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Purification of two isofunctional hydrolases (EC 3.7.1.8) in the degradative pathway for dibenzofuran in *Sphingomonas* sp. strain RW1

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Abstract

Sphingomonas sp. strain RW1, when grown in salicylate-salts medium, synthesized the enzymes for the degradation of dibenzofuran. The reaction subsequent to *meta* cleavage of the first benzene ring was found to be catalyzed by two isofunctional hydrolases, H1 and H2, which were purified by chromatography on anion exchange, hydrophobic interaction and gel filtration media. Each enzyme was able to hydrolyze 2-hydroxy-6-oxo-6-(2-hydroxyphenyl)hexa-2,4-dienoate and 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate to produce salicylate and benzoate, respectively. SDS/PAGE of each purified enzyme showed a single band of M_r 31 000 (H1) or 29 000 (H2). The N-terminal amino acid sequences of the two proteins showed 50% homology.

Abbreviations: DHB – 2,3-dihydroxybiphenyl, DSM – German Culture Collection (Braunschweig), FPLC – protein liquid chromatograph(y), HOHPDA – 2-hydroxy-6-oxo-6-(2-hydroxyphenyl)hexa-2,4-dienoate, HOPDA – 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate, THB – 2,2',3-trihydroxybiphenyl

Introduction

Degradative pathways of dibenzofuran (Engesser et al. 1989; Fortnagel et al. 1989, 1990; Strubel et al. 1991; Wilkes et al. 1992; Wittich et al. 1992) and biphenyl (Higson 1992) have been elucidated (Fig. 1).

Whereas there is an increasing understanding of multi-component dioxygenases (Correll et al. 1992; Harayama et al. 1992; Mason & Cammack 1992) and *meta*-cleavage enzymes (Lipscomb et al. 1988; Harayama et al. 1992) much less is known about the hydrolytic cleavage of C-C bonds (EC 3.7.1.-) in the degradation of aromatic compounds. Three 2-hydroxy-6-oxo-2,4-heptadienoate hydrolases (EC 3.7.1.9; Bayly & Di Berardino 1978; Duggleby & Williams 1986) from strains of *Pseudomonas putida* and a HOPDA-hydrolyzing enzyme from the biphenyl-degrading *Pseudomonas cruciviae* strain S93B1 (EC 3.7.1.8; Omori et al. 1986a) have been reported. In this paper we describe the purification of two isofunctional enzymes (EC 3.7.1.8) from *Sphingomonas* sp. strain RW1, an organism which is able to utilize dibenzofuran and dibenzo-*p*-dioxin as sole source of carbon and energy for growth (Wittich et al. 1992).

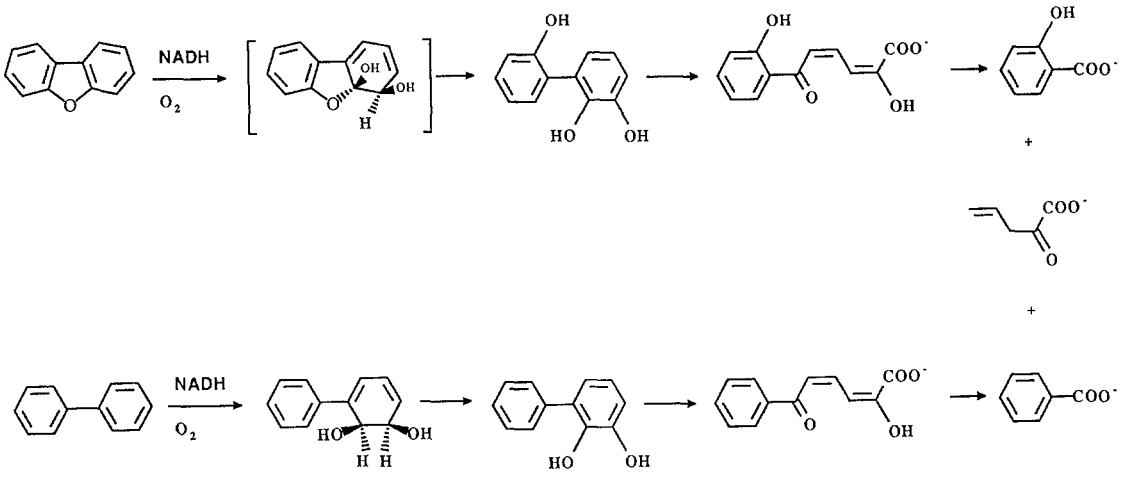

Fig. 1. Initial reactions in selected degradative pathways of dibenzofuran and biphenyl. The initial reaction in each case is catalyzed by a multi-component dioxygenase (Bünz & Cook 1993; Haddock et al. 1993). Biphenyl 2,3-dioxygenase yields a stable *cis*-diol which is converted to the catechol (2,3-dihydroxybiphenyl) by a dehydrogenase (Gibson et al. 1973; Higson 1992). Dibenzofuran-4,4a-dioxygenase forms an unstable hemiacetal which decays spontaneously to the catechol (2,2',3-trihydroxybiphenyl). The catechol is subject to *meta* ring cleavage yielding the chemically reactive intermediates 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (HOPDA, from biphenyl) and 2-hydroxy-6-oxo-6-(2-hydroxyphenyl)hexa-2,4-dienoate (HOHPDA, from dibenzofuran); the depiction of the *cis,cis* isomer is purely formal, given the rapid spontaneous keto-enol tautomerism (Omori et al. 1986a,b; Whitman et al. 1991). There follows a hydrolytic step which converts HOPDA (HOHPDA) to benzoate (salicylate) and 2-oxopentenoate (Omori et al. 1986a; Strubel et al. 1991). Whereas this is the only characterized degradative pathway for dibenzofuran, alternative fates for (chloro)biphenyl are known, e.g. the reduction of HOPDA (Omori et al. 1986b; Ahmad et al. 1991; Higson 1992).

Materials and methods

Materials

Dibenzofuran (Aldrich, Milwaukee, WI, USA), sodium salicylate (Fluka, Buchs, Switzerland), 2,3-dihydroxybiphenyl (Wako Chemicals, Neuss, Germany), DNase I (Boehringer Mannheim GmbH, Germany) and aprotinin (Sigma, St. Louis, MO, USA) were from commercial sources. THB was kindly provided by Dr. H. Harms (cf. Fortnagel et al. 1990). HOHPDA (HOPDA) was prepared in 10-ml reaction mixtures from 0.5 mmol THB (1 mmol 2,3-dihydroxybiphenyl) by oxygenation with 0.2 mg of partially purified THB 1,2-dioxygenase from *Sphingomonas* sp. strain RW1 (see below) in 50 mM potassium phosphate buffer, pH 7.4. The proteins were removed by membrane filtration (Centriprep-10). The concentration of the yellow product was determined photometrically; a molar absorption coefficient of 13 000 l. $(mol.cm)^{-1}$ at 432 nm was used (Omori et al. 1986a). HOPDA was stable at 4° C for one week, whereas HOHPDA showed a loss of colour after 15 min. The FPLC columns DEAE-Sepharose CL-6B (26 by 145 mm), and commercially-packed Mono Q HR (16 by 100 mm), Phenyl Superose HR (5 by 50 mm) and Superose 12 or 6 HR (10 by 300 mm) were from Pharmacia (Uppsala, Sweden) which also provided Blue Dextran 2000, molecular weight standards for gel filtration and PD-10 columns of Sephadex G-25. Molecular weight standards for PAGE, reagents for SDS/PAGE and premade gradient PAGE gels (4 to 20%) were from BIO-RAD, Richmond, CA, USA. Membrane filtration units were from Amicon (Danvers, MA, USA). The sources of other chemicals are in Locher et al. (1991).

Analytical methods

Spectrophotometric determinations were done with a Uvikon 820 (Kontron, Zürich, Switzerland). Protein concentration was assayed by the method of Bradford (1976) with bovine serum albumin as the standard. Reversed-phase HPLC was done at room temperature with 50% (v/v) methanol in 10 mM potassium phosphate buffer, pH 2.2, as the

mobile phase (Locher et al. 1989). FPLC was done with apparatus from Pharmacia (the DEAE column at 4° C) or with HPLC apparatus at room temperature (Locher et al. 1991). The mobile phase was sparged with oxygen-free helium and fractions were collected on ice under a stream of oxygen-free N_2.

The progress of protein purifications and the M_r of proteins under denaturing conditions was determined by SDS/PAGE; gels were routinely stained with Coomassie brilliant blue and occasionally with silver (Laemmli 1970; Merril et al. 1983; Locher et al. 1991). The estimation of M_r under native conditions was done routinely by gel filtration chromatography with a Superose column at flow rates of 0.3 to 0.6 ml/min. The eluent was 50 mM Tris sulphate, pH 7.5, containing 150 mM Na_2SO_4. The molecular weight standards were ferritin (440 000), aldolase (158 000), bovine serum albumin (67 000), cytochrome c (12 500) and aprotinin (6 800). On occasion, gradient gels under native conditions were used. The broad range standard from BIO-RAD was used for molecular weight determination.

The N-terminal amino acid sequence of a purified and desalted enzyme, or of a blotted protein, was determined by automated Edman degradation (Locher et al. 1991).

HOHPDA-hydrolase was routinely assayed at 25° C as the decrease in absorbance of the substrate at 432 nm (Omori et al. 1986a). The reaction mixture (1.0 ml) contained 37.5 µmole of potassium phosphate buffer, pH 7.4, 0.05 to 1 mg of protein and 50 nmole of HOPDA (or HOHPDA), with which the reaction was started. The hydrolases were sometimes determined by HPLC as benzoate (salicylate) formed from HOPDA (HOHPDA).

THB-1,2-dioxygenase was detected as the formation of HOHPDA from THB; the enzyme was routinely assayed at 432 nm as formation of HOPDA from 2,3-dihydroxybiphenyl, using a modified method of Ishigooka et al. (1986) without ferrous sulfate. Alternatively, oxygen uptake was determined in a Clarke-type oxygen electrode with a thermostated (30° C) 1-ml reaction vessel (Rank Bros., Bottisham, Cambs., UK). The reaction mixture (1.0 ml) contained 49 µmole of potassium phosphate buffer, pH 7.4, 5 to 200 µg of protein and 300 nmole of 2,3-dihydroxybiphenyl (dissolved in acetone) with which the reaction was started.

Catechol-2,3-dioxygenase (EC 1.13.11.2) was assayed as production of 2-hydroxymuconate semialdehyde from catechol (Bird & Cain 1974).

Organism, growth conditions and preparation of cell-free extract

Sphingomonas sp. strain RW1 (DSM 6014) was stored at 4° C on slants of dibenzofuran-salts medium, from which 25-ml precultures (in 100-ml Erlenmeyer flasks) containing 5 mM salicylate-salts medium [the salts medium of Fortnagel et al. (1990) supplemented with trace elements (5 ml/l; Drews 1983)] were inoculated. Cultures were grown at 30° C on an orbital shaker and cultures in the mid-exponential phase of growth were used to inoculate (1.5% v/v) the main cultures (1.5 l in 5-l Erlenmeyer flasks). The main culture was grown for 18 h, when cells were harvested by centrifugation (5 000 g, 20 min, 4° C), washed twice in chilled 50 mM Tris-HCl buffer, pH 7.5, (10 000 g, 15 min, 4° C) and stored at – 20° C.

Frozen cells (20 g) were suspended (1 g/ml) in 50 mM Tris-HCl, pH 7.5, containing 1 mM DTT. After addition of 0.2 mg/ml DNase and PMSF to 0.1 mM, cells were disrupted by three passages through a chilled French press (130 MPa). Undisrupted cells and other particulate matter were removed by centrifugation (20 000 g, 30 min, 4° C) and ultracentrifugation (150 000 g, 60 min, 4° C), and cell extract was used immediately. Fractionated proteins were routinely frozen at – 20° C.

Results

Purification of two isofunctional HOHPDA hydrolases

HOHPDA hydrolase was initially detected as the enzyme-catalyzed disappearance of HOHPDA, whereby substrate disappearance was followed photometrically and salicylate formation was detected by HPLC. When isofunctional enzymes were

Table 1. Buffer systems, all pH 7.5, with 1 mM DTT, used as mobile phases in FPLC.

Column	Buffer A	Buffer B
DEAE	A1: 50 mM Tris-HCl	B1: 0.8 M Tris-HCl
Mono Q	A2: 20 mM Tris-SO_4	B2: A2 + 1 M Na_2SO_4
Phenyl Superose	A3: A2 + 0.8 M $(NH_4)_2SO_4$	B3: A2
Superose	A4: 50 mM Tris-SO_4 + 150 mM Na_2SO_4	none

detected, it was observed that both HOHPDA and HOPDA were substrates for each enzyme. HOHPDA was much less stable than HOPDA, and HOPDA was prepared from a commercially-available substrate, so each enzyme was routinely assayed with HOPDA as the substrate.

Cell-free extract (about 500 mg of protein in 15 ml buffer A1; Table 1) was applied to a DEAE-column equilibrated with buffer A1 at a flow rate of 2.5 ml/min, and 7-ml fractions were collected. The flow of buffer A1 was maintained for 60 min when a linear gradient to 100% buffer B1 in 120 min was started. Hydrolase activity was detected in a non-resolved double peak between fractions 42 and 49, about 450 to 550 mM Tris-HCl (not shown). Fractions 43 to 48 were pooled and concentrated by membrane filtration.

Proteins from the DEAE column were separated on a Mono Q column (Fig. 2). Two HOHPDA hydrolases were clearly separated as sharp peaks of activity and they were designated H1 and H2. Hydrolase H1 eluted at about 20 mM Na_2SO_4 and fractions 28 to 31 were pooled and concentrated for further purification. Hydrolase H2 eluted at about 65 mM Na_2SO_4 and fractions 46 to 49 were pooled and concentrated.

Hydrolase H1 from the Mono Q column was brought to 0.8 M $(NH_4)_2SO_4$ by addition of 3 M $(NH_4)_2SO_4$, the pH was adjusted to 7.5 with 3 M KOH and the protein applied to a Phenyl Superose column equilibrated with buffer A3; fractions of 0.5 ml were collected. After 10 min at a flow rate of 0.5 ml/min, the concentration of $(NH_4)_2SO_4$ was decreased to 0 M over 40 min. Active fractions eluted at about 210 mM $(NH_4)_2SO_4$ with the major protein peak observed and were concentrated (not shown).

Hydrolase H2 precipitated on the addition of $(NH_4)_2SO_4$ in the range of 0.4 to 1.7 M. The supernatant fluid was discarded, the precipitate was dissolved in buffer A2 containing 0.3 M-$(NH_4)_2SO_4$,

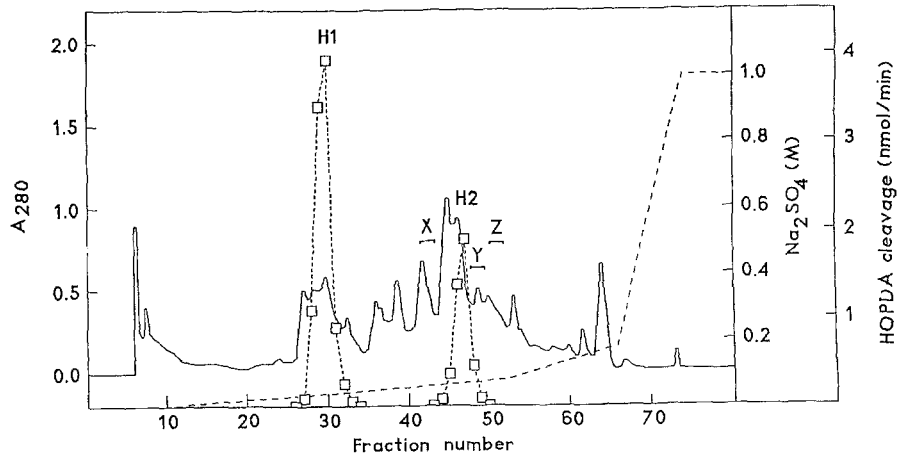

Fig. 2. Separation of two HO(H)PDA-hydrolyzing enzymes by anion exchange chromatography. A Mono Q column was equilibrated with buffer A2 (Table 1) at 4 ml/min and 4-ml fractions were collected. Concentrated eluate (2 ml) from the DEAE column was applied. After 5 min a gradient of buffer B2 was started (0 to 8% buffer B2 over 35 min, 8 to 18% B2 over 10 min; the flow rate was reduced to 2.5 ml/min for the latter portions of the gradient, 18 to 100% B2 over 10 min and 10 min 100% B2). The bars labelled X, Y and Z represent THB-1,2-dioxygenase I, catechol 2,3-dioxygenase and THB-1,2-dioxygenase II, respectively.

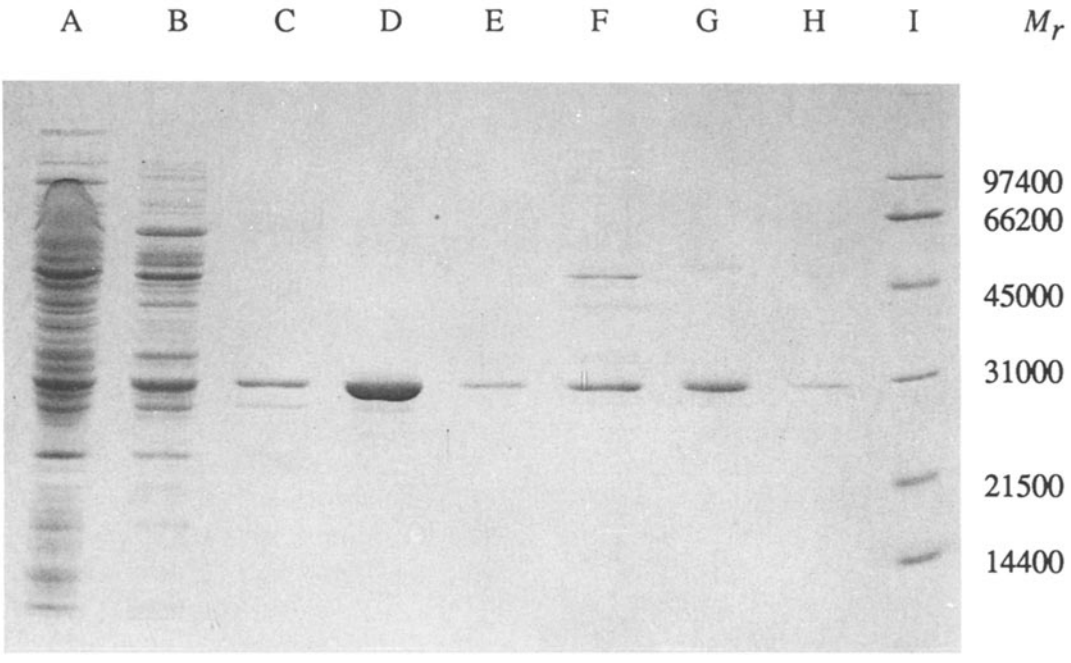

Fig. 3. SDS/PAGE of different steps during the purifications of HOHPDA-hydrolases H1 and H2. Proteins were detected by staining with Coomassie Brilliant Blue R250. Lanes: A, crude extract (10 μg of protein); B, pooled fractions of both hydrolases eluted from the DEAE column (6 μg); C, pooled fractions of hydrolase H1 eluted from the Mono Q column (1 μg); D, pooled fractions of hydrolase H1 eluted from the Phenyl Superose column (2 μg); E, pooled fractions of hydrolase H1 eluted from the Superose 12 column (1 μg); F, pooled fractions of hydrolase H2 eluted from the Mono Q column (2 μg); G, pooled fractions of hydrolase H2 eluted from the Phenylsuperose column (2 μg); H, pooled fractions of hydrolase H2 eluted from the Superose 12 column (1 μg); I, standard marker proteins (each about 1 μg).

applied to the Phenyl Superose column equilibrated with buffer A3 and eluted as for H1. Hydrolase H2 eluted at about 360 mM $(NH_4)_2SO_4$ with the largest of the three protein peaks observed and was concentrated (not shown).

The last step in the purification of each hydrolase was gel filtration (Superose 12) with buffer A4 at a flow rate of 0.4 ml/min as the mobile phase; fractions of 0.5 ml were collected. Each hydrolase gave a single symmetrical protein peak which co-eluted with the activity peak (not shown). Each pure enzyme was concentrated and stored at – 20° C prior to establishing some of its properties.

The effectivity of the different purification steps is illustrated in Fig. 3. Neither hydrolase contains significant impurities after gel filtration.

Purified hydrolases H1 and H2 could be stored at 4° C for 48 h with little loss of activity (10% H1, 20% H2). No activity was lost after one week at – 20° C or – 70° C in buffer containing 30% glycerol. The

Table 2. Purification of hydrolase H1.

Step	Volume (ml)	Total protein (mg)	Total activity (mU)	Specific activity (mU/mg)	Yield (%)	Purification (fold)
Crude extract[a]	15	375	2920	7.8	100	1
DEAE[a]	18	69	752	10.9	25.8	1.4
Mono Q	4	5	533	107	18.3	13.7
Phenyl Superose	2	1.31	360	274	12.3	35.3
Superose	1.5	0.8	226	282	7.7	36.2

The activity of hydrolase H1 was measured as HOPDA consumption.[a] Activity of hydrolase H2 was also present.

Table 3. Purification of hydrolase H2.

Step	Volume (ml)	Total protein (mg)	Total activity (mU)	Specific activity (mU/mg)	Yield (%)	Purification (fold)
Crude extract[a]	15	375	2920	7.8	100	1
DEAE[a]	18	69	752	10.9	25.8	1.4
Mono Q	2.1	2.78	121	43.5	4.1	5.6
Phenyl Superose	1.5	0.92	52	56.5	1.8	7.2
Superose	1.5	0.505	31.3	61.9	1.1	7.9

The activity of hydrolase H2 was measured as HOPDA consumption. [a] Activity of hydrolase H1 was also present.

purification procedure for the two hydrolases has been repeated five times and representative purifications are presented (Tables 2 and 3). Typically 15 g (wet weight) of cells yielded 0.8 mg of pure hydrolase H1 and 0.5 mg of pure hydrolase H2.

Detection of two isofunctional THB 1,2-dioxygenases

The fractions collected from the DEAE column to purify hydrolases H1 and H2 were observed to contain a major portion of an unresolved double peak of THB 1,2-dioxygenase activity. The presence of two isofunctional THB dioxygenases was confirmed on the Mono Q column, where baseline separation was obtained. These dioxygenases eluted at about 50 mM and 75 mM Na_2SO_4, straddling, but not significantly overlapping, hydrolase H2. Fractions of these dioxygenases from either anion exchange column were stable at – 20° C, but further purification was not attained, because the enzymes rapidly and irreversibly lost activity or were not separated. A catechol-2,3-dioxygenase, which had neither THB nor DHB activity was detected in the eluate from the Mono Q column at 70 mM Na_2SO_4. No catechol-1,2-dioxygenase activity could be found. THB dioxygenase 1 (Fig. 2) was used to prepare HO(H)PDA (see Methods section).

Some properties of hydrolases H1 and H2

The purified enzymes H1 and H2 are HOHPDA hydrolases, because each converted the substrate to salicylate; similarly, each enzyme converted HOPDA to benzoate. The reaction products were not formed stoichiometrically. The recoveries of salicylate and benzoate were 66% and 89%, respectively, with H1 and 45% and 75%, respectively, with H2. Unidentified, but presumably aromatic products were detected by HPLC. We had no satisfactory assay for 2-oxopentenoate.

The hydrolases have very similar M_r-values, 31 000 (H1) and 29 000 (H2), under denaturing conditions (Fig. 3). M_r-values for native proteins were estimated by gel filtration (Superose 12 and Superose 6) and native PAGE: 37 000, 43 000 and 40 000, respectively, were obtained for H1, and 41 000, 48 000 and 45 000, respectively, for H2. We presume the enzymes to be monomeric.

The amino acid sequence of the N-terminus of hydrolase H1 was:
Thr-Gln-Leu-Pro-Ser-Asn-Phe-Ile-Ala-Thr-Xaa-Gly-Tyr-Arg-Thr-His-Tyr-Ile-Glu-Met
whereas
Met-Phe-Glu-Gln-Phe-Glu-Ser-Lys-Phe-Ile-Asp-Xaa-Asp-Gly-Ile-Arg-Thr-His-Tyr-Ile
was determined for H2 and identical amino acids are underlined in the H1 sequence. There is 50% identity between the isoenzymes at the N-termini. No similar sequence was found in the SWISSPROT, PIR and MIPSX databases.

Discussion

HOHPDA hydrolases H1 and H2 are similar (M_r) but independent isofunctional enzymes with different properties (separation by ion exchange and hydrophobic interaction; different N-termini). They are representatives of a rare group of hydrolytic en-

zymes, those which cleave a C-C bond (EC 3.7.1.-). Only ten representatives (including EC 3.7.1.8 and 3.7.1.9), not all of which have been purified, are given in Enzyme Nomenclature 1992 (Webb 1992). One keto group and another labile bond are essential to facilitate the reaction and Omori et al. (1986a) present a reasonable reaction scheme for EC 3.7.1.8. Omori et al. (1986a) identified the nature of the C5 moiety cleaved from HOPDA as 2-oxopent-4-enoate, and Strubel et al. (1991) were able to detect the oxopentenoate from HO(H)PDA with partially purified enzymes. We thus presume that hydrolases H1 and H2 also release the C5-moiety as the oxopentenoate (and correspondingly that the hydrolases can be ascribed to EC 3.7.1.8), but this has yet to be confirmed in strain RW1. The reactivity of HOPDA, and more especially of HOHPDA, has the consequence that spontaneous reactions occur independent of the enzymic reaction. This has the consequence that the enzymic reaction apparently does not proceed stoichiometrically. This effect is thus not limited to enzyme reactions in vitro: Fortnagel et al. (1990), Strubel et al. (1991), Wittich et al. (1992) and Kohler et al. (1993) report the formation of chromanone derivatives from HOHPDA via a spontaneous intramolecular Michael addition in growing cells. So complete turnover of substrate does not mean complete mineralization of the compound.

The HOPDA hydrolase isolated by Omori et al. (1986a) is a homomultimer, possibly a hexamer (M_r 160 000) of subunit M_r 29 000. The 2-hydroxy-6-oxo-2,4-heptadienoate hydrolases from Bayly & Di Berardino (1978) appear to be homotetramers (M_r 118 000 and 100 000), with subunits of M_r 28 000 and M_r 25 000, respectively, whereas the hydrolase described by Duggleby & Williams (1986), encoded on a TOL plasmid, is a homodimer (M_r 65 000) with subunits of M_r about 32 000. Duggleby & Williams (1986) discuss the differences between these three hydrolases (EC 3.7.1.9) as well as the degrees of immunological relatedness. The enzymes we isolated have essentially the same M_r under denaturing conditions as those cited in EC 3.7.1.8 and EC 3.7.1.9, but we believe the enzymes from *Sphingomonas* to be monomeric. We have no other means of comparing our enzymes with those in EC 3.7.1.8 and EC 3.7.1.9, apart from the absence of cofactors in each case, and a search in a sequence database gave no related proteins. This will presumably reflect the low number of enzymes in EC 3.7.1.- and the difficulty of working biochemically or genetically with an enzyme whose labile substrate has to be synthesized enzymically.

In contrast to the failure to find in the database any proteins related to the HOHPDA hydrolases from *Sphingomonas* sp. strain RW1, the isoenzymes H1 and H2 share 50% sequence homology at the N-terminus. These enzymes are produced essentially constitutively, as are the two THB dioxygenases (see above) and two reductases for the dibenzofuran dioxygenase system (Bünz & Cook 1993). The reason for this is open to conjecture, but possibly expression of not one but two similar degradative pathways is constitutive. The other could be a portion of a biphenyl degradative pathway, because biphenyl-utilizing mutants of this organism occur spontaneously (Wittich et al. 1992).

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