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Purification and Characterization of an Esterase Hydrolyzing Monoalkyl Phthalates from *Micrococcus* sp. YGJ1

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An esterase that specifically hydrolyzes medium-chain (C_3–C_5) monoalkyl phthalates was purified from phthalate-grown *Micrococcus* sp. YGJ1. The enzyme activity was split into two fractions by hydrophobic chromatography on Phenyl Sepharose, and the enzymes were purified to homogeneity from each fraction. The purified enzymes showed similar properties with respect to molecular mass (60 kDa), subunit molecular mass (27 kDa), N-terminal amino acid sequence, optimal pH (about 7.5), temperature-dependence, substrate specificity, and inhibitor susceptibility. The enzymes showed no activity toward various dialkyl phthalates or aliphatic carboxyl esters. 2-Mercaptoethanol effectively protected the enzymes from spontaneous inactivation. Diethylpyrocarbonate, *p*-chloromercuribenzoate, Hg^{2+}, and Cu^{2+} strongly inhibited the enzymes, while phenylmethylsulfonyl fluoride produced weak inhibition, and various metal chelating reagents were ineffective. These findings show that the enzymes bear a close resemblance to the putative phthalate ester hydrolase (PehA) of *Arthrobacter keyseri* 12B.

Key words: esterase, hydrolase, *Micrococcus*, monoalkyl phthalate, phthalic acid ester.

Abbreviations: DAP, dialkyl phthalate; KEM, potassium phosphate buffer, pH 7.0, containing 10% (v/v) ethylene glycol and 10 mM 2-mercaptoethanol; MAP, monoalkyl phthalate; *p*-CMB, *p*-chloromercuribenzoate.

Dialkyl phthalates (DAPs) are used to prepare various chemicals. Particularly, dibutyl phthalate and di(2-ethylhexyl) phthalate are manufactured in large scale for use as plasticizers for polyvinyl chloride resin. DAPs tend to accumulate in the environment, and are regarded as environmental pollutants (*1*, *2*). A variety of microorganisms are responsible for the degradation of DAPs in the natural environment (*3–9*). DAPs are hydrolyzed to phthalate and alcohol at the early stage of bacterial metabolism (*10–12*). Anaerobic bacteria use the alcohol to live (*13*, *14*), while aerobic bacteria degrade phthalate further for assimilation (*7*, *8*, *11*). *Nocardia erythropolis* S-1 releases a lipase into culture broth to hydrolyze DAPs to phthalate (*15*). In other bacterial strains, DAPs are hydrolyzed stepwise by the action of two distinct enzymes (*10–12*) (Fig. 1). The first enzyme, designated DAP esterase, hydrolyzes DAP to monoalkyl phthalate (MAP), and is constitutively produced in bacterial cells (*11*, *12*). DAP esterase has been purified from *Micrococcus* sp. YGJ1, and has been shown to hydrolyze various aliphatic and aromatic carboxyl esters other than MAPs (*16*). The second enzyme, designated MAP esterase, hydrolyzes MAP to phthalate. MAP esterase has been reported to be inducible with phthalate in *Arthrobacter* sp. DSM 20389 or *Pseudomonas* sp. DSM 5030 (*12*). However, the instability of MAP esterase hinders its purification, and the molecular and catalytic properties of the MAP esterase have been little documented until now. The MAP esterase appears to be unique, since most carboxylic ester hydrolases [EC 3.1.1] act on non-charged esters and not on charged esters such as MAPs. In this paper, we describe the first purification of the MAP esterase from *Micrococcus* sp. YGJ1 grown on phthalate.

MATERIALS AND METHODS

Chemicals—Monomethyl phthalate was obtained from Aldrich (Milwaukee, WI, USA). MAPs of ethanol, 1-propanol, 2-propanol, 2-butanol, 1-pentanol, 1-heptanol, 1-octanol, and 1-nonanol were synthesized as described by Goggans and Copenhaver (*17*). MAPs of 1-butanol, 1-hexanol and 2-ethyl-1-hexanol were obtained from Tokyo Kasei (Tokyo). Lubrol PX (nonaethylene glycol monododecyl ether) was purchased from Nacalai Tesque (Kyoto).

Microorganism—Micrococcus sp. YGJ1 (Faculty of Engineering, Gifu University) was cultured in phthalate medium as described previously (*16*). Liquid cultures were maintained at 30°C in 500-ml Sakaguchi flasks containing 250 ml medium. The flasks were shaken at 100 rpm for 1 day.

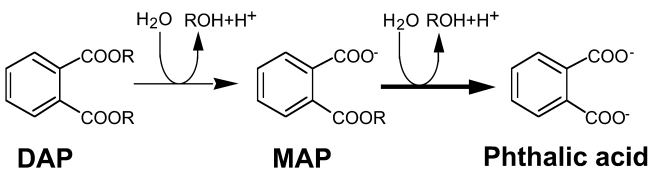

Fig. 1. **Initial hydrolytic steps in the metabolism of phthalate esters by *Micrococcus* sp. YGJ1.** "R" indicates an alkyl group.

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Determination of Enzyme Activity—MAP esterase activity was determined by measuring the formation of phthalate from MAP. The reaction mixture (1 ml) contained 4 mM MAP dissolved in 0.1 ml of ethanol, 50 mM potassium phosphate buffer (pH 7.5), and enzyme (2–3 μg). Monobutyl phthalate was used as a substrate for routine assays. The reaction was carried out at 30°C for 30 min, and stopped by the addition of 20 μl of 3 M H_2SO_4. The mixture was extracted with 3 ml of ether, 1 ml of the extract was evaporated, and the residue was dissolved in 0.5 ml of 80% (v/v) methanol. Part of this sample (20 μl) was subjected to HPLC, which was performed at 45°C on a Shim-pack C_{18} reversed-phase column (0.6 × 15 cm) (Shimadzu, Kyoto) and monitored at A_{254}. The column was eluted with a methanol-H_2O gradient. The activity toward DAPs, benzoate ester, and salicylate ester was also measured by HPLC. Monomethyl phthalate and monoethyl phthalate were not well separated from phthalate under these conditions. Therefore, the formation of acid was also measured with an acid-base indicator. The reaction mixture (1.5 ml) was similar to that described above, except that 5 mM potassium phosphate buffer (pH 7.5) was used and 0.001% (w/v) Bromthymol Blue was added. The decrease in A_{620} was measured with an UV-300 spectrophotometer (Shimadzu). Under these conditions, a change in A_{620} of 0.01 corresponded to 0.18 mM of acid formed. The activity toward aliphatic esters was determined by measuring the formation of carboxylic acid with 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (*18*). The DAP esterase activity was determined with *p*-nitrophenyl acetate as described previously (*16*). One unit of enzyme activity was defined as that amount catalyzing the degradation of 1 μmol of substrate per min under the assay conditions. The specific activity was defined as units per mg of protein. Protein was determined by the method of Lowry *et al*. (*19*) as modified by Bennett (*20*). Bovine serum albumin was used as a standard.

Enzyme Purification—About 96 g (wet weight) of bacterial cells were prepared from a 24-liter culture. Enzyme purification was carried out at 0–4°C with 50 mM KEM [50 mM potassium phosphate buffer, pH 7.0, containing 10% (v/v) ethylene glycol and 10 mM 2-mercaptoethanol], unless otherwise noted. The bacterial cells were suspended in 300 ml of KEM, and disrupted by shaking three times for 1 min at 1-min intervals in a CO_2-refrigerated MSK cell homogenizer (B. Braun Biotech International, Melsungen, Germany). Glass beads (ϕ = 0.1 mm) and cell debris were removed by centrifugation. The supernatant (crude extract) was treated with solid ammonium sulfate. The precipitate obtained between 60–75% saturation was collected by centrifugation, and dissolved in about 30 ml of KEM. After dialysis against 500 ml of KEM, the solution (ammonium sulfate fraction) was divided into two parts, and each part was passed through a column (4 × 26 cm) of Bio-Gel A-1.5m (Bio-Rad, Hercules, CA, USA), previously equilibrated with KEM, at a flow rate of 12 ml/h, and 4.7-ml fractions were collected. The active fractions were pooled and applied to a column (3 × 20 cm) of DEAE-cellulose (Serva, Heidelberg, Germany), previously equilibrated with KEM. The column was washed with 500 ml of KEM, and the enzyme was eluted with a linear gradient, established with 200 ml of KEM and 200 ml of the same buffer containing 0.5 M ammonium sulfate, at a flow rate of 16 ml/h, and 5.8-ml fractions were collected. The active fractions were pooled, and solid ammonium sulfate was added to a final concentration of about 1.5 M. Then, the solution was loaded onto a column (3 × 8 cm) of Phenyl Sepharose (Amersham Biosciences, Uppsala, Sweden) previously equilibrated with KEM containing 1.5 M ammonium sulfate. The column was washed successively with 200 ml of the equilibration buffer, a linear gradient established with 130 ml of the equilibration buffer and 130 ml of KEM, 100 ml of KEM, and, finally, 100 ml of KEM containing 0.4% (w/v) Lubrol PX. The flow rate was adjusted to 40 ml/h and 6.1 ml fractions were collected. A part of the enzyme was eluted in the KEM wash, and the remainder was eluted in the Lubrol wash (Fig. 2). These fractions were pooled separately, dialyzed against 1 liter of 25 mM KEM, and applied to a column (1.6 × 5.5 cm) of hydroxyapatite (Bio-Rad) equilibrated with 25 mM KEM. After the column was washed with 40 ml of 25 mM KEM, the enzyme was eluted with a linear gradient established with 50 ml of 25 mM KEM and 50 ml of 500 mM KEM at a flow rate of 10 ml/h, and 1.2-ml fractions were collected. The active fractions were pooled, dialyzed against KEM, and stored at -25°C.

Analytical Methods—Disc PAGE was performed by the method of Davis (*21*). After electrophoresis, the protein was stained with Coomassie Brilliant Blue R-250. SDS-PAGE was carried out in 12.5% polyacrylamide gels by the method of Laemmli (*22*). To determine the subunit molecular mass, molecular mass marker proteins (Nacalai Tesque) were used. After SDS-PAGE, the protein band was electroblotted onto a polyvinylidene difluoride membrane (Atto, Tokyo), and the N-terminal amino acid sequence was analyzed with a Procise 492 protein sequencer (Perkin-Elmer, Norwalk, CA, USA). The molecular mass of the native enzyme was determined by analytical gel filtration on Sephadex G-200 (Pharmacia Biotech, Uppsala, Sweden), previously equilibrated with 50 mM KEM and calibrated with standard proteins including bovine liver catalase (M_r = 250 kDa), yeast alcohol dehydrogenase (140 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and soybean trypsin inhibitor (21 kDa).

RESULTS

Purification of MAP Esterase—Micrococcus sp. YGJ1 cells were grown aerobically with various growth substrates (each 0.1%, w/v), and crude extracts were prepared to measure the MAP esterase activity. The activity was high (0.091–0.117 μmol/min/mg) in phthalate- or dibutyl phthalate-grown bacteria, and low (0.003–0.007 μmol/min/mg) in glucose- or succinate-grown bacteria. Such variations in enzyme activity depending on the growth substrates are consistent with the inductive production of MAP esterase. In the subsequent study, phthalate-grown bacteria were used as the enzyme source.

The MAP esterase in the crude extract was unstable in 50 mM potassium phosphate buffer, pH 7.0, and lost almost all activity during storage for 3-days at 4°C. However, the addition of 10% (v/v) ethylene glycol and 10 mM 2-mercaptoethanol or 1mM DTT markedly stabilized the

Table 1. **Purification of MAP esterase from *Micrococcus* sp. YGJ1.**

Step	Protein (mg)	Activity (unit)	Specific activity (unit/mg)	Purification (fold)	Yield (%)
Crude extract	614	66.3	0.108	1	100
Ammonium sulfate	389	49.0	0.126	1.17	73.9
Bio-Gel A-1.5m	193	38.4	0.199	1.84	57.9
DEAE-cellulose	43.2	33.7	0.779	7.21	50.8
Phenyl Sepharose					
KEM wash	0.91	8.95	9.84	91.1	13.5
Lubrol wash	0.60	11.4	19.0	176	17.2
Hydroxyapatite					
E1	0.24	5.21	21.7	201	7.9
E2	0.33	6.50	19.7	182	9.8

enzyme, and no loss of enzyme activity could be found after incubation as above. Therefore, the enzyme purification was performed in KEM at 0–4°C. The steps used for enzyme purification are summarized in Table 1. The MAP esterase activity was eluted as a single activity peak in the Bio-Gel A-1.5m step and the DEAE-cellulose step (data not shown). The bulk of the DAP esterase activity was removed in the DEAE-cellulose step. During hydrophobic chromatography on Phenyl Sepharose, the MAP esterase activity was recovered in two well-separated fractions, one peak of enzyme activity in the KEM wash, and the other in the Lubrol PX wash (Fig. 2). Each of the fractions was further treated with a hydroxyapatite. After adsorption to hydroxyapatite at low phosphate concentration, the enzymes from these fractions were eluted at a similar phosphate concentration (about 0.14 M). The MAP esterases thus purified from the KEM wash and the Lubrol PX wash fractions were designated E1 and E2, respectively. They were finally purified to a similar extent (about 180–200 fold; specific activity 19.7–21.7 μmol/min/mg) from the crude extract.

General Properties of the Purified Enzymes—On disc PAGE at 4°C, the purified enzymes E1 and E2 showed single protein bands with similar mobilities (Fig. 3A). Activity staining with 1-naphthyl acetate and Fast Blue BB (*23*) yielded no band, indicating that the two enzymes do not hydrolyze 1-naphthyl acetate. E1 and E2 gave single protein bands at a position of 27 kDa on SDS-PAGE (Fig. 3B), and their molecular masses were determined to be about 60 kDa by gel filtration on Sephadex G-200, suggesting that E1 and E2 are dimeric proteins (data not shown). They also had the same N-terminal amino acid sequence of SATAAREEYQRKRSQFIEIG in Edman degradation. They showed similar absorption spectra with a maximum at 280 nm (ε_{280} = 66,000 $M^{-1}\cdot cm^{-1}$) with a shoulder at 290 nm, a minimum at 250 nm, and no significant absorption in the visible region. Thus, E1 and E2 resemble each other in their physical properties, and their catalytic properties are also similar as described below.

The purified enzymes were rapidly inactivated at 25°C in the absence of 2-mercaptoethanol. Actually, the enzymes lost almost all activity on simple passage through a Sephadex G-25 column (1.6 × 6 cm) to remove 2-mercaptoethanol, and the inactivated enzymes recovered about 20% of the original activity by subsequent incubation with 10 mM DTT. Therefore, the purified enzymes were always stored in KEM. Under the standard assay conditions, the enzyme reaction progressed linearly for at least 30 min, and the reaction rate was proportional to the amount of enzyme used (less than 3.5 μg). In potassium phosphate buffer, E1 and E2 showed an optimum pH at about 7.5 with 50% activity at around pH 6.5 and 7.8 (Fig. 4A). They also showed similar temperature-dependence. When the assay was done at various temperatures, the enzyme activity was maximum at about 40°C with 50% activity at about 27°C and 55°C

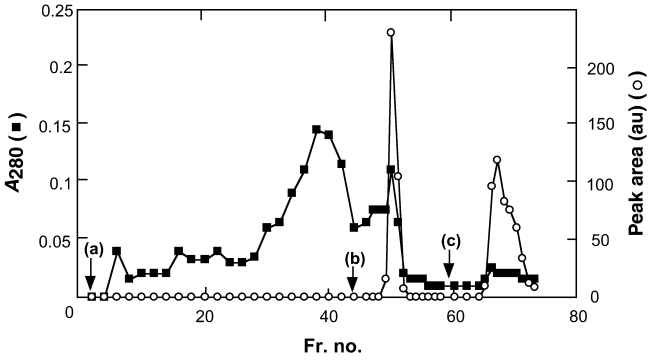

Fig. 2. **Elution from a Phenyl Sepharose column.** After the unadsorbed materials were washed out, the column was further washed successively with (a) 260 ml of a linear ammonium sulfate gradient (1.5 M to 0 M), (b) 100 ml of 50 mM KEM, and (c) 100 ml of 50 mM KEM containing 0.4% (w/v) Lubrol PX. Arrows show the positions of the buffer changes. Protein was measured at A_{280} (filled squares). The enzyme activity (open circles) was measured with an aliquot (20 μl) of each fraction under the standard assay conditions, and is expressed in terms of the peak area of phthalate determined by HPLC. au, arbitrary unit.

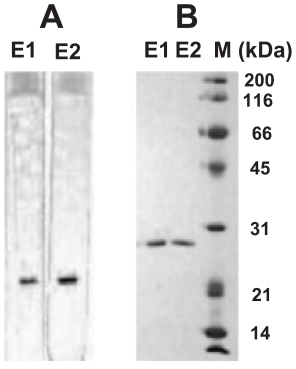

Fig. 3. **Disc PAGE (A) and SDS-PAGE (B) of the purified enzymes.** The purified preparations of E1 and E2 (each 4 μg) were analyzed. Lane M, marker proteins.

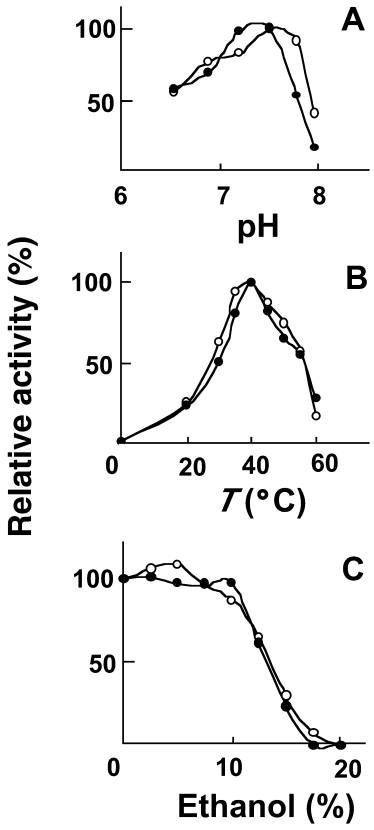

Fig. 4. **Effects of pH, temperature, and ethanol on MAP esterase activity.** The activities of E1 (filled circles) and E2 (open circles) were assayed under the standard conditions, except that pH (A), temperature (B), or ethanol concentration (C) was varied as indicated.

(Fig. 4B). Ethanol, which was used to prepare the ester solution, had no effect on the enzyme activity at concentrations below 10% (v/v), but was inhibitory at higher concentrations. E1 and E2 were inhibited to similar extents (Fig. 4C).

Substrate Specificity—The enzyme activities toward various carboxylic esters (each 4 mM) were examined by measuring the formation of acid. El and E2 showed similar specificities. The following esters were hydrolyzed: monobutyl phthalate, relative activity 100%; monopentyl phthalate, 50–51%; monopropyl phthalate, 12–19%; butyl benzoate, 5.1–6.0%; butyl salicylate, 0.1–0.2%. In contrast, none of following esters were hydrolyzed: MAPs of methanol, ethanol, 2-propanol, 2-butanol, 1-hexanol, 1-heptanol, 2-ethyl-1-hexanol, 1-octanol, or 1-nonanol; DAPs of C_1–C_9 *n*-alkyl chains; butyrate esters of C_1–C_7 *n*-alkyl chains; butyl esters of C_2–C_{10} *n*-acyl chains; glycerol esters, including triacetin, tripropionin and tributyrin; chromogenic esters such as *p*-nitrophenyl acetate and 1-naphthyl acetate. The kinetic constants determined by double reciprocal plots are listed in Table 2, showing that monobutyl phthalate is the most active substrate. Toward MAPs of C_3–C_5, the K_m and V_{max} values of E1 were somewhat lower than those of E2.

Table 3. **Effects of various reagents on MAP esterase activity.** E1 or E2 was preincubated at 25°C for 5 min with various reagents (each 1 mM) in 50 mM potassium phosphate, pH 7.5, and the residual activity was measured as described under "MATERIALS AND METHODS." When the enzyme was treated with *p*-CMB, the activity was determined with Bromthymol Blue. In other cases, the activity was determined by HPLC.

Reagent	Residual activity (%)	
	E1	E2
Diethylpyrocarbonate	1.6	2.4
$HgCl_2$	nd[c]	nd
$CuSO_4$	23	10
Phenylmethylsulfonyl fluoride	79	70
p-CMB[a]	nd	nd
p-CMB, DTT[b]	49	44

[a]Because of its reaction with the 2-mercaptoethanol added into the mixture with the enzyme solution, extra *p*-CMB was added. [b]The *p*-CMB–treated enzyme was further incubated at 25°C for 5 min with 10 mM DTT. [c]Not detectable.

Inhibitors—E1 and E2 showed similar susceptibilities to various inhibitors (Table 3). Diethylpyrocarbonate, which modifies histidine residues, and *p*-chloromercuribenzoate (*p*-CMB) strongly inactivated the enzymes. The *p*-CMB–treated enzymes were partly reactivated by incubation with DTT. Phenylmethylsulfonyl fluoride, a modifier of reactive serine residues, caused weak inhibition. Hg^{2+} and Cu^{2+} strongly inhibited the enzymes, possibly due to reaction with cysteine residues, since other metal ions (each 1 mM), such as Mg^{2+}, Ca^{2+}, Co^{2+}, Ni^{2+} and Fe^{3+}, had no effect. None of the metal chelating reagents tested (each 1 mM), including EDTA, 2,2′-dipyridyl, 1,10-phenanthroline, 8-hydroxyquinoline, and Tiron, caused inhibition.

DISCUSSION

Kinetic studies of the non-enzymatic base-catalyzed hydrolysis of phthalate esters have shown that MAPs are hydrolyzed at a much slower rate than DAPs (*24*). MAPs are also hard to hydrolyze enzymatically by rat and human liver carboxylesterases [EC 3.1.1.1] (*25*). Obviously, the carboxylate anion near the ester bond makes MAPs hard to hydrolyze, possibly due to the electrostatic repulsion toward the catalytic nucleophile attacking the ester bond. Thus, there may be a positively charged amino acid residue in the catalytic site of MAP esterase to bind the carboxylate anion and neutralize its negative charge. This may partly explain the narrow substrate specificity of the MAP esterases. Both a benzene ring and

Table 2. **Kinetic constants of MAP esterases.**

MAP	E1		E2	
	K_m (mM)	V_{max} (μmol/min/mg)	K_m (mM)	V_{max} (μmol/min/mg)
Monopropyl	5.88	7.81	6.25	11.1
Monobutyl	1.67	40.1	2.70	49.5
Monopentyl	1.39	15.6	2.63	17.9

carboxylate group appear to be essential structural features for the substrate ester, since various DAPs and aliphatic carboxylic esters did not serve as substrates, and butyl benzoate was a poor substrate as compared with monobutyl phthalate. E1 and E2 hydrolyzed MAPs with C_3–C_5 chains, with maximum activity toward monobutyl phthalate. This seems to be reasonable, since *Micrococcus* sp. YGJ1 was originally isolated from the soil by enrichment culture with dibutyl phthalate as the carbon source (*16*). In other bacterial strains, there may be other MAP esterases with different chain length specificities. Bacteria grown on dimethyl phthalate or diethyl phthalate are expected to have esterases acting on MAPs with shorter alkyl chains (*26*, *27*). On the other hand, esterases acting on MAPs with longer chains may be found in microorganisms that degrade di-*n*-octyl phthalate and di(2-ethylhexyl) phthalate (*10*). In contrast to MAP esterases, the lipase purified from the culture broth of *Nocardia erythropolis* S-1 shows broad specificity, and hydrolyzes various aliphatic and aromatic carboxylic esters, including DAPs (*15*). The lipase can also hydrolyze MAPs as well as DAPs, since DAPs are converted to phthalate and no accumulation of MAPs is found.

It should be emphasized that DAPs are not hydrolyzed by MAP esterase, because the term phthalate ester esterase (hydrolase) is often used without careful discrimination between DAP esterase and MAP esterase. Eaton found a gene, *pehA*, in the nucleotide sequence of the *Arthrobacter keyseri* 12B catabolic plasmid pRE1 (GenBank accession no. AF331043) and inferred that it encodes a putative phthalate ester hydrolase, since the deduced amino acid sequence of *pehA* shows some sequence homology (34%) to the *N*-carbamoylsarcosine amidohydrolase [EC 3.5.1.59] from *Arthrobacter* sp (*28*). Although PehA has not been characterized by biochemical methods, the properties reported for PehA suggest that PehA may be a MAP esterase rather than a DAP esterase. First, PehA expressed in *Escherichia coli* could not hydrolyze dimethyl, diethyl or dibutyl phthalate. Second, the calculated molecular mass (24 kDa) of PehA is consistent with the subunit molecular mass (27 kDa) of E1 and E2. Third, the deduced N-terminal amino acid sequence (MSATAAREEYQKLRSQFKEKG) of PehA is highly homologous to the chemically determined amino acid sequences of E1 and E2: Except for the N-terminal methionine, 16 of 20 amino acid residues are identical. Moreover, phylogenetic analysis of the bacterial 16S rRNA gene showed that *Micrococcus* sp. YGJ1 is a close relative of *A. keyseri* 12B (Yamada, S., *et al*., GenBank accession no. AB127968). Fourth, Eaton's supposition that PehA has a catalytic cysteine residue is consistent with the finding that thiols or thiol reagents markedly influence MAP esterase activity. Since phenylmethylsulfonyl fluoride caused only weak inhibition, the serine residue may not be essential for catalysis.

Although there is some difference in the kinetic constants (Table 2), E1 and E2 closely resemble each other in many physical and catalytic properties, including their UV-absorption spectra, mobility in disc PAGE, molecular mass, subunit molecular mass, N-terminal amino acid sequence, pH- and temperature-dependence, substrate specificity and susceptibility to various inhibitors. E1 and E2 were separated based on their different behaviors in hydrophobic chromatography (Fig. 2): E2 was more strongly bound to Phenyl Sepharose than E1, and could be released from the resin only in the presence of a detergent such as Lubrol PX or Triton X-100. Accordingly, the purified enzymes were applied to a second Phenyl Sepharose column to confirm their molecular difference. Then, E1 was eluted in the KEM wash fraction as before, and E2, unlike the strong binding on the first Phenyl Sepharose column, was also eluted in the KEM wash fraction without detergent (data not shown). E2 gave a somewhat broad elution profile with a shallow downward slope, unlike the sharp elution profile of E1. These results suggest that E1 is the original enzyme and that E2 is a derivative of E1. The purified preparations of E1 and E2 are probably identical despite a small difference in their properties. Possibly, E1 is converted to E2 by the binding of some hydrophobic compound(s) that was removable by the adsorption chromatography on hydroxyapatite. This possibility should be investigated further to know the physiological meaning of the two enzyme forms of MAP esterase.

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