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Macrophomate Synthase: Characterization, Sequence, and Expression in *Escherichia coli* of the Novel Enzyme Catalyzing Unusual Multistep Transformation of 2-Pyrones to Benzoates[1]

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***Macrophoma commelinae* isolated from spots on leaves of *Commelina communis* has the ability to transform 5-acetyl-4-methoxy-6-methyl-2-pyrone (1) to 4-acetyl-3-methoxy-5-methylbenzoic acid (macrophomic acid, 2). This biotransformation includes the condensation of the 2-pyrone ring with a C3-unit precursor to form a substituted benzoic acid. We optimized conditions for induction of enzyme activity in *M. commelinae*, identified oxalacetate as a C3-unit precursor with cell extract, and purified the novel enzyme, macrophomate synthase. Oxalacetate inhibited the enzyme activity at a concentration higher than 5 mM, and magnesium chloride stimulated the enzyme activity. Kinetic analyses gave K_m of 1.7 mM for 1 at 5 mM oxalacetate, K_m of 1.2 mM for oxalacetate at 5 mM 1, and k_{cat} of 0.46 s^{-1} per subunit. Pyruvate was a weak substrate, with K_m of 35.2 mM and k_{cat} of 0.027 s^{-1} at 5 mM 1. We cloned and sequenced a cDNA encoding the macrophomate synthase. The cDNA of 1,225 bp contained an open reading frame that encoded a polypeptide of 339 amino acid residues and 36,244 Da, the sequence of which showed no significant similarity with known proteins in a homology search with BLAST programs. Transformed *E. coli* cells carrying the cDNA encoding the mature protein of macrophomate synthase overproduced macrophomate synthase under the control of the T7 phage promoter induced by IPTG. The purified enzyme showed the same values of K_m and optimum pH as the native macrophomate synthase.**

Key words: cDNA structure, *Macrophoma commelinae*, macrophomate synthase, overexpression, oxalacetate, 2-pyrone.

The fungal strain *Macrophoma commelinae* (*1*), isolated from spots on leaves of *Commelina communis*, produced various 2-pyrone derivatives (*2*). From biosynthetic studies of these metabolites, Sakurai *et al.* (*3*) reported that the fungus converted 5-acetyl-4-methoxy-6-methyl-2-pyrone (**1**) and an unidentified C3-unit precursor to 4-acetyl-3-methoxy-5-methylbenzoic acid (macrophomic acid, **2**) (*4*). Interestingly, this metabolite **2** was found in culture of *M. commelinae* as a minor constituent, but the precursor **1** was not detected in a number of 2-pyrone metabolites (*4*). In further study on this novel type of aromatic ring formation, Sakurai *et al.* (*3*, *5*) elucidated that the whole carbon skeleton of **1** was converted to **2** except for the loss of C-2 as a carbon dioxide (Fig. 1). For this unusual conversion, they proposed stepwise anion-based condensation between **1** and phosphoenolpyruvate (*5*). Interestingly, they also found that the fungus converted a broad range of 2-pyrones to the corresponding benzoates (*5*).

Our research group independently reported the isolation of phytotoxic pyrenocines A (**3**) (*6*, *7*) and B (**5**) and their benzoate derivatives, pyrenochaetic acids A (**4**) (*8*) and B (**6**), in the culture of the causal fungus of onion pink root disease, *Pyrenochaeta terrestris*. The interesting observation shown above prompted us to study this unusual transformation. In previous papers, we established intact incorporation of [*U*-^{13}C]glycerol, and proposed that **2** was constructed from **1** and phosphoenolpyruvate by concerted C-C bond formation between **1** and phosphoenolpyruvate *via* the Diels-Alder reaction (*9*, *10*).

In a preliminary study, we identified of oxalacetate as a substrate for the C3-unit precursor and purified the extraordinary enzyme macrophomate synthase which mediates unusual five-step conversion from **1** to **2** as a preliminary form (*11*). Subsequently, we investigated the substrate diversity of this enzyme using various 2-pyrone analogs (*12*). Herein, we describe purification and characterization of the macrophomate synthase from *M. commelinae*, and identification of the purified enzyme and by expression of the cDNA in *E. coli*.

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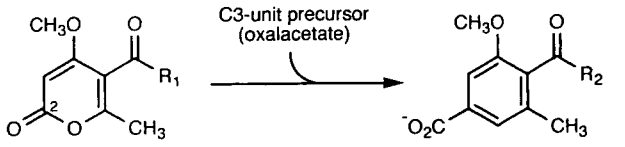
Abbreviations: BSA, bovine serum albumin; DIG, digoxygenin; IPTG, isopropyl-1-thio-β-D-galactoside; PAGE, polyacrylamide gel electrophoresis; PIPES, piperazine-1,4′-bis(2-ethanesulfonate); PVDF, polyvinylidene difluoride.



1 $R_1 = CH_3$
3 $R_1 = CH{=}CHCH_3$
5 $R_1 = CH_2CHOHCH_3$

2 $R_2 = CH_3$
4 $R_2 = CH{=}CHCH_3$
6 $R_2 = CH_2CHOHCH_3$

Fig. 1. **Enzymatic conversion of 2-pyrones to benzoates.**

MATERIALS AND METHODS

Growth of M. commelinae and Induction of Macrophomate Synthase—M. commelinae IFO 9570 was cultured under stirring (200 rpm) at 30°C for 72 h in a jar-fermenter (5 liters, EYELA M-160) containing modified Pfeffer medium consisting of corn steep liquor (100 g), yeast extract (10 g), sucrose (200 g), NH_4NO_3 (40 g), KH_2PO_4 (20 g), $MgSO_4{\cdot}7H_2O$ (10 g), $FeSO_4{\cdot}6H_2O$ (4 mg), and tap water (4 liters). The pH of the medium was adjusted to 7.0 with 5 M KOH. Mycelial cells (fresh weight 1 kg) were collected by filtration and washed with 500 ml of 50 mM potassium phosphate, pH 7.2, containing 60 mM KCl. The cells were suspended in 4 liters of the same buffer, and 20 ml of 200 mM **1** in *N,N*-dimethylformamide was added to give a final concentration of 1 mM. The suspension was incubated at 30°C for 3 h with stirring, then filtered.

Assay of Macrophomate Synthase—The enzyme in 100 µl of 50 mM piperazine-1,4′-bis(2-ethanesulfonate) (PIPES) containing 5 mM $MgCl_2$, pH 7.2, was preincubated with 5 µl of 0.1 M **1** in *N,N*-dimethylformamide at 30°C for 5 min. The reaction was initiated by the addition of 5 µl of 0.1 M oxalacetate, and stopped by the addition of 100 µl of 2-propanol after 10 min of incubation at 30°C. The enzyme solution boiled at 100°C for 10 min was used as a control. Reaction product **2** was measured by capillary electrophoresis (P/ACE 5000, Beckman) with a capillary column [ϕ75 µm × 57 cm, free zone 20 nl electrophoresis, 100 mM borate buffer, pH 8.1, and 50 mM SDS, constant current 90 µA, temp. 15°C, UV 200 nm, 5 nl injection, retention time (min) **1** 4.1, **2** 4.7]. In the reaction with **3** and **5** the same analytical conditions were used [retention time (min) **3** 6.8, **5** 5.4]. Concentration of protein was measured by absorbance at 280 nm using bovine serum albumin (BSA) as a standard.

Purification of Macrophomate Synthase—For preparation of cell extract, 1 kg of wet cells was suspended in 1 liter of 50 mM PIPES, pH 7.2, containing 60 mM KCl and 4% poly (ethylene glycol) #6,000, and homogenized by mortar and pestle at 4°C. The homogenate was centrifuged at 8,000 ×*g* for 60 min, and the supernatant (1.8 liters) was used for enzyme purification.

The cell extract was applied to a column of DEAE Sepharose Fast Flow (ϕ60 × 80 mm, Pharmacia) that had been equilibrated with 50 mM PIPES, pH 7.2, containing 30% glycerol. The column was washed with the same buffer, and proteins were eluted with the buffer containing 0.5 M KCl. The active fraction was dialyzed against 50 mM PIPES, pH 7.0, containing 30% glycerol. The enzyme solution was centrifuged at 17,000 ×*g* for 60 min, and the supernatant was filtered with an EB-Disk 13 (Cica).

The enzyme solution (150 ml) was applied to a column of HiLoad 26/10 Q Sepharose High Performance (ϕ 26 × 100 mm, Pharmacia) that had been equilibrated with 50 mM PIPES, pH 7.0, containing 30% glycerol. The column was washed with the same buffer, and proteins were eluted with a linear gradient of zero to 1 M KCl.

The enzyme solution (27 ml) was applied to a column of Butyl Sepharose 4 Fast Flow (ϕ5 × 50 mm, Pharmacia) which had been equilibrated with 50 mM PIPES, pH 7.0. The column was washed with the same buffer, and the enzyme was eluted with water.

Molecular Mass Measurement—Molecular mass was estimated by SDS-PAGE (*13*) and by gel filtration chromatography (YMC-Pack Diol-200, YMC). Standard proteins were phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, and soybean trypsin inhibitor for the SDS-PAGE, and myosin, β-galactosidase, phosphorylase *b*, BSA, and ovalbumin for the gel filtration.

Protein Sequence Analysis—The enzyme solution (4 µg/4 ml water) was blotted on a polyvinylidene difluoride (PVDF) membrane with a ProSpin cartridge (Applied Biosystems). The PVDF membrane was removed from the cartridge and analyzed with an Applied Biosystems Protein Sequencer 492A.

Enzymatic Digestion for Protein Sequence Analysis—The purified enzyme (50 pmol) was denatured in 100 µl of 6 M guanidine-HCl containing 0.3% EDTA at room temperature for 8 h, mixed with 1.5 µl of 14.5 µM 4-vinylpyridine, and left at room temperature for 1 h. The denatured and modified protein was dialyzed against 100 ml of 20 mM Tris-HCl, pH 9.0, and incubated with lysylendpeptidase (Wako) (1:100, w/w) at 37°C for 15 h.

Separation of Peptides—The digests were separated into peptides by reverse-phase HPLC (Nanospace SI-1, Shiseido) on a column of Capcell Pak C_{18} UG120 (5 µm, ϕ1.5 × 150 mm, Shiseido) with a gradient of acetonitrile in 0.1% trifluoroacetic acid. The isolated peptides were analyzed with an Applied Biosystems Protein Sequencer 492 A.

Construction of a cDNA Library—The mycelial cells were suspended in 100 ml of the same buffer, and 0.5 ml of 200 mM **1** in *N,N*-dimethylformamide was added to give final concentration of 1 mM. The suspension was incubated at 30°C for 2 h (Fig. 2B) with stirring, then filtered. The mycelia were ground in liquid nitrogen with a mortar and pestle. The resultant powder was put in a 50-ml tube, and total RNA was extracted using Isogen (Nippon Gene). The poly-$(A)^+$ RNA was purified by using Oligotex™-dT30 (super) (TaKaRa). To construct a cDNA library, 250 ng of double-stranded cDNA was synthesized from 10.2 µg of poly$(A)^+$ RNA by using the ZAP-cDNA Gigapack III Gold Cloning Kit according to the manufacturer's protocols (Stratagene). Synthesized cDNAs were ligated into an *Eco*RI–*Xho*I–predigested and dephosphorylated ZAP cloning vector, and packaged with Gigapack III Gold packaging extracts (Stratagene). A library of 2.4×10^6 recombinants was screened by plaque hybridization methods (*14*).

Preparation of a Probe—Two oligonucleotide primers, sense primer 5′-GA(A/G)CA(A/G)CCIGA(A/G)(C/T)TICA(C/T)GCIAA(A/G)GCICCITA(C/T)(A/C)G-3′ and the antisense primer 5′-AC(C/T)TC(C/T)TCIGCIGCICC(A/G)TCIACIGT-(C/T)TC(A/G)TC-3′, were designed using the peptide sequences E-Q-P-E-L-H-A-K-A-P-Y and D-E-T-V-D-G-A-A-E-E-V, respectively. The PCR product from these primers and

the cDNA library was labeled with digoxygenin (DIG) using a DIG DNA Labeling and Detection Kit (Boehringer Mannheim) and used as a probe.

Sequence Analysis of cDNA—The cDNA fragments were sequenced with an automated DNA sequencer (Model 4000L, LI-COR). Computer analyses of the DNA sequence and amino acid sequence were done with DNASIS software (Hitachi Soft Engineering) and BLAST programs (*15*).

Construction of Expression Vector—To insert the open reading frame of the macrophomate synthase into an expression vector, PCR primers 5′-CACAACACCGTCCATATGGCAAAGTCC-3′ and 5′-GAAGCGGCCGCAAAGTCTAAGCCTTG-3′ were designed to create *Nde*I and *Not*I sites. The *Nde*I and *Not*I fragment of the PCR product was inserted into the *Nde*I–*Not*I site of pET30b containing T7 phage promoter to construct plasmid pETMS. The pETMS plasmid was introduced into *E. coli* BL21 (DE3) (Stratagene), and the transformant, *E. coli*–MS was selected.

Purification of Macrophomate Synthase from E. coli—Cells from 200 ml of *E. coli*–MS culture were washed with 50 mM PIPES, pH 7.2, containing 30% glycerol, and homogerized with a French Press in 20 ml of the same buffer. The cell extract was obtained by centrifugation at 15,000 ×*g* for 60 min and put on a column of Butyl Sepharose 4 Fast Flow (5 × 50 mm, Pharmacia) equilibrated with 50 mM PIPES, pH 7.0. The column was washed with the same buffer, and the enzyme was eluted with water.

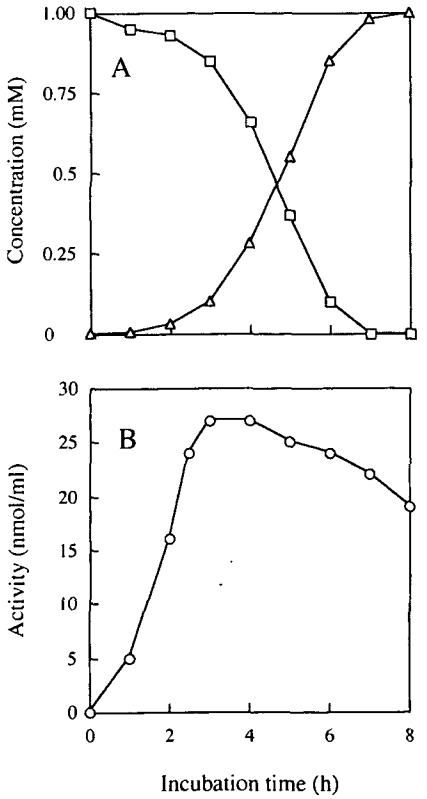


Fig. 2. **Induction of the macrophomate synthase.** A: Mycelial cells grown in the modified Pfeffer medium were incubated in 50 mM potassium phosphate, pH 7.2, containing 60 mM KCl and 1 mM **1** at 30°C as described in "MATERIALS AND METHODS." Concentrations of **1** (□) and **2** (△) in the buffer were measured. B: Mycelial cells incubated with 1 mM **1** for various periods were collected by filtration and homogenized in 50 mM potassium phosphate, pH 7.2, containing 60 mM KCl. The extract obtained was assayed for the activity forming **2** from 1 mM **1** by incubation for 2 h at 30°C.

RESULTS

Induction of Macrophomate Synthase—On incubation with 1 mM **1** as described in "MATERIALS AND METHODS," mycelial cells of *M. commelinae* showed a sigmoidal curve of the formation of **2** from **1** (Fig. 2A), and **1** was converted into **2** almost completely in 8 h. Activity of the formation of **2** from **1** was measured with the extract of the cells incubated with 1 mM **1** and found to reach maximum in 3 h (Fig. 2B). These results indicate that the enzyme catalyzing the formation of **2** from **1** is induced by the addition of the substrate **1**. Higher concentrations of **1** than 1 mM in the medium did not increase the maximum enzyme activity induced in 3 h. Therefore, incubation conditions for enzyme induction were optimized as follows: 50 mM potassium phosphate buffer, pH 7.2, containing 60 mM KCl and 1 mM **1**, at 30°C for 3 h.

TABLE I. **Effects of various compounds on the formation of 2 by cell extract of *M. commelinae*.** Extracts from the induced cells were incubated with 1 mM **1** and 5 mM compound at 30°C for 2 h.

Compound (5 mM)	Relative activity
None	100
L-alanine	99
D-alanine	97
L-serine	103
D-serine	107
Lactate	85
Pyruvate	396
Phosphoenolpyruvate	112
D-(+)-2-Phosphoglycerate	122
D-(−)-3-Phosphoglycerate	92
DL-Glyceraldehyde 3-phosphate	94
Oxalacetate	4,090
L-Malate	108

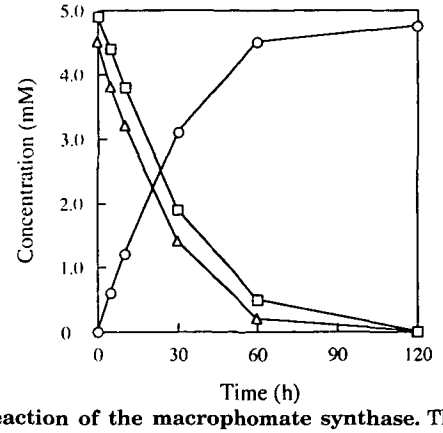


Fig. 3. **Reaction of the macrophomate synthase.** The enzyme partially purified by DEAE Sepharose and Q Sepharose chromatographies was incubated at 30°C, pH 7.0, containing 5 mM **1**, 5 mM oxalacetate, and 5 mM $MgCl_2$. Concentrations of **1** (□) and **2** (○) were measured as described in "MATERIALS AND METHODS," and that of oxalacetate (△) was measured with malate dehydrogenase and NADH.

Reaction of Macrophomate Synthase—According to the results of Sakurai *et al.* (*5*) and Oikawa *et al.* (*9*), various C3-unit precursors were tested for the formation of **2** from **1** by the cell extract from the induced *M. commelinae*. The enzyme activity was increased by addition of several compounds, as shown in Table I, and the highest activity was obtained with oxalacetate in the presence of 5 mM $MgCl_2$. Activities with all compounds except oxalacetate were apparently lost after anion exchange chromatography of the cell extract. This observation indicated that the cell extract contained enzymatic activities converting the compounds in Table I to oxalacetate and these were removed in the first chromatography step. The stoichiometric relationship between increase of **2** and decrease of **1** and oxalacetate is shown in Fig. 3. Consequently, the stoichiometry of the reaction catalyzing the macrophomate synthase was as follows: **1** + oxalacetate → **2** + $2CO_2$ + H_2O. The substrate, oxalacetate, was also an inhibitor of the enzyme activity at concentrations higher than 5 mM (data not shown).

Purification of Macrophomate Synthase—The purification process involving a series of chromatographies is summarized in Table II. Macrophomate synthase was eluted with a major peak from HiLoad 26/10 Q Sepharose HP. The peak fraction showed one major band on SDS-PAGE (*13*) and a few minor bands. Hydrophobic column chromatography with Butyl Sepharose was used to remove the minor components, and macrophomate synthase was eluted not with 50 mM PIPES, pH 7.0, but with water. The resultant enzyme fraction exhibited a single band on SDS-PAGE as shown already (*11*). Native PAGE (*16*) of the enzyme fraction exhibited a single band which corresponded to the gel section of the macrophomate synthase activity (data not shown). The enzyme in water was stored at 4°C for at least 3 months without loss of the activity. The molecular mass of the purified enzyme was approximately 40 kDa by SDS-PAGE and 90 kDa by gel filtration, indicating the enzyme to be a dimer. Amino acids analysis of about 100 pmol of the purified enzyme gave a single N-terminal sequence by

TABLE II. **Purification of the macrophomate synthase from *M. commelinae*.**

Step	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min/mg)	Yield (%)
Cell extract	49,300	54,000	1.1	100
DEAE Sepharose FF	5,430	15,000	2.8	28
HiLoad 26/10 Q Sepharose HP	252	11,000	44	20
Butyl Sepharose 4FF	0.62	82	130	0.15

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GCGAGCTCCACCGCGGTGCGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGGAATTCCACGAGCTCAATTCTTATCTTCTCCTC     87
TTCAATCACAACACCGTCACA ATG GCA AAG TCC TAC AGT GAA CAG CCG GAA CTG CAC GCG AAG GCG CCG        156
                      M   A   K   S   Y   S   E   Q   P   E   L   H   A   K   A   P         16
                                                                    (K-1)
TAT CGC TCT GCG ATG CTC ACG TAC CCT GGC AAT CTT CGT CAA GCA CTG AAA GAC GCC ATG GCC GAT     222
 Y   R   S   A   M   L   T   Y   P   G   N   L   R   Q   A   L   K   D   A   M   A   D      38
CCC TCA AAG ACA CTT ATG GGT GTT GCT CAC GGC ATT CCA AGT ACA TTC GTG ACC AAG GTG CTC GCA     288
 P   S   K   T   L   M   G   V   A   H   G   I   P   S   T   F   V   T   K   V   L   A      60
GCA ACG AAG CCC GAT TTC GTC TGG ATC GAT GTA GAA CAC GGC ATG TTC AAC CGT CTC GAG CTC CAC     354
 A   T   K   P   D   F   V   W   I   D   V   E   H   G   M   F   N   R   L   E   L   H      82
GAC GCT ATA CAC GCA GCA CAG CAC CAC TCC GAG GGC CGA TCT CTC GTC ATC GTT CGT GTG CCC AAG     420
 D   A   I   H   A   A   Q   H   H   S   E   G   R   S   L   V   I   V   R   V   P   K     104
CAC GAT GAG ATC TCG CTC TCA ACA GCC CTC GAC GCA GGC GCA GCC GGT ATC GTT ATT CCA CAT GTT     486
 H   D   E   I   S   L   S   T   A   L   D   A   G   A   A   G   I   V   I   P   H   V     126
GAA ACC GTC GAG GAA GTG CGT GAG TTT GTC AAA GAA ATG TAC TAC GGA CCC ATC GGA CGC CGC TCC     552
 E   T   V   E   E   V   R   E   F   V   K   E   M   Y   Y   G   P   I   G   R   R   S     148
TTC AGC CCC TGG ACA TTC TCC CCC GGC ATC GCA GAT GCA TCG CTC TTC CCC AAC GAC CCG TAC AAC     618
 F   S   P   W   T   F   S   P   G   I   A   D   A   S   L   F   P   N   D   P   Y   N     170
GTA GCC ACG AGC AAC AAC CAC GTA TGT ATC ATC CCG CAG ATC GAG TCT GTC AAA GGC GTC GAG AAC     684
 V   A   T   S   N   N   H   V   C   I   I   P   Q   I   E   S   V   K   G   V   E   N     192
GTC GAT GCG ATT GCC GCC ATG CCC GAA ATC CAC GGC CTC ATG TTC GGC CCG GGA GAC TAC ATG ATC     750
 V   D   A   I   A   A   M   P   E   I   H   G   L   M   F   G   P   G   D   Y   M   I     214
GAT GCC GGT CTT GAT CTG AAT GGC GCG CTG AGC GGC GTT CCG CAC CCG ACT TTT GTG GAG GCC ATG     816
 D   A   G   L   D   L   N   G   A   L   S   G   V   P   H   P   T   F   V   E   A   M     236
ACT AAG TTC TCA ACG GCG GCG CAG AGG AAC GGG GTT CCG ATT TTT GGT GGT GCG TTG AGC GTT GAT     882
 T   K   F   S   T   A   A   Q   R   N   G   V   P   I   F   G   G   A   L   S   V   D     258
ATG GTT CCC TCG TTG ATT GAG CAA GGG TAC CGC GCT ATC GCT GTG CAA TTT GAT GTC TGG GGT CTG     948
 M   V   P   S   L   I   E   Q   G   Y   R   A   I   A   V   Q   F   D   V   W   G   L     280
TCG AGA CTG GTG CAT GGG TCT TTG GCC CAG GCA AGG GCT TCA GCA AAA CAG TTC GCT GGT CAA GGT    1014
 S   R   L   V   H   G   S   L   A   Q   A   R   A   S   A   K   Q   F   A   G   Q   G     302
                                                                    (K-2)
AAA GCA GCG ACT GAC GGA ACT ACA GAC GAA ACC GTC GAC GGC GCC GCC GAA GAG GTG GCC AAT GGG    1080
 K   A   A   T   D   G   T   T   D   E   T   V   D   G   A   A   E   E   V   A   N   G     324
                                                                            (K-3)
GTC TCC AAA GTG AAG CTC GAC GAA GCT GGC GAC GAA GAC AAG GCT TAG ACTTTCTTATCGTTC            1143
 V   S   K   V   K   L   D   E   A   G   D   E   D   K   A   *                           339
TAGTAGATGCTTCCTTGTTGCTACGTCAGATGTGAAAGAATACATATAGATTGCGAGTATCGAAAAAAAAAAAAAAAAAAAA        1225
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Fig. 4. **Nucleotide sequence of the cDNA encoding the macrophomate synthase and deduced amino acid sequence.** Underlines indicate sequences of isolated peptides.

the initial amino acid 42.3 pmol, as follows: A-K-S-Y-S-E-Q-P-E-L-H-A-K-A-P-Y-R-S-R-M-L-T-Y-P-G-N.

Properties of Purified Macrophomate Synthase—Optimum pH of the macrophomate synthase reaction was 7.0 with a sharp peak: the activity was 10% of the optimum at pH 5.9 and 40% at pH 7.6. The macrophomate synthase was stable in the narrow pH range of 6.3 to 7.4 on incubation at 30°C for 10 min and 6.3 to 7.2 on storage at 4°C for 16 h. Storage at pH 6.0 and 8.0 reduced the original activity to 40 and 10%, respectively. Optimum temperature of the macrophomate synthase activity was 30°C on incubation for 10 min at pH 7.0. The enzyne was stable at temperatures below 50°C on incubation for 10 min at pH 7.0. At 60°C, the activity was reduced to 25%. Therefore, reduction of the activity at 30 to 50°C is unlikely to depend on heat inactivation.

The activity of macrophomate synthase was markedly reduced by 5 mM $HgCl_2$, $ZnSO_4$, $CuSO_4$, and $CoCl_2$, slightly reduced by 5 mM $MnCl_2$, and stimulated by 5 mM $MgCl_2$, $SnCl_2$, and $CaCl_2$. The highest stimulation was afforded by 5 mM $MgCl_2$, while higher concentration of $MgCl_2$ was inhibitory.

Kinetic analysis was done by use of equal concentrations of oxalacetate and $MgCl_2$, and gave K_m of 1.7 mM for **1** at 5 mM oxalacetate and 1.2 mM for oxalacetate at 5 mM **1**. At 5 mM **1**, V_{max} was 0.69 μmol per min per mg of protein, k_{cat} 0.46 s^{-1} per subunit, and oxalacetate showed substrate inhibition at higher concentration than 5 mM. Substrate **1** was replaced by compounds **3** and **5** to give corresponding products **4** and **6**, with K_m of 26.8 mM and k_{cat} of 0.061 s^{-1} for **3** and K_m of 11.6 mM and k_{cat} of 0.21 s^{-1} for **5** at 5 mM oxalacetate. Pyruvate served as a substrate in place of oxalacetate, having 0.16% of the activity of oxalacetate at 5 mM, with K_m of 35.2 mM and k_{cat} of 0.027 s^{-1} at 5 mM **1**. This was supported by the competitive inhibition of oxalacetate by pyruvate, with K_i of 104 mM. Other acids in Table I, 2-oxoglutarate, 2-oxobutyrate, L-aspartate, 3-bromopyruvate, and 3-hydroxypyruvate, were not substrates.

Amino Acid Sequences of Macrophomate Synthase—Lysylendpeptidase digests of the purified macrophomate synthase were separated by reverse-phase HPLC and three isolated peptides, which were named K-1, K-2, and K-3, were sequenced.

The PCR primers for preparation of a probe were designed from sequences of peptides K-1 and K-3. Screening of the cDNA library from the induced *M. commelinae* with the probe gave four positive clones which had a 1.2-kbp insert. These clones showed the identical sequence containing a 1,020-bp open reading frame, a 108-bp 5′-untranslated region, and a 77-bp 3′-untranslated region with a poly(A) tail (Fig. 4). Figure 4 shows the deduced amino acid sequence of 339 residues with a molecular mass of 36,244. The primary structure had no significant similarity with other reported structures.

Overexpression in E. coli—*E. coli* BL 21 was transformed with expression vector pETMS, and transformants were screened by comparison of the band on SDS-PAGE corresponding to macrophomate synthase induced by the addition of 2.5 mM IPTG. Of 30 recombinant colonies, *E. coli*–MS was selected as showing the highest enzyme activity. Cells of *E. coli*–MS were incubated at 25°C, and 2.5 mM IPTG was added after 9 h. Macrophomate synthase was accumulated in the highest level at 12 h after IPTG addition (Fig. 5). The cell extract was found to contain a high concentration of macrophomate synthase (Fig. 6).

Purification and Characterization of Macrophomate Synthase from E. coli—Macrophomate synthase was purified by only one step of Butyl Sepharose 4 Fast Flow column chromatography from *E. coli*–MS, which was incubated at 25°C for 12 h after the addition of 2.5 mM IPTG (Table III).

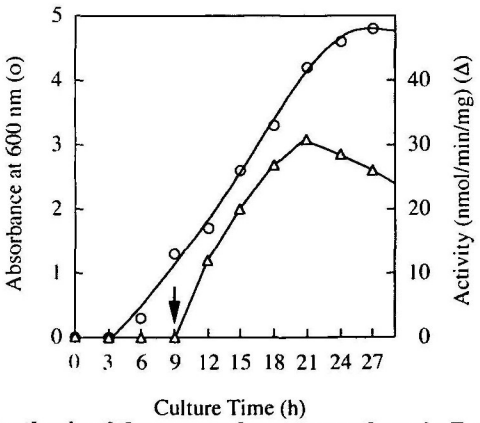

Fig. 5. **Synthesis of the macrophomate synthase in *E. coli*–MS.** *E. coli*–MS was incubated in Luria-Bertani medium at 25°C, and 2.5 mM IPTG was added at 9 h (arrow). Absorbance of culture at 600 nm (○) and macrophomate synthase activity (△) were measured.

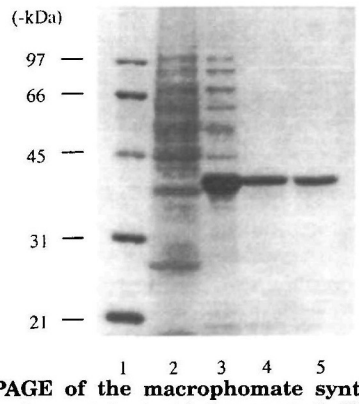

Fig. 6. **SDS-PAGE of the macrophomate synthase from *E. coli*–MS.** The gel was stained with Coomassie Brilliant Blue. Lane 1: molecular mass standard. Lane 2: extract from *E. coli* harboring pET30b (control). Lane 3: extract from *E. coli*–MS. Lane 4: macrophomate synthase purified from *E. coli*–MS. Lane 5: macrophomate synthase purified from *M. commelinae*.

TABLE III. **Purification of the macrophomate synthase from *E. coli*–MS.**

Step	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min/mg)	Yield (%)
Cell extract	390	12,000	30.8	100
Butyl Sepharose 4FF	60.8	7,380	121.4	61.5

The yield of purified macrophomate synthase from 200 ml of culture was 60.8 mg. The purified enzyme gave a single band of 40 kDa on SDS-PAGE (Fig. 6), and a peak of 90 kDa by gel filtration, indicating the enzyme to be a dimer. Sequence analysis of the purified enzyme gave the same *N*-terminal sequence as the enzyme purified from *M. commelinae*, A-K-S-Y-S-E-Q-P-E-L, in which the initial methionine residue is lost. The purified enzyme had essentially the same properties and kinetic parameters as the *M. commelinae* enzyme: optimum pH of 7.0, specific activity of 121 nmol/min/mg, K_m of 1.7 mM for **1** at 5 mM oxalacetate and 1.2 mM for oxalacetate at 5 mM **1**, and k_{cat} of 0.42 s^{-1} per subunit at 5 mM **1**.

DISCUSSION

At the beginning of this work, we addressed the major question of whether a single enzyme or several enzymes mediate the five-step conversion of **1** to **2**. The study described in this paper unambiguously established that a single enzyme is responsible for this unusual multistep transformation.

Sakurai *et al.* showed that the resting cells of *M. commelinae* preincubated with **1** began to transform **1** to **2** without a time lag. On the basis of their results, we searched for the best conditions to induce a high enzyme activity for this transformation (Fig. 2). The extract obtained from the resultant induced cells showed the enzymatic activity in the presence of various compounds as a C3-unit precursor (Table I), of which oxalacetate exhibited remarkable increase on the activity. Activities with all compounds except oxalacetate were apparently lost on anion exchange chromatography of the cell extract. At this stage, we established that oxalacetate is another substrate of the macrophomate synthase. Only slight activity with pyruvate remained in the purified enzyme, and later pyruvate was found to be an alternative substrate. Through the measurement of **1**, **2**, and oxalacetate, we settled the stoichiometry of the transformation of **1** to **2** as the reaction in Fig. 4. Overall, the transformation included two C-C bond formations, two decarboxylations, and a dehydration. Although the last step of the purification process resulted in a low yield of the enzyme, it was effective to remove minor bands, and the final fraction gave a single band on SDS-PAGE and native PAGE. Sequence analysis of the purified enzyme gave a single N-terminal sequence in significant yield of the initial residue. These data indicated that the purified single protein is macrophomate synthase, which catalyzes the proposed reaction in Fig. 4. The function of this protein was further confirmed by cloning of the cDNA using protein sequence data and expression of the enzyme activity in *E. coli* containing the cDNA. Overexpression of the macrophomate synthase in *E. coli* would provide enough of the enzyme, which has low abundance in *M. commelinae*, to allow a detailed characterization of the enzyme reaction mechanism. Homology search gave no information on proteins homologous to the amino acid sequence deduced from the cDNA.

The finding that oxalacetate is the intact precursor for the biosynthesis of **2** makes the decarboxylation essential, and the finding that pyruvate acts as an alternative substrate suggests that decarboxylation occurs at the β-carboxyl group of the oxalacetate. Its lower efficiency in the conversion and the higher K_m of pyruvate, however, suggest that pyruvate is not an intermediate in the enzymatic transformation. To date, two kinds of enzymatic decarboxylation of oxalacetate have been reported. Biotin-dependent oxalacetate decarboxylase from *Klebsiella pneumoniae* requires a sodium ion for release of the carboxyl group from the *N*-carboxylbiotin and is accompanied by the transport of the sodium ion across the membrane (*17*). Other enzymes depend on divalent metal ions for the oxalacetate decarboxylation activity, namely, pyruvate kinase (*18*), malic enzyme (*19*), and oxalacetate decarboxylases from *Acetobacter xylinum* (*20*) and *Pseudomonas putida* (*21*). The physiological role of divalent-metal-cation–dependent decarboxylation is not clear.

Sakurai *et al.* proposed a stepwise anion-based condensation mechanism which involves unlikely complex reactions catalyzed by the single and relatively small enzyme. We previously proposed an alternative mechanism that involves two concerted C-C bond formations (Diels-Alder reaction) affording a bicyclic intermediate which decomposes in subsequent dehydration–decarboxylation to yield the corresponding benzoate **2** (*9*). Experimental evidence (*22*) for this bicyclic intermediate has recently been obtained, although involvement of the Diels-Alder reaction is not essential in this mechanism. Details of the reaction mechanism of this unusual transformation remain to be solved.

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