metal chelating reagents to the assay mixture had no apparent effect on the enzyme activity (Table 3). *o*-Phenanthroline, a potent metal chelating reagent, had no inhibitory effect on the enzymes even when preincubated for 1.5 hr. 2-Mercaptoethanol alone also caused only slight inhibition. However, strong inhibition was observed by *o*-phenanthroline in the presence of 2-mercaptoethanol (Table 4). Similar inhibition was observed in the case of lipoxygenases which require ferric iron as cofactor [23, 24].

Table 3. Effect of inhibitors on emodinanthrone oxygenase activity

Reagent	Concentration (mM)	EAO inhibition (%)
Ancymidol	1.0	0
L-Ascorbic acid	1.0	33.6
p-Chloromercuriphenyl-sulphonic acid	1.0	74.5
Cytochrome *c*	0.05	51.1
Diethyldithiocarbamate	1.0	0
N-Ethylmaleimide	0.1	82.0
Pentachlorophenol	1.0	54.2
Potassium cyanide	1.0	0
Potassium ferricyanide	0.1	93.4
SKF 525A	1.0	0
Sodium azide	1.0	0
Superoxide dismutase	0.07 (mg ml^{-1})	0
Uniconazole	1.0	0

Steady-state kinetics

Steady-state kinetic analysis of the *A. terreus* emodinanthrone oxygenase was carried out assuming that the O_2 concentration remained constant in the assay mixtures. The K_m value for emodinanthrone was calculated to be 6.8×10^{-6} M from double reciprocal plots.

DISCUSSION

The purification and characterization of emodinanthrone oxygenase from *A. terreus* has been carried out.

Table 4. Effect of iron chelating reagents on emodinanthrone oxygenase activity

Reagent	Concentration (mM)	EAO inhibition (%)
α,α'-Dipyridyl	1.0	0
o-Phenanthroline	1.0	0
**o*-Phenanthroline	1.0	0
*2-Mercaptoethanol	2.0	14.5
*2-Mercaptoethanol + *o*-phenanthroline	2.0 + 1.0	63.3

*Enzyme activity was measured after preincubation with the reagent for 1.5 hr at 4°.

The enzyme was found to be membrane bound and was very unstable after solubilization. The heterogeneous state of the solubilized enzyme hindered its purification. For example, isoelectric focusing of the enzyme preparations at different purification stages gave activity peaks with different p*I* values. There was a tendency for the p*I* to increase with each chromatographic step. Even in the final purification step (Mono Q column), minor activity peaks were observed. However, the main peak appeared to be a single band on SDS–PAGE (Fig. 4). These data suggested that phospholipids associated with the solubilized enzyme caused the enzyme heterogeneity, and their removal by chromatographic procedures led to a large loss of activity and higher p*I*s.

Emodinanthrone oxygenase incorporates one atom of atmospheric oxygen into emodinanthrone to form emodin without any requirement for an external electron donor. Generally, oxygenases are classified into monooxygenases and dioxygenases. Monooxygenases are further categorized into two classes, i.e. internal monooxygenases and external monooxygenases, according to their electron donor requirements [10]. One group of internal monooxygenase is comprised of lactate monooxygenase and amino acid oxidases [11, 12]. These are flavoproteins and catalyse the oxidative decarboxylation of their substrates. Others catalyse epoxidation of hydroquinones, e.g. the two hydroquinone monooxygenases (epoxidizing) from *Streptomyces* LL-C10037 and MPP 3051 [14], the mammalian dihydrovitamin K-dependent carboxylase [15, 25], and a particulate preparation from the fungus *Penicillium patulum* [13], all of which are reported not to have flavins or haems as their prosthetic groups. Epoxidation of hydroquinones by these enzymes requires only molecular oxygen. Substrate hydroquinones serve as the reducing equivalents. All of the latter type of enzymes require metal ions or can be activated by metal ions.

Recently, Shen and Hutchinson reported the purification and characterization of tetracenomycin (Tcm) F1 monooxygenase from *Streptomyces glaucescens* WMH1068 [26]. This enzyme catalyses the oxidation of the naphthacenone Tcm F1 to the naphthacenequinone Tcm D3 in the biosynthesis of the anthracycline antibiotic Tcm C. The Tcm F1 monooxygenase only requires molecular oxygen for activity and can be classified as an internal monooxygenase. The Tcm F1 monooxygenase catalyses a very similar reaction to that of emodinanthrone oxygenase, but does not possess any of the prosthetic groups of known monooxygenases.

Inhibition by *o*-phenanthroline in the presence of 2-mercaptoethanol was reported for soybean lipoxygenase [23, 24]. Lipoxygenases are dioxygenases which catalyse the hydroperoxidation of polyunsaturated lipids containing a *cis*-1,*cis*-4-pentadiene moiety. These enzymes require ferric ion as a cofactor and incorporate one molecular oxygen into the substrate. The ferric iron is the active form and is strongly bound to the enzyme. It can only be removed from the enzyme after reduction to the ferrous state or by denaturation [27–29]. The mechanism of the lipoxygenase reaction has been the subject of much discussion with radical and organometallic species proposed as viable intermediates [27, 30–33]. The recent observation of enzyme-bound pentadienyl and peroxy radicals in purple lipoxygenase supported the radical mechanism of oxygenation of polyunsaturated fatty acid by lipoxygenase [34]. This mechanism, similar to that of the autoxidation of polyunsaturated fatty acids [35], proposes that the active site ferric iron Fe^{3+} oxidizes the 1,4-diene unit of the substrate to a pentadienyl radical, which reacts smoothly with molecular oxygen, giving a peroxy radical [36]. Reduction by the ferrous iron Fe^{2+} would result in the peroxide anion.

Mechanistically, the reaction catalysed by emodinanthrone oxygenase is considered to be similar to that of lipoxygenases. In both cases, the enzyme activity is not inhibited by Fe^{2+} chelating reagents but can be strongly inhibited in the presence of reducing reagents such as 2-mercaptoethanol, indicating ferric ion is involved in the enzyme reaction. Assuming that the ferric iron is a prosthetic group of emodinanthrone oxygenase, it is possible to propose a reaction mechanism for the reaction of emodinanthrone oxygenase (Fig. 5). The hydrogen at C-10 of emodinanthrone is very active because of the

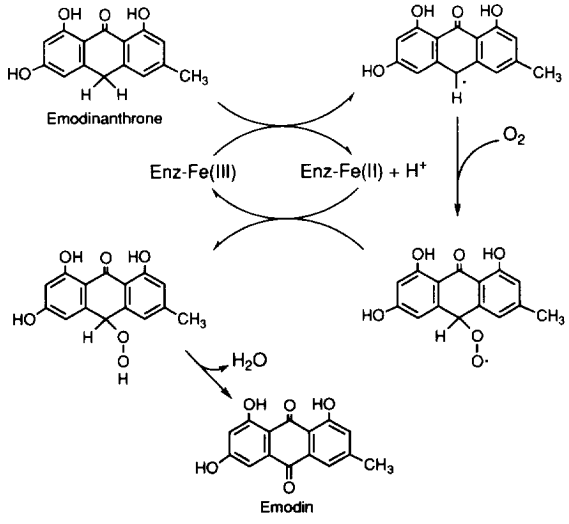


Fig. 5. Proposed mechanism of emodinanthrone oxygenase reaction.

electron withdrawing effect of the C-9 carbonyl and benzene rings and subsequent stabilization of the resultant radical by delocalization over the benzene rings. The reaction is initiated by the removal of hydrogen at C-10 of emodinanthrone to form the emodinanthrone radical with concomitant reduction of the active ferric enzyme to the ferrous state. Molecular oxygen then binds to the emodinanthrone radical–enzyme complex. To the peroxy radical thus formed, one electron transfer from the ferrous iron occurs to give the emodinanthrone peroxy anion. Then, the free active enzyme is regenerated and the emodinanthrone peroxide is formed. The emodinanthrone peroxide is structurally very unstable and decomposes to form emodin.

EXPERIMENTAL

Fungal strain and growth condition. Aspergillus terreus IMI 16043 was maintained on potato-dextrose agar slants. Modified Czapek–Dox medium [glucose 50 g, $NaNO_3$ 2 g, K_2HPO_4 1 g, KCl 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.01 g, $ZnSO_4 \cdot 7H_2O$ 0.01 g, $CuSO_4 \cdot 5H_2O$ 0.005 g, malt extract (Difco) 0.5 g, yeast extract (Difco) 0.2 g, distilled water 1 l, adjusted to pH 6.8 before autoclaving] was used for surface cultures which were grown for 7 days at 28°. Mycelia were collected by filtration and washed with distilled water. They could be stored at −80° for several months without loss of enzyme activity.

Preparation of cell-free extract and enzyme solubilization. The frozen mycelia were thawed and homogenized in a Waring blender with 50 mM Tris–HCl buffer, pH 7.5, containing 15% (v/v) glycerol. After standing at 0° for 20 min, the homogenate was filtered through 5 layers of gauze and then centrifuged at 12 000 *g* for 30 min to remove unbroken cells and cell debris. The cell-free extract thus obtained was then subjected to ultracentrifugation at 100 000 *g* for 60 min at 4°. The resultant microsomal precipitates were suspended in 50 mM Tris–HCl buffer, pH 7.5, containing 15% (v/v) glycerol and the detergent for solubilization, and then stirred gently for 1 hr at 4°. The solubilized enzyme was recovered in the supernatant by ultracentrifugation at 100 000 *g* for 60 min.

Purification of emodinanthrone oxygenase from A. terreus. All steps were carried out at 4° except for the HPLC steps which were performed at room temp. Buffer I [50 mM Tris–HCl buffer, pH 7.5, containing 15% (v/v) glycerol and 0.2% Triton X-100], buffer II [30 mM K-Pi buffer, pH 7.5, containing 15% (v/v) glycerol and 0.2% Triton X-100], and buffer III [50 mM Tris–HCl buffer, pH 7.5, containing 15% (v/v) glycerol and 0.1% Triton X-100] were used.

The 1.0% Triton X-100 solubilized enzyme preparation (46 ml) obtained from 290 g mycelia was applied to a DEAE-cellulose column (12.5 × 4 cm) equilibrated with buffer I. The enzyme was eluted with 0.25 M NaCl in buffer I. The active fractions collected were applied to a Hydroxyapatite column (9 × 3 cm) equilibrated with buffer I. The buffer II eluate from the Hydroxyapatite column was applied to a Q Sepharose column (14 × 2.5 cm) equilibrated with buffer I. Elution was carried out with 900 ml of a linear gradient from 0 to 0.25 M NaCl in buffer I. Active fractions (470 ml) were concd by adsorption and stepwise elution from a Hydroxyapatite column (3.5 × 2.5 cm). After further concn by Centriprep-10, suitable portions were applied to a TSK G-3000 SW HPLC gel filtration column (600 × 7.5 mm) equilibrated with buffer III. The active fractions were concd by Centriprep-10 and then applied to a Mono Q HR 5/5 column which had been equilibrated with buffer III. Elution was carried out with a linear gradient from 0 to 0.15 M NaCl in buffer III. This final preparation of active enzyme was stored and used for its characterization.

Emodinanthrone oxygenase asssay. Emodinanthrone oxygenase activity was assayed spectrophotometrically by measuring the increase of absorbance at 490 nm at 30°. The standard assay mixture contained 1.8 ml of 0.5 M K-Pi buffer, pH 6.5, 200 nmol emodinanthrone dissolved in 1 ml ethyleneglycol monomethylether, and a suitable amount of enzyme preparation, in a total vol. of 2.9 ml. The reaction was initiated by the addition of enzyme soln together with 50 μl phosphatidyl choline (5.8 mg ml^{-1}). The $\Delta\varepsilon$ of the product (emodin) and the substrate (emodinanthrone) at 490 nm was 6.35×10^3 M^{-1} cm^{-1}.

SDS–PAGE. Performed on 12% (w/v) acrylamide separating gel with 4% (w/v) stacking gel according to the procedures of Laemmli [37]. Gels were fixed and stained with silver reagents by the procedure of Morrisey [38] using a silver stain kit from Kanto Chemical Co. Inc.

Isoelectric focusing. Carried out in a Rotofor cell (Bio-Rad) according to the instruction manual of the manufacturer. A mixture of the enzyme preparation, 2% (w/v) pharmalyte, 0.1% detergent used for the enzyme solubilization, and 15% (v/v) glycerol in a total vol. of 45–50 ml was loaded into a Rotofor cell and the focusing was carried out at 10 W constant power at 4° for 4 hr. Enzyme preparations containing ion strengths higher than 0.5 mmol NaCl were desalted by passing through PD-10 columns before loading. After focusing for 4 hr, 20 fractions were harvested and their enzyme activities and pH values were measured.

Determination of protein concentration. Protein concentrations were determined by the modified Lowry's procedure of Bensadoun and Weinstein [39] using bovine serum albumin as the standard.

HPLC analysis for substrate specificity study. Reaction products were analysed by means of the following HPLC conditions. ODS-80 Tm column (150 × 4.6 mm) developed with a 0–90% (v/v) MeCN linear gradient containing 2% (v/v) HOAC in 45 min at a flow rate of 1.0 ml min^{-1}. Elution was monitored at 280 nm.

Chemicals. Triton X-100 and Lubrol PX were purchased from Nacalai Tesque and Sigma, respectively; DEAE-cellulose (DE-52) from Whatman Chemical Separation, Ltd; Q Sepharose, Chelating Sepharose, Mono Q HR 5/5 and PD-10 columns from Pharmacia-LKB; Hydroxyapatite from Seikagaku Corp; Toyopearl HW-55 and TSK G-3000 SW, and ODS-80 Tm columns from Tosoh; cardiolipin, phosphatidyl glycerol, and phosphatidyl ethanolamine from Sigma; phosphatidyl

choline from Wako Pure Chemical Industries Ltd; pharmalyte from Pharmacia-LKB; Centriprep concentrators from Amicon; molecular weight markers for SDS–PAGE from Sigma and those for gel filtration from Oriental Yeast Co., Ltd.

Emodinanthrone was prepared by reduction of emodin as follows. The reaction mixture containing emodin and excess HI in glacial HOAC was refluxed in the dark for 3 hr. After cooling, the reaction mixture was poured into H_2O and the I_2 formed was decomposed with sodium metasulphite. The emodinanthrone formed was extracted with Et_2O and recrystallized from Me_2CO.

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