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Purification and Properties of 2-Hydroxy-6-Oxo-2,4-Heptadienoate Hydrolase from Two Strains of *Pseudomonas putida*

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Growth on phenol of two strains of *Pseudomonas putida* biotype A, NCIB 10015 and NCIB 9865, elicits the synthesis of an enzyme that hydrolyzes 2-hydroxy-6-oxo-2,4-heptadienoate to 2-oxopent-4-enoate. The purified enzyme from *Pseudomonas* NCIB 10015 has a molecular weight of 118,000 and dissociates in sodium dodecyl sulfate to a species of molecular weight 27,700; the enzyme from *Pseudomonas* NCIB 9865 has a molecular weight of 100,000 and dissociates to a species of 25,000 molecular weight. The hydrolases from both strains have similar K_m values, pH optima, and thermal labilities and attack the same range of substrates. Neither hydrolase was stimulated by Mg^{2+} or Mn^{2+}, and both were inhibited by *p*-chloromercuribenzoate and iodoacetamide. Immunodiffusion studies with the purified enzymes and antibodies formed against them show some cross-reaction of *Pseudomonas* NCIB 9865 enzyme with antibodies to *Pseudomonas* NCIB 10015, but not vice versa.

In the pathway of the degradation of *m*-cresol by *Pseudomonas putida* NCIB 10015, the product of ring cleavage of 3-methylcatechol, namely, 2-hydroxy-6-oxo-2,4-heptadienoate, is hydrolyzed to 2-oxopent-4 enoate (2). The corresponding product of ring cleavage from 4-methylcatechol is metabolized exclusively by an oxidized nicotinamide adenine dinucleotide-dependent aldehyde dehydrogenase (4), whereas the product from catechol is metabolized by both routes (4). Another strain, *P. putida* NCIB 9865, metabolizes *m*-cresol by a pathway identical to that for *P. putida* NCIB 10015 (Bayly, unpublished data). This report describes the purification of the enzymes from two strains of *P. putida* that catalyze the hydrolysis of 2-hydroxy-6-oxo-2,4-heptadienoate and gives a comparison of several of their properties.

MATERIALS AND METHODS

Organisms and methods of cultivation. The strains of *P. putida* biotype A used were NCIB 10015, hereafter referred to as PsU, and NCIB 9865, referred to as P23X1. The former strain has been classified as *P. putida* biotype A by Stanier et al. (21), who designated it as strain 144. Stanier et al. (21) used several growth characteristics to differentiate biotypes A and B of *P. putida*, and we have classified P23X1 as biotype A on the basis of the results shown in Table 1. Points of comparison in the ability of the two strains to use aromatic compounds as a carbon source are that they grow on *o*-, *m*-, and *p*-cresol, 2,3-xylenol, *p*-hydroxybenzoate, and quinate. PsU grows on benzoate, but P23X1 does not. Maintenance of cultures and the basal medium used for growth have been described previously (4). Cells were inoculated into 40-liter batches of basal medium that contained (10 mM) sodium fumarate and were incubated at 30°C with forced aeration. When the optical density was 0.1 to 0.2 at 580 nm (Spectronic 20, Bausch & Lomb, Inc., Rochester, N.Y.), phenol was added to give a concentration of 2.5 mM and incubation was continued for 4 to 5 h, at which time the optical density was 0.6 to 0.8. Cells were harvested, washed twice with phosphate buffer (0.1 M KH_2PO_4 adjusted to pH 7.4 with NaOH), and stored at −20°C.

Preparation of cell extracts. Washed cells were suspended in twice their volume of the same phosphate buffer at 0 to 4°C and disrupted in a French pressure cell (American Instrument Co., Silver Spring, Md.) under a pressure of 10,000 lb/in^2. Cell debris was removed by centrifugation at 26,000 × *g* for 60 min, and the cell extract was held at 0 to 4°C until used on the same day.

Enzyme assays. All assays for activity of 2-hydroxy-6-oxo-2,4-heptadienoate hydrolase were carried out in silica cuvettes of 1-cm path length, using a Hitachi 124 recording spectrophotometer. The final reaction volume was 3 ml, and the temperature was 23°C.

For assays against 2-hydroxy-6-oxo-2,4-heptadienoate (I), 2-hydroxymuconic semialdehyde (II), and 2-hydroxy-5-methylmuconic semialdehyde (III), the reaction mixtures contained 100 μmol of phosphate buffer (pH 7.6), 0.15 to 0.2 μmol of substrate (prepared as described previously [4]), and enzyme. The disappearance of the substrate was followed at the wavelengths of maximum absorbance reported previously (3), and specific activities were calculated by using

reported extinction coefficients (3). For tests of the substrate specificity of the purified hydrolase, other substrates were prepared as follows: 5-carboxymethyl-2-hydroxymuconic semialdehyde (IV) from 3,4-dihydroxyphenylacetic acid (20) and maleylpyruvate (V) from gentisate (8). The products of *meta* cleavage of 3-isopropylcatechol and 3,4-dimethylcatechol were prepared by the same method used for I (4). If these two catechols are cleaved as are catechol, 3-methylcatechol, and 4-methylcatechol, then the products formed would be 2-hydroxy-7-methyl-6-oxo-2,4-octadienoate (VI) from 3-isopropylcatechol and 2-hydroxy-5-methyl-6-oxo-2,4-heptadienoate (VII) from 3,4-dimethylcatechol. Compounds VI and VII have maximum absorbance in 0.1 M phosphate buffer (pH 7.6) at 393 and 388 nm, respectively. The molar coefficient for VII under those conditions was determined as described by Bayly et al. (3) and found to be 7,500. The molar extinction coefficient for VI was taken to be 10,000 (24). Assays for activity against VI and VII were carried out at 393 and 388 nm, respectively, using the conditions described above. Enzymatic activity against V was determined by following the decrease in absorbance at 330 nm, which is its wavelength of maximum absorbance in 0.1 M phosphate buffer (pH 7.1). The reaction mixture contained 0.1 to 0.15 μmol of substrate in 0.1 M phosphate buffer (pH 7.1), and the reaction volume was 3 ml. Activity against IV was determined by using the wavelength and extinction coefficient reported by Sparnins et al. (20). The reaction mixture contained 100 μmol of phosphate buffer (pH 7.0) and 0.15 to 0.2 μmol of substrate. 2-Oxopent-4-enoic acid was prepared, and the enzymatic activity against it was determined, as described by Collinsworth et al. (7).

Protein determination. The concentration of protein in cell extracts and fractions was determined by the method of Lowry et al. (14), using bovine albumin as a standard.

Physicochemical examination of purified hydrolase preparations. Molecular weights were determined by gel filtration on Sephadex G-200 (1.6 by 90 cm) as described by Andrews (1). Analytical disc gel electrophoresis was by the method of Davis (10), using 7.5% polyacrylamide gels at pH 8.9. Gels were stained with Coomassie brilliant blue R (Sigma Chemical Co., St. Louis, Mo.) for 4 to 5 h and then destained electrophoretically in 18% methanol-9% acetic acid. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried out as described by Weber and Osborn (25), using 10% gels. Staining and destaining were as before. Reduced and nonreduced samples for SDS gels were prepared as described by Tack et al. (23).

Heat stability of the hydrolases was determined on preparations that had been purified approximately 50-fold. The samples (1 ml), containing approximately 1 mg of protein, were held at the required temperatures, cooled in ice, and assayed.

Amino acid analysis of the purified enzymes was carried out on samples that contained 0.5 to 1 mg of protein, which had been prepared as follows. The sample was dialyzed against water to remove salts, lyophilized, and hydrolyzed with 6 M HCl in a sealed tube for 20 h at 110°C. Analysis was carried out in a JEOL automatic acid analyzer (model no. 6AH) by the method of Moore and Stein (16).

Cystine was determined in dialyzed samples that had been treated with performic acid.

TABLE 1. *Growth characteristics of PsU and P23X1*[a]

Characteristic	PsU	P23X1	% of positive strains	
			Biotype A	Biotype B
Growth at 4°C	−	−	34	100
D-Xylose	−	±	16	67
L-Arabinose	−	−	19	89
D-Mannose	−	−	19	78
D-Galactose	−	−	0	78
Mannitol	+	−	9	56
o-Hydroxybenzoate	−	−	9	67
Phenol	+	+	16	67
L-Tryptophan	−	−	0	100
L-Kynurenine	−	−	0	100
Anthranilate	−	−	6	100
Nicotinate	−	±	66	0

[a] Data from Stanier et al. (21). Tests were carried out as described by Stanier et al. (21). +, Growth; ±, slight growth; −, no growth. Testosterone, α-aminovalerate, and tryptamine were not tested.

Immunological studies. Antisera against the purified hydrolases were prepared by subcutaneous inoculation of Monash-bred rabbits with the purified enzyme (approximately 1 mg) emulsified in Freund complete adjuvant. Rabbits were bled 3 weeks after inoculation and then at two 2-week intervals. Serological reaction between enzyme preparations and antisera was determined by immunodiffusion, using a 1% Ionagar gel in 0.15 M NaCl.

Chemicals. 3,4-Dimethylcatechol and 3-isopropylcatechol were gifts from P. J. Chapman and Coalite and Chemical Products, Bolsover, England, respectively. Catechol, 3-methylcatechol, and 4-methylcatechol were from Aldrich Chemical Co. Inc., Milwaukee, Wis. All catechols were purified by vacuum sublimation before use.

Purification of 2-hydroxy-6-oxo-2,4-heptadienoate hydrolases. The procedures used for PsU and P23X1 differed only in details such as the volume of samples applied to columns, the volume of fractions collected, and the flow rate of eluants. All steps were carried out at 2 to 4°C. After determination of enzyme activity in eluted fractions, only those in which the specific activity was >10% of the most active fraction were pooled for the subsequent step unless stated otherwise.

Step 1. Cell extracts were treated with 0.1 volume of 2% (wt/vol) protamine sulfate. After equilibration for 30 min, the precipitate was removed by centrifugation at 25,000 × *g* for 20 min.

Step 2. The supernatant fluid from step 1 was brought to 30% saturation by the slow addition of solid ammonium sulfate. After equilibration for 30 min, the precipitated protein was removed by centrifugation at 20,000 × *g* for 15 min. The supernatant solution was

brought to 50% ammonium sulfate saturation and, after equilibration, the precipitate was collected by centrifugation and dissolved in 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, pH 7.6. The solution was dialyzed against the same buffer until free of ammonium ions.

Step 3. The dialyzed solution was applied to a diethylaminoethyl (DEAE)-cellulose column (5 by 50 cm) that had been equilibrated with 0.05 M Tris buffer, pH 7.6; the column was washed with the same buffer, and the protein was eluted with 2 liters of a linear gradient of 0 to 1 M NaCl in the same buffer. Pooled enzyme fractions were dialyzed against 0.05 M Tris buffer, pH 8.0.

Step 4. The dialyzed protein solution was applied to a DEAE-cellulose column (2.5 by 30 cm) equilibrated with 0.05 M Tris buffer, pH 8.0, and washed with the same buffer, and the protein was eluted with 800 ml of a linear gradient of 0 to 0.8 M NaCl in the same buffer.

Step 5. Fractions with activity of at least 20% of that in the most active fraction were pooled, concentrated to 10 ml by ultrafiltration (PM-30 Amicon membrane), applied to the top of a Sephadex G-150 column (2.6 by 60 cm), and eluted with 0.05 M Tris buffer, pH 7.6.

Step 6. Pooled fractions were dialyzed against 0.05 M Tris buffer, pH 7.1, applied to a DEAE-cellulose column (2.5 by 30 cm), and washed with the same buffer containing 0.15 M NaCl, and the protein was eluted with a 0.2 to 0.8 M NaCl linear gradient in the same buffer (200 ml for PsU and 1 liter for P23X1).

Step 7. Those fractions in which the specific activity was at least 50% of that in the fraction with the highest activity were pooled, dialyzed against 0.05 M Tris buffer, pH 8.0, and applied to a DEAE-cellulose column (2.5 by 20 cm). After washing with the same buffer, the protein was eluted with 1 liter of a 0 to 0.5 M NaCl linear gradient in the same buffer.

Fractions in which the elution profile for protein was coincident with enzyme activity were examined by polyacrylamide gel electrophoresis, and those fractions in which only one band was detected were pooled. A portion of the pooled fractions that was to be used for amino acid analysis and for production of antisera was dialyzed to remove salts and then lyophilized. The remainder of the pooled material was kept at 2 to 4°C, and examinations for substrate specificity, K_m values, pH optima, inhibition/activation studies, molecular weight, and subunit structure were completed within 5 to 6 days.

RESULTS

A summary of the purification procedures and yields obtained for the enzymes of both strains is shown in Table 2.

Molecular weight and subunit structures. Molecular weights as determined on Sephadex G-200 were 118,000 for the hydrolase from PsU and 100,000 for the hydrolase from P23X1.

Polyacrylamide gel electrophoresis of both enzymes (run separately) showed only one band in each case (Fig. 1). Examination of each enzyme by polyacrylamide gel electrophoresis in the presence of SDS was carried out on samples before and after reduction with mercaptoethanol. With both hydrolases no difference was found after reduction, a single band being obtained in each case (Fig. 2). With bovine serum albumin, ovalbumin, chymotrypsinogen, and cytochrome *c* as standards, the molecular weight of the subunits for the hydrolase from PsU was 27,700 and that from P23X1 was 25,000. Therefore, both hydrolases appear to be tetramers with four subunits of similar molecular weight.

TABLE 2. *Purification steps of 2-hydroxy-6-oxo-2,4-heptadienoate hydrolase*

Purification step	Vol (ml)	Protein (mg)	Sp act[a]	Total activity	Purification	Recovery (%)
P. putida NCIB 10015 (PsU)						
Crude extract	1,385	48,129	0.093	4,477		100
Protamine sulfate	1,445	44,433	0.093	4,132	1	92
Ammonium sulfate (30 to 50%)	392	11,172	0.27	3,016	2.9	67
DEAE-cellulose, pH 7.6	460	8,326	0.30	2,514	3.2	56
DEAE-cellulose, pH 8	20	978	1.51	1,476	16.2	33
Sephadex G-150	9.7	182	4.56	829	49	18
DEAE-cellulose, pH 7.1	14.5	107	5.9	631	63	14
DEAE-cellulose, pH 8	10	29	12.5	368	134	8
P. putida NCIB 9865 (P23X1)						
Crude extract	1,345	48,823	0.34	16,600		100
Protamine sulfate	1,440	47,088	0.38	17,890	1.1	108
Ammonium sulfate (30 to 50%)	216	11,664	0.82	9,506	2.4	57
DEAE-cellulose, pH 7.6	200	4,200	2.04	8,568	6.0	52
DEAE-cellulose, pH 8	8.2	1,232	4.24	5,225	12.4	31
Sephadex G-150	20.5	164	28.1	4,613	82.6	28
DEAE-cellulose, pH 7.1	10	24	115.9	2,752	340	16
DEAE-cellulose, pH 8	15	10	204	2,040	600	12

[a] Micromoles of substrate utilized per minute per milligram of protein.

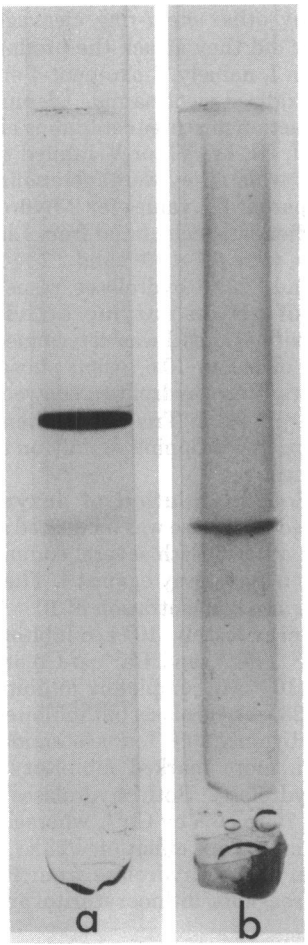

FIG. 1. *Polyacrylamide gel electrophoresis of purified 2-hydroxy-6-oxo-2,4-heptadienoate hydrolases. Protein (30 μg) was applied to the gels, and electrophoresis was carried out in Tris-glycine buffer, pH 8.9. (a) P23X1 hydrolase; (b) PsU hydrolase.*

Immunological specificity. Antisera prepared against each purified hydrolase were examined by immunodiffusion against both purified enzymes (Fig. 3). A precipitin band was found between P23X1 hydrolase and its homologous antiserum, whereas there was a major and a minor band between PsU hydrolase and its homologous antiserum. No cross-reaction was detected between the PsU hydrolase and antiserum to P23X1 hydrolase, but there was a precipitin line between P23X1 hydrolase and antiserum to PsU hydrolase, which appeared to be a line of identity between PsU hydrolase and its homologous antiserum. No precipitin reaction was detected between anti-PsU hydrolase and extracts of uninduced cells of either PsU or P23X1. A very faint precipitin band was detected between anti-P23X1 hydrolase and both uninduced cell extracts.

Amino acid composition. The amino acid compositions of each hydrolase are given in Table 3, and they show an overall similarity. The low cysteic acid values are in agreement with the observation that the subunits of the enzymes are unlikely to be linked by disulfide bands, as no difference was found after SDS electrophoresis of reduced and nonreduced samples.

Heat stability. The results shown in Fig. 4 indicate that, under the conditions used, there is little difference in the heat stability of the two purified hydrolases.

Kinetic properties. Specificity of the hydrolases to products of *meta*-ring cleavage of several

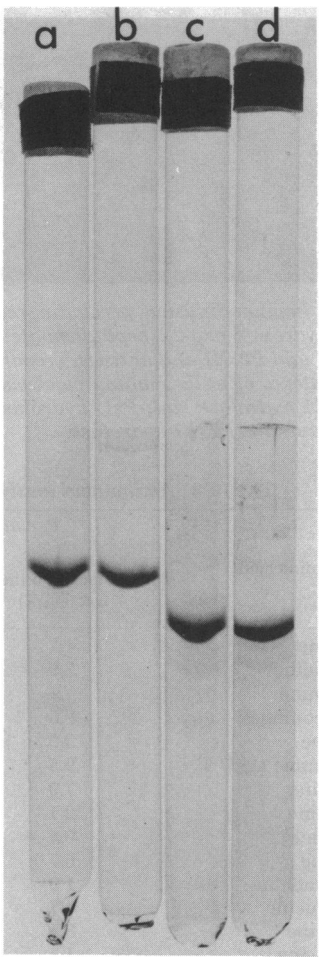

FIG. 2. *SDS-polyacrylamide gel electrophoresis of purified 2-hydroxy-6-oxo-2,4-heptadienoate hydrolases. Samples were run before and after reduction with mercaptoethanol. (a) PsU nonreduced; (b) PsU reduced; (c) P23X1 nonreduced; (d) P23X1 reduced.*

dihydroxy aromatic compounds is shown in Table 4. Both preparations attacked the same substrates, but there were differences in the comparative rates of attack on some compounds. Compounds II and III were attacked at about the same rate by the hydrolase from PsU, which was 3 to 7% of the rate at which compound I was attacked. The hydrolase from P23X1 attacked II and III at 1 to 3% of the rate of attack on I, whereas activity against VII was 67% of that against I, compared with 14% for the PsU enzyme. The comparative rate of attack on VI by PsU hydrolase was four times that of the P23X1 hydrolase. Neither preparation had activity against any other *meta*-ring cleavage product tested, nor did they attack the product of their reaction on I, namely, 2-oxo-pent-4-enoate. Addition of oxidized nicotinamide adenine dinucleotide to reaction mixtures containing either compound I, II, III, IV, VI, or VII failed to increase the rate at which they were metabolized.

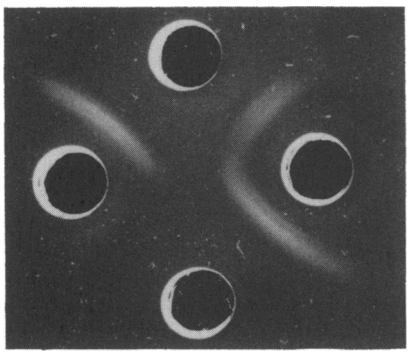

FIG. 3. *Immunodiffusion precipitin reactions between 2-hydroxy-6-oxo-2,4-heptadienoate hydrolases from PsU and P23X1 and antisera prepared against them. Contents of wells reading clockwise from the top: P23X1 hydrolase, anti-PsU hydrolase, PsU hydrolase, and anti-P23X1 hydrolase.*

The apparent K_m values for 2-hydroxy-6-oxo-2,4-heptadienoate, calculated from Lineweaver-Burk plots, were 6.7×10^{-6} and 2.7×10^{-5} M for the PsU and P23X1 hydrolases, respectively.

Effect of pH on enzyme activity. Enzymatic activity against I was determined over the pH range of 5.0 to 10.6, using phosphate and Tris buffers. Both hydrolases showed maximal activity at pH 8.6 in Tris buffer, the activity of the PsU enzyme dropping rapidly on either side of that range.

Inhibition/stimulation of enzyme activity. Purified hydrolase was incubated separately for 30 min at 20°C with several compounds and metal ions before assay against I. The following metal ions at a concentration of 10^{-3} M showed slight (approximately 10%) inhibitory effects: Mg^{2+}, Mn^{2+}, Fe^{2+}, and Ca^{2+}. *p*-Chloromercuribenzoate (10^{-3} M) completely inhibited the activity of P23X1 hydrolase, but inhibited the PsU hydrolase by only 20%. Iodoacetamide (10^{-3} M) also had a more marked inhibitory effect on P23X1 hydrolase. Both hydrolases were inhibited to 75 to 80% by Cu^{2+}, whereas Zn^{2+} had a greater inhibitory effect on P23X1 hydrolase (40%) than on the hydrolase from PsU (20%). Reducing reagents, dithioerythritol and reduced

TABLE 3. *Amino acid analysis of 2-hydroxy-6-oxo-2,4-heptadienoate hydrolases*

Amino acid	*P. putida* NCIB 10015		*P. putida* NCIB 9865	
	Composition (mol fraction %)	No. of residues per 27,700 molecular weight[a]	Composition (mol fraction %)	No. of residues per 25,000 molecular weight[a]
Lysine	1.7	5	1.3	3
Histidine	2.9	8	1.9	5
Arginine	7.1	20	4.7	12
Threonine	2.5	7	2.9	7
Serine	3.9	11	4.9	12
Glutamic acid	9.5	26	9.9	25
Glycine	7.9	22	8.6	22
Alanine	12.1	34	11.0	28
Cystine[b]	0.4	1	0.3	1
Valine	5.7	16	5.6	14
Methionine	1.9	5	1.9	5
Isoleucine	4.7	13	4.4	11
Leucine	8.5	24	7.9	20
Tyrosine	1.7	5	1.7	4
Phenylalanine	3.7	10	3.7	9
Aspartic acid	8.8	24	8.2	22
Proline	4.4	12	4.2	11

[a] To the nearest integer.
[b] Determined as cysteic acid.

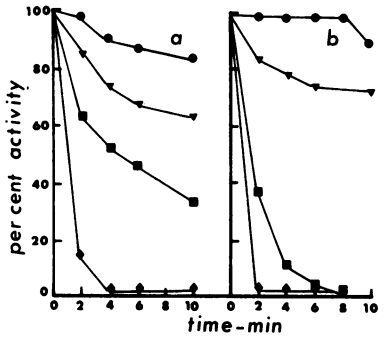

FIG. 4. *Thermal inactivation of 2-hydroxy-6-oxo-2,4-heptadienoate hydrolase. Samples (1 ml) of semipurified (50-fold) enzyme were heated at 40°C (●), 45°C (▼), 50°C (■), and 55°C (◆) for various times, chilled in ice, and assayed. (a) PsU; (b) P23X1.*

glutathione, both at 10^{-3} M, had less than a 5% inhibitory effect on the activity of either hydrolase.

DISCUSSION

The purified hydrolases from the two strains of *P. putida* used in this study have several properties that are very similar. Their molecular weights differ only slightly, and both are composed of four subunits that are identical within each enzyme and that do not appear to be linked by disulfide bonds. The subunit size of 25,000 to 27,700 is similar to that reported by Collinsworth et al. (7) for the hydratase that catalyzes the subsequent step in the degradation of *m*-cresol. Kinetic properties such as K_m values, pH optima, and the range of substrates attacked are also very similar, significant rates of metabolism occurring only of substrates that contain a keto group as a result of *meta* cleavage of catechols substituted in the 3 position. The major difference in this area is the much higher activity against 2-hydroxy-5-methyl-6-oxo-2,4-heptadienoate of the P23X1 hydrolase compared with the hydrolase from PsU. This compound is the product presumed to result from *meta* cleavage of 3,4-dimethylcatechol, which has been suggested by Chapman (5) to be a metabolite of 2,3-xylenol, which was used for selective isolation of P23X1 by D. Hopper (personal communication). PsU can utilize 2,3-xylenol as a sole carbon source, but no studies have been carried out to determine whether the lower activity of its hydrolase against VII results in PsU having a slower growth rate on 2,3-xylenol than has P23X1. Although the activity of the PsU hydrolase against 2-hydroxy-5-methylmuconic semialdehyde is low, it is sufficient to metabolize this compound to 2-oxohex-4-enoate in an in vitro situation, as Bayly and Dagley (2) demonstrated the formation of the latter compound with semipurified hydrolase preparations. However, this activity apparently is too low to support the growth of PsU on *p*-cresol when either the aldehyde dehydrogenase, tautomerase, or decarboxylase is absent, as it has been shown that mutant strains defective in any of these enzymes cannot utilize *p*-cresol for growth even though such strains still have an active hydrolase (4, 26). The greater sensitivity of the P23X1 hydrolase to *p*-chloromercuribenzoate and iodoacetamide may indicate either a greater availability of sulfhydryl groups to react with these compounds than is the case with the PsU hydrolase, even though the concentration of sulfur-containing amino acids is almost the same in both enzymes (Table 2), or that the PsU hydrolase possesses a relatively labile sulfhydryl group that has been modified during the purification procedures.

What may be a significant difference between the purified hydrolases and other *meta*-cleavage pathway enzymes that have been studied is the lack of stimulation of the hydrolase activity by divalent metal ions such as Mn^{2+} and Mg^{2+}. Collinsworth et al. (7) showed that the hydratase, which is sequential to the hydrolase, is activated by Mn^{2+}, as is the aldolase that cleaves the product formed by the action of the hydratase (3). Although the decarboxylase of this pathway has not been purified, it has been shown that it is stimulated by Mg^{2+} and Mn^{2+} (18, 19). Catechol 2,3-oxygenase has been shown to contain Fe^{2+}, which is essential for activity

TABLE 4. *Substrate specificity of 2-hydroxy-6-oxo-2,4-heptadienoate hydrolase from strains of P. putida*

Substrate	Activity[a]	
	P. putida NCIB 10015	*P. putida* NCIB 9865
2-Hydroxy-6-oxo-2,4-heptadienoate (I)	100 (12.5)[b]	100 (204)[b]
2-Hydroxymuconic semialdehyde (II)	7	3
2-Hydroxy-5-methylmuconic semialdehyde (III)	3	1
2-Hydroxy-5-methyl-6-oxo-2,4-heptadienoate (VII)	14	67
2-Hydroxy-7-methyl-6-oxo-2,4-octadienoate (VI)	20	5
5-Carboxymethyl-2-hydroxymuconic semialdehyde (IV)	0	0
Maleylpyruvate (V)	0	0
2-oxopent-4-enoate	0	0

[a] Expressed as a percentage of the activity determined with 2-hydroxy-6-oxo-2,4-heptadienoate.

[b] Specific activity expressed as micromoles of substrate used per minute per milligram of protein.

(11). In other *meta*-cleavage pathways, Leung et al. (13) have shown Mn^{2+} and Mg^{2+} stimulation of 4-hydroxy-2-oxopimelate aldolase from an *Acinetobacter* sp., and Tack et al. (23) showed a similar stimulation of the aldolase that cleaves 4-carboxy-4-hydroxy-2-oxoadipate, a metabolite of the catabolism of gallic acid by a strain of *P. putida*. The possible role and evolutionary significance of divalent metal ions in *meta*-pathway degradation of aromatic compounds has been discussed by Dagley (9).

Immunological specificity of enzymes has been used to compare enzymes acting in the same and analogous pathways (6, 15, 22). In the present study there was some cross-reaction between P23X1 hydrolase and the antiserum prepared against PsU hydrolase, but no cross-reaction was detected between PsU hydrolase and antiserum to P23X1 hydrolase. Stanier et al. (22) reported that the muconolactone isomerase and muconate-lactonizing enzyme of 14 strains of *P. putida* biotype A gave immunodiffusion patterns that indicated identity or near identity of their isomerases or lactonizing enzymes, respectively. They also reported that microcomplement-fixation studies showed that immunological homology of enzymes within the biotype A strains tested was not complete. The amino acid composition of the two hydrolases in this study showed differences, for example, in histidine and arginine, which suggests that their sequences may be sufficiently different to account for their comparative lack of immunological homology, even though both strains are biotype A *P. putida*. On the basis of the reaction of identity between the two hydrolases and antibody to PsU hydrolase, they must have at least one antigenic determinant in common. No detailed studies on *meta*-pathway hydrolases from other species of *Pseudomonas* or other genera appear to have been reported; therefore, comparisons with analogous enzymes cannot be made at this time. Ribbons (17) has described a strain of *P. aeruginosa* T1 that degrades *o*-cresol by a route involving hydrolysis of 2-hydroxy-6-oxo-2,4-heptadienoate, and strain 345 of *Alcaligenes eutrophus*, used by Johnson and Stanier (12), has been reported by Bayly et al. (R. C. Bayly, P. L. Venegas, and P. J. Chapman, Abstr. Annu. Meet. Am. Soc. Microbiol. 1974, P133, p. 166) to utilize the same route for metabolism of phenol; examination of the hydrolases from these organisms is proceeding.

Comparative study of the hydrolases with the oxidized nicotinamide adenine dinucleotide-dependent aldehyde dehydrogenase that acts in the degradative pathways of phenol and *p*-cresol in P23X1 and PsU would be of value, as it has been suggested by Dagley (9) that the divergence of *meta* pathways at this point may be of evolutionary interest. Repeated attempts to isolate the dehydrogenase from these strains has failed, due to the apparent instability of the enzyme under the purification procedures used, and such comparison must await some method of stabilization of the enzyme.

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