Biochemical and molecular characterization of a periplasmic hydrolase for oxidized polyvinyl alcohol from *Sphingomonas* sp. strain 113P3

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Oxidized polyvinyl alcohol hydrolase (OPH) and polyvinyl alcohol dehydrogenase were found to be constitutively present in the periplasm of *Sphingomonas* sp. strain 113P3 (formerly *Pseudomonas* sp. 113P3). The OPH was purified to homogeneity with a yield of 40 % and a 5.9-fold increase in specific activity. The enzyme was a homodimer consisting of 35 kDa subunits. Its activity was inhibited by PMSF, Hg2+ and Zn2+. The enzyme hydrolysed oxidized polyvinyl alcohol (oxidized PVA) and p-nitrophenyl acetate (PNPA), but did not hydrolyse any of the mono- or diketones tested. *K*<sub>m</sub> and *V*<sub>max</sub> values for oxidized PVA and PNPA were 0.2 and 0.3 mM, respectively. The gene for OPH was cloned and sequenced. Sequencing analysis revealed that the open reading frame consisted of 1095 bp, corresponding to a protein of 364 amino acids residues, encoding a signal peptide and a mature protein of 34 and 330 amino acids residues, respectively. The presence of a serine-hydrolase motif (a lipase box; Gly-X-Ser-X-Gly) strongly suggested that the enzyme belongs to the serine-hydrolase family. The protein exhibited homology with OPH of the *Pseudomonas* sp. strain VM15C (63 % identity) and the polyhydroxybutyrate depolymerases from *Mesorhizobium loti*, *Rhizobium* sp. and *Sinorhizobium meliloti* (29–32 % identity). The *oph* gene was expressed in *Escherichia coli* under the control of the lac promoter. The recombinant protein had the same molecular mass and N-terminal amino acid sequence as the purified OPH from strain 113P3.

INTRODUCTION

Polyvinyl alcohol (PVA) is a water-soluble synthetic polymer, which is used in paper and textile mills and in copolymers as a biodegradable segment. PVA is the only known xenobiotic carbon-chain polymer to biodegrade at high molecular masses (Kawai, 1995), although natural rubber is a natural biodegradable carbon-chain polymer. The biodegradability of PVA is greatly dependent on pendant hydroxyl groups, which confer water-solubility and susceptibility to biological oxidation. Several groups have reported microbial degradation of PVA. Suzuki (1976, 1978) isolated *Pseudomonas* sp. O-3, which assimilates PVA as a sole carbon source, and purified PVA oxidase from its culture supernatant. Watanabe *et al*. (1975, 1976) and Sakai *et al*. (1985, 1986) isolated PVA-utilizing *Pseudomonas vesicularis* PD, and purified PVA oxidase and *β*-diketone hydrolase (oxidized PVA hydrolase, OPH). Both groups demonstrated that PVA was depolymerized by extracellular enzymes and the depolymerized molecules were incorporated and metabolized in cells. Sakazawa *et al*. (1981) and Shimao *et al*. (1984) reported that PVA is efficiently degraded by *Pseudomonas* sp. VM15C in the presence of pyrroloquinoline quinone (PQQ) or a PQQ-producing strain. The bacterium produces PVA oxidase and PVA dehydrogenase (PVADH). Although the role of PVA oxidase cannot be ruled out, PVADH and OPH (both membrane-associated proteins) are thought to be the major metabolic enzymes in the degradation of PVA (Shimao *et al*., 1996, 2000). A cell-free extract of *Escherichia coli* harbouring the

Abbreviations: OPH, oxidized polyvinyl alcohol hydrolase; PHB, polyhydroxyalkanoate; PNPA, p-nitrophenyl acetate; PQQ, pyrroloquinoline quinone; PVA, polyvinyl alcohol; PVADH, polyvinyl alcohol dehydrogenase.

The GenBank/EMBL/DDBJ accession number for the sequence reported in this paper is AB190288.
gene for OPH had only weak activity on 4,6-nonenedione (Shimao et al., 2000). Another PVA-utilizing pseudomonad, *Pseudomonas* sp. 113P3, was isolated by Hatanaka et al. (1995a, b, 1996) and its PVADH was purified from cell-free extract and characterized as a quinohaemoprotein. OPH, however, has been neither purified nor characterized from the strain. On the other hand, Matsumura et al. (1998, 1999) suggested that PQQ-dependent PVADH from *PVA*-utilizing *Alcaligenes faecalis* KK314 produced a monoketone structure in *PVA*, which was subsequently cleaved by an aldolase-type enzyme (apoPVADH). Thus the PVADHs from various *PVA*-utilizing bacteria have been shown to be quinohaemoproteins, but details of the second enzyme, either a diketone hydrolase or an aldolase-type enzyme, await further characterization.

In this paper, we report the purification and characterization of OPH from *Sphingomonas* sp. strain 113P3 (formerly *Pseudomonas* sp. 113P3). Cloning and sequencing of the gene was also performed. The results show that OPH from strain 113P3 has certain unique characteristics such as its localization, substrate specificities and molecular mass, as well as the gene itself.

**METHODS**

**Materials.** *PVA* 117 [number-average molar mass (‘average molecular weight’, *Mn*) 75 000] used in this study was a product of the Kuraray Co. PQQ was purchased from Wako Pure Chemical Industries. DEAE-Sepharose, CM-Sepharose, Phenyl Sepharose and Superdex 200 were from Amersham Pharmacia. Oxidized PVA was prepared as described previously (Shimao et al., 2000) except that the reaction was done in a total volume of 7.5 ml and *PVA* 500 (polymerization degree 500, *Mn* 22 000) was used (or omitted as a control). The oxidation rate of hydroxyl groups was calculated to be approximately 4-0 % from \( \varepsilon = 14.6 \text{ M}^{-1} \text{ cm}^{-1} \) (Shimao et al., 2000). All other chemicals were commercial products of the highest grade available.

**Bacterial strains and cultivation.** *Sphingomonas* sp. strain 113P3 (formerly identified as *Pseudomonas* sp. 113P3) was used throughout. The strain has the accession number FERM P-13483 in the International Patent Organism Depositary (IPOD) (Tsukuba, Japan). The strain was grown on *PVA* medium (pH 7.5) as reported previously (Hatanaka et al., 1995a). The *glucose* medium contained the same components as *PVA* medium except that glucose was added. The cells were harvested by centrifugation, washed twice with 0.85 % NaCl and kept at -80 °C until use. *E. coli* DH5x and *E. coli* transformants were grown at 37 °C in LB medium, supplemented with 50 μg ampicillin ml⁻¹ when necessary.

**Taxonomic identification.** As genetic evidence for identification, the partial 16S rDNA (tDNA) sequence of strain 113P3 was analysed, based on the methods of Rochelle et al. (1995). Nucleotide sequencing was carried out using an ABI PRISM 377-18 DNA sequencer and a BigDye Terminator Cycle sequencing Kit (Applied Biosystems) according to the manufacturers’ instruction manuals.

**Enzyme assay.** Preliminary studies on the substrate specificity of the purified enzyme revealed that OPH acted on oxidized *PVA* and *p*-nitrophenyl acetate (PNPA). Therefore OPH activity was routinely measured by using PNPA as a substrate. The reaction mixture contained 1 mM PNPA and 50 mM potassium citrate buffer (pH 6.0), which was preincubated at 37 °C for 1 min. The reaction was started by the addition of enzyme solution and carried out at 37 °C for 20 min. The enzyme activity was assayed in duplicate by measuring the decrease of A_{415} (due to *p*-nitrophenol liberated; \( \varepsilon = 2591 \text{ M}^{-1} \text{ cm}^{-1} \)) with a Shimadzu UV-160 spectrophotometer (1 cm light path). A reaction mixture without enzyme solution was used as a reference. One unit of enzyme activity was defined as the amount of enzyme that catalysed the hydrolysis of 1 μmol PNPA min⁻¹ under the assay conditions. Activity toward oxidized *PVA* was measured by the decrease of A_{490} in carbonate buffer (pH 10.0: \( \varepsilon = 14.6 \text{ M}^{-1} \text{ cm}^{-1} \)), as described previously (Shimao et al., 2000). The calculation for oxidized *PVA* was done as follows. As the oxidation rate of hydroxyl groups in *PVA* 500 was 4 %, 10 mol diketone structures must exist among 500 hydroxyl groups in 1 mol *PVA* 500. Thus the hydrolysis of 1 mol diketone corresponded to 0.1 mol oxidized *PVA*. Based on this calculation, specific activity and \( V_{\text{max}} \) values were measured. The activity toward mono- and diketones and esters was assayed by decrease in pH and determination of the carboxylic acid formed, which was spectrophotometrically analysed according to the method of Kasai et al. (1975), measuring the colour development with ferric hydroxamate of carboxylic acids. PVADH activity was measured as described previously (Shimao et al., 1986).

**Preparation of cell-free extracts, and periplasmic, cytoplasmic and membrane fractions.** Cells grown on *PVA* were suspended in 50 mM Tris/HiCl buffer (pH 8.0) and sonicated with a UD-200 ultrasonic disruptor (Tomiy Seiko Co.) at 20 kHz for 10 min below 4 °C, and the sonicate was centrifuged at 16 000 g for 30 min to remove unbroken cells and cell debris. The resultant supernatant was used as cell-free extract. The method of Anraku & Heppel (1967) was modified for preparation of the periplasmic, cytoplasmic and membrane fractions. The cell paste (wet weight 190 g) obtained from 5 L culture on *PVA* medium was suspended in an appropriate amount of 30 mM Tris/HiCl buffer (pH 8.0) containing 30 % (w/v) sucrose and 1 mM EDTA, and gently stirred at room temperature for 15 min to cause plasmolysis. The mixture was centrifuged at 16 000 g for 30 min at 4 °C to obtain the plasmolysed cells. The supernatant was used as the sucrose-EDTA fraction. The plasmolysed cells were suspended in 10 vols cold water containing 1 mM MgCl₂ and the suspension was gently stirred for 30 min on ice. The mixture was centrifuged at 16 000 g for 30 min at 4 °C to remove the osmotically shocked cells, and the supernatant was used as the cold-water fraction. The sucrose-EDTA and the cold-water fractions were combined and used as the periplasmic extract. The osmosylated cells were sonicated with a UD-200 ultrasonic disruptor at 20 kHz for 10 min below 4 °C and centrifuged at 68 000 g for 90 min at 4 °C. The supernatant and pellets were used as the cytoplasmic and membrane fractions, respectively.

Activities of glucose-6-phosphate dehydrogenase (Bergmeyer et al., 1974) and alkaline phosphatase (Garden & Levinthal, 1960) were also measured as cytosolic and periplasmic marker enzymes, respectively.

**Purification of OPH.** The periplasmic extract was dialysed against 10 mM Tris/HiCl buffer (pH 8.5) at 4 °C overnight, with several changes of the buffer. The pellets formed during dialysis were discarded by centrifugation. The supernatant was applied to a DEAE-Sepharose column (2.5 x 10 cm) pre-equilibrated with 20 mM Tris/HiCl buffer (pH 8.5). The column was washed with two bed volumes of the same buffer and the elution was performed with a linear gradient from 0 to 0.5 M NaCl in the same buffer. The activity was found only in the unbound fractions, which were collected and concentrated by ultrafiltration with a YM 30 Diaflo membrane (Millipore). PVADH activity was eluted from the bound fractions and used for preparation of oxidized *PVA*. The concentrated enzyme was dialysed against 10 mM acetate buffer (pH 6.0) overnight and applied to a CM-Sepharose column (1.8 x 6 cm), pre-equilibrated with 50 mM sodium acetate buffer (pH 6.0), washed twice with 0.85 % NaCl and finally eluted with 500 mM sodium acetate buffer (pH 6.0). The enzyme activity was assayed by measuring the decrease of A_{415} at 37 °C following the addition of enzyme solution and carried out at 37 °C for 10 min. The enzyme activity was assayed in duplicate by measuring the decrease of A_{490} (due to *p*-nitrophenol liberated; \( \varepsilon = 2591 \text{ M}^{-1} \text{ cm}^{-1} \)) with a Shimadzu UV-160 spectrophotometer (1 cm light path). The reaction mixture without enzyme solution was used as a reference. One unit of enzyme activity was defined as the amount of enzyme that catalysed the hydrolysis of 1 μmol PNPA min⁻¹ under the assay conditions. Activity toward oxidized *PVA* was measured by the decrease of A_{490} in carbonate buffer (pH 10.0: \( \varepsilon = 14.6 \text{ M}^{-1} \text{ cm}^{-1} \)), as described previously (Shimao et al., 2000). The calculation for oxidized *PVA* was done as follows. As the oxidation rate of hydroxyl groups in *PVA* 500 was 4 %, 10 mol diketone structures must exist among 500 hydroxyl groups in 1 mol *PVA* 500. Thus the hydrolysis of 1 mol diketone corresponded to 0.1 mol oxidized *PVA*. Based on this calculation, specific activity and \( V_{\text{max}} \) values were measured. The activity toward mono- and diketones and esters was assayed by decrease in pH and determination of the carboxylic acid formed, which was spectrophotometrically analysed according to the method of Kasai et al. (1975), measuring the colour development with ferric hydroxamate of carboxylic acids. PVADH activity was measured as described previously (Shimao et al., 1986).
with the dialysis buffer. The column was washed with two bed volumes of the buffer and eluted with a linear gradient from 0 to 0-3 M NaCl in the same buffer. The active fractions were pooled and ammonium sulfate was added to 0-3 M. Then the enzyme solution was applied to a Phenyl Sepharose column (2.5×10 cm) pre-equilibrated with 50 mM Tris/HCl buffer (pH 7-6) containing 0-3 M ammonium sulfate. The column was washed with two bed volumes of the buffer and eluted with a linear gradient from 0-3 to 0 M ammonium sulfate. The active fractions were pooled and used as the purified enzyme preparation. The purified enzyme was stable at 4 °C for several weeks.

**Analyses.** The protein concentration was determined by a Bio-Rad Protein Assay kit with bovine serum albumin as the standard. The homogeneity of the protein and the molecular mass of the enzyme subunit were confirmed by SDS-PAGE based on the method of Laemmli (1970). The molecular mass of the native OPH was determined by a SMART System (Amersham Pharmacia) on a Superdex 200 column (3.2×300 mm) equilibrated with 50 mM Tris/HCl buffer (pH 7-5) containing 100 mM NaCl and 1 mM MgCl2, with phosphorylase b (94 kDa); 2, bovine serum albumin (67 kDa); 3, ovalbumin (43 kDa) as size standards. The N-terminal amino acid sequence of the purified enzyme was determined with a Procise 491 protein sequencer (Applied Biosystems). Internal amino acid sequences were analysed by the method of Aebersold (1987).

Homology searches were performed with the BLAST program (http://blast.genome.ad.jp/).

The Mn of oxidized PVA was measured by HPLC, performed with a Tosoh CCPM-II liquid chromatograph. The analytical conditions were as follows: detection, Tosoh RI-8020; columns, Tosoh TSK-GEL2500PW connected with TSK-GEL3000PW; eluent, 0-3 M NaNO3; flow rate, 1 ml min⁻¹; column temperature, 40 °C. Molecular masses were measured using ethylene glycol, its oligomers and polyethylene glycols, and TSK standard polyethylene oxides (Tosoh).

**Cloning of the OPH-encoding gene (oph).** DNA purification, transformation and electrophoresis were performed as described by Sambrook & Russell (2001). Ex Taq DNA polymerase was routinely used for PCR under the conditions recommended by the manufacturer (Takara Bio Co.). The PCR products were sequenced for both strands. To prepare a probe DNA for screening the OPH-encoding gene, 5'- and 3'- degenerate primers were designed based on the N-terminal and internal amino acid sequences of the purified enzyme, followed by nested PCR (Olsvik et al., 1991) to amplify the specific fragment. In the first PCR, the primers used were Nt1 [5'-GA(A)/G(A)/TG(G/G/CG(G/ A/C)/TG(G/C/G/G/G/G-3')] and In1 [5'-CC/C/T/TG- (G/A)TA(G/A)/TG(G/A)/TC(C/T/G)/GT(G/A)AA-3']. For the nested PCR, 1 μl of the first PCR reaction mixture was used and the primers Nt2 [5'-TG(G/G/G/G(A/C)/TG(G/C/G/G/G/G/G/A/C)/TTG(G/A)-3'] and In2 [5'-GG-(G/A)/C(A/C/T/G)/GT(G/A/C/T/G)/TA(A/C/G/C)/G(A)/AT(G/A/CT-3')] were used for the reaction. The product of 500 bp was purified and ligated into a pGEM-T easy vector (Promega). The plasmid (pOPH-p) was transformed and extracted from transformant E. coli.

Inverse PCR (Ochman et al., 1988) was performed to amplify the colony surrounding oph. Naed-digested and self-ligated chromosomal DNA was used as a template and amplified with the primer pair OPH-Inv-F (5'-GACCATCGGAACCCCAACCGG-3') and OPH-Inv-R (5'-GCGCTGGAATCGCCACCTCTC-3'). The amplified 2.5 kb DNA fragment was ligated into a pGEM-T easy vector (pOPH-i) and sequenced. In the downstream region of oph, a gene encoding PVADH was found, which was already deposited in GenBank under accession no. D83772. Further downstream region (1 kb EcoRI fragment) was cloned into pBluescript II SK+ (Stratagene) by the colony hybridization method (Sambrook & Russell, 2001). The sequence including oph, the PVADH-encoding gene (pwaA) and the putative cytochrome c gene (cytC) was deposited in GenBank under accession no. AB190288.

**Construction of the expression vector for oph.** The ORF of oph except for the putative signal peptide region was amplified by PCR using the primers ExOPH-N (5'-GACCCTAAGGAGTTTTATATGAAGGCGAATGGGCCTGCCCCG-3') and ExOPH-C (5'-AAGGTTTCTTGTGAATTGATC-3'), which contained SacI and HindIII sites (underlined) and the Shine–Dalgarno sequence (italized). The amplified fragment was first ligated into a pGEM-Teasy vector and cut by SacI and HindIII, and then ligated into the corresponding position of a pUC118 vector. The resultant plasmid (pUC-oph) was transformed into E. coli DH5α. The transfectants were grown on LB medium at 37 °C for 2 h and the expression of oph was induced by the addition of 1-0 mM IPTG.

**RESULTS**

**Reidentification of Pseudomonas sp. strain 113P3 as Sphingomonas sp.**

*Pseudomonas* sp. strain 113P3 was reidentified as a member of the genus *Sphingomonas*, based on the DNA sequence of 16S rRNA as shown in Methods. The strain has 95.8–93.0% identity with *Sphingomonas* species (*S. chlorophenolica*, *S. yanoikuyae*, *S. macrogolitabidus*, *S. terrae*, *S. adhaesiva*, *S. parapaucimobilis*, *S. sanguinis*, *S. parapaucimobilis*; among these, *S. chlorophenolica* was closest to strain 113P3. This result was supported by the finding that PCR amplified a specific band of the total DNA extracted from the strain when primers were designed on the sequence (16S rRNA) of *S. chlorophenolica*, but not when designed on the sequence (16S rDNA) of *Pseudomonas aeruginosa*.

**Inductivity and localization of OPH and PVADH**

The inductivity of OPH and PVADH was studied with cells grown on PVA medium, glucose medium and a nutrient broth, and the effect of the addition of PQQ was studied as well. The organism could not grow on PVA in the absence of PQQ. OPH activity was detected in cell extracts obtained after growth on different media. ApoPVADH was expressed in all the media, but its activity was detected only if PQQ was added. Thus both OPH and PVADH were constitutively formed. Since the best medium was PVA medium supplemented with PQQ, the following experiments were performed with cells grown on this medium. Most of the PVADH and OPH activities were present in the periplasmic fraction (Table 1). Shimao et al. (2000) provided evidence that PVADH and OPH are membrane-bound enzymes in *Pseudomonas* sp. VM15C. Therefore, we conclude that the reaction sites of PVA degradation in *Sphingomonas* sp. strain 113P3 and in *Pseudomonas* sp. VM15C are in the periplasm.

**Purification of OPH**

The OPH was purified from the periplasmic extract prepared from 190 g cell paste (wet wt) obtained from a 5-litre
The culture of PVA medium supplemented with PQQ (Table 2). The purified enzyme was homogeneous on SDS-PAGE after three purification steps, with a yield of 40 % and a 5.9-fold increase in specific activity (Table 2). OPH activity was separated from PVADH on a DEAE-Sepharose column. The molecular mass of the native enzyme was estimated to be approximately 70 kDa by gel filtration (not shown). On SDS-PAGE, the molecular mass of a monomer protein was estimated to be 35 kDa. Thus the purified enzyme was considered to be a homodimeric protein with a molecular mass of approximately 70 kDa by gel filtration (not shown). On searching withBlast these sequences did not exhibit homology with any known protein.

**Characterization and amino acid sequence of the OPH**

The pH stability was assayed by measuring the residual activity after keeping the enzyme solutions (0.14 mg ml⁻¹) in various buffers for 24 h on ice. The enzyme was stable in a narrow pH range of 6.5–8.5 (in 50 mM sodium phosphate or Tris/HCl buffer). The thermal stability was assayed by measuring the residual activity after keeping the enzyme at various temperatures for 30 min in 50 mM Tris/HCl buffer (pH 8.0). The enzyme was stable below 30 °C, but the activity rapidly decreased above 40 °C and was almost nil at 60 °C. The optimal pH and temperature under our assay conditions were 8.0 and 37 °C, respectively. The enzyme activity was strongly inhibited by 1 mM PMSF, but not by other inhibitors tested, including chelating agents, thiol agents and inhibitors of the respiratory chain. The enzyme was slightly activated by Mg²⁺ and Fe³⁺ and appreciably inhibited by Hg²⁺ and Zn²⁺. The inhibition by PMSF strongly suggests that the enzyme is a serine hydrolase.

\( K_m \) and \( V_{max} \) values for oxidized PVA and PNPA were 0.2 and 0.3 mM, and 0.1 and 3.4 μmol min⁻¹ mg⁻¹, respectively. Whether the OPH was really active toward oxidized PVA was confirmed by decrease in \( A_{300} \) (for diketone structure) (Silverstein et al., 1991; Shimao et al., 2000) and the shift of Mn of oxidized PVA. An increase in \( A_{300} \) up to 60 min due to PVADH activity and then a decrease due to OPH activity were found (Fig. 1a, b). Both reactions were dependent on the amounts of the enzymes, suggesting that these enzymes catalysed both reactions. Oxidized PVA was hydrolysed by OPH, which was analysed by HPLC (Fig. 1c). Oxidized PVA prepared by PVADH showed two peaks on HPLC, corresponding to \( M_m \) values of approximately 11 000 (47 %) and 1400 (53 %). The former peak was shifted to \( M_n \) values ranging from 7500 to 3700 and the latter peak increased in height after hydrolysis by OPH.

The enzyme had no activity on other substrates tested, including monoketones (2-hexanone, 2-heptanone, 4-heptanone, acetone dicarboxylic acid, dihydroxyacetone and hydroxycetone), diketones (2,3-butanedione, 2,4-pentanediene, 2,4-hexanediene, 2,5-hexanediene, 2,4-nonanediene, 5,5’-dimethyl-1,3-cyclohexadiene, 1,3-diphenyl-1,3-propanedione) and esters (methyl and ethyl butyric acid and ethyl propionic acid, methyl 2-butoxacetate, ethylene glycol monoacetate, triacetyl glyceride, methyl- and ethyl-β-hydroxyvaleric acid, 4,4-dimethyl-3-oxopentanoate and methyl p-hydroxybenzoic acid). The N-terminal amino acid sequence of the purified OPH was KSEWACPEGF-TDKAG. Two internal amino acid sequences, (In1) IGRF-TDHYG and (In2) DDGSTVPFQ, were also determined, as described in Methods. On searching with Blast these sequences did not exhibit homology with any known protein.

**Cloning and sequencing of the oph gene**

Based on the N-terminal and internal amino acid sequences, nested PCR with degenerate primers was performed and a fragment of 500 bp was amplified (pOPH-p). The region surrounding oph was amplified by inverse PCR. The 2.5 kb

### Table 1. Localization of OPH and PVADH

<table>
<thead>
<tr>
<th>Enzyme preparation*</th>
<th>Total activity (U)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PVADH</td>
</tr>
<tr>
<td>Culture supernatant</td>
<td>0.1</td>
</tr>
<tr>
<td>Periplasm</td>
<td>6.3</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>2.2</td>
</tr>
<tr>
<td>Membrane</td>
<td>0.071</td>
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</table>

ND, Not detected.

*Cells grown on PVA medium were osmolysed and fractionated, as described in Methods.

†Alkaline phosphatase (ALP) activity as a periplasmic marker enzyme was assayed as described in Methods.

‡Glucose-6-phosphate dehydrogenase (G6PDH) activity as a cytoplasmic marker enzyme was assayed as described in Methods.

### Table 2. Purification of OPH from *Sphingomonas* sp. strain 113P3

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Protein (mg)</th>
<th>Total activity (units)</th>
<th>Specific activity (units mg⁻¹)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
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</thead>
<tbody>
<tr>
<td>Periplasmic extract</td>
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<td>30</td>
<td>0.34</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>DEAE-Sepharose</td>
<td>26</td>
<td>29</td>
<td>1.1</td>
<td>3.2</td>
<td>97</td>
</tr>
<tr>
<td>CM-Sepharose</td>
<td>8.0</td>
<td>14</td>
<td>1.9</td>
<td>5.6</td>
<td>47</td>
</tr>
<tr>
<td>Phenyl Sepharose</td>
<td>6.0</td>
<td>12</td>
<td>2.0</td>
<td>5.9</td>
<td>40</td>
</tr>
</tbody>
</table>
fragment was obtained and cloned into a cloning vector (pOPH-i). Further downstream region was cloned by the colony hybridization method (pOPH-c). Joining the three plasmid sequences (pOPH-p, pOPH-i and pOPH-c) resulted in a DNA sequence (3827 bp) containing three open reading frames, encoding OPH (oph), PVADH (pvaA) and a putative cytochrome c (cytC) (Fig. 2). The ORF of oph consists of 1095 bp, corresponding to a protein of 364 amino acid residues, encoding a signal peptide and a mature protein of 34 and 330 amino acid residues, respectively. The deduced amino acid sequence was in accordance with the N-terminal and internal amino acid sequences of the purified OPH. The presence of the serine-hydrolase motif (lipase box, Gly-X-Ser-X-Gly) strongly suggests that the oph-encoded protein belongs to the serine-hydrolase family (Pelletier et al., 1995). The putative amino acid sequence of oph exhibited homology to OPH from Pseudomonas sp. strain VM15C (63 % identity), and to the polyhydroxyalkanoate (PHA) depolymerases from Mesorhizobium loti, Rhizobium sp. and Sinorhizobium meliloti strain 1021 (29–32 % identity).

The recombinant gene expressed an approximately 35 kDa protein corresponding to the same size as the purified OPH in cell-free extracts in the form of inclusion bodies. The recombinant enzyme did not exhibit any activity toward PNPA or oxidized PVA but N-terminal amino acid analysis of the recombinant protein revealed the same sequence as the purified OPH (MKSEWACPEGFTPKA-).

In the downstream region of oph, a gene encoding PVADH (pvaA) was located. The putative amino acid sequence of the gene exhibited homology with PVADH from strain VM15C (52 % identity). Furthermore, a putative cytochrome c gene located downstream of pvaA was sequenced.

**Fig. 2.** Gene organization of the cloned regions including oph, the PVADH gene (pvaA) and a putative cytochrome c gene (cytC). Arrows indicate the orientation of each gene.

**Fig. 1.** In vitro degradation of PVA by PVADH and OPH. (a) Spectrophotometric analysis of oxidized PVA formation and hydrolysis. The reaction mixture (total volume 3 ml) contained PVADH (0–3 units, bound fraction on DEAE-Sepharose), PVA117 (10 mg), 1 mM CaCl₂, 0.1 mM phenazine ethosulfate and 50 mM carbonate buffer (pH 10). The A₃₅₀ (for diketone structure) was monitored. The reaction mixture was incubated at 30 °C. After 60 min, 2 mM EDTA (final concentration) was added to the reaction mixture to stop the reaction, followed by incubation for a further 10 min at 30 °C, and then by incubation in the presence (○) or absence (○) of OPH (unbound fraction on DEAE-Sepharose, 0–1 unit). (b) Absorption spectra of oxidized PVA formed in the reaction mixture. A portion of the reaction mixture was withdrawn at appropriate time intervals, diluted sixfold with 50 mM carbonate buffer (pH 10–0) and its absorption spectrum measured. (c) HPLC analysis of molecular shift by hydrolysis of oxidized PVA; solid line, oxidized PVA; dashed line, hydrolysed oxidized PVA. The Mᵣ values of oxidized PVA before and after hydrolysis by OPH were measured, as described in Methods.
**DISCUSSION**

Based on 16S rRNA analysis, *Pseudomonas* sp. 113P3 was reidentified as a *Sphingomonas* species; it was closest to *S. chlorophenolica*. Kim et al. (2003) also isolated a PVA-utilizing strain of *Sphingomonas* sp., SA3, with the closest match with *S. adhaesiva* and *S. terrae*, but did not report information on the PVA-metabolic enzymes of that strain. Many *Sphingomonas* strains are involved in the degradation of either natural or synthetic polymers (Kawai, 1999). Their unique membrane structures are suggested to be relevant to the incorporation of large molecules into the cells and their metabolism on membranes (in the periplasmic space) or in the cytoplasm, as found in PEG-utilizing (Kawai & Enokibara, 1996; Sugimoto et al., 2001), polyaspartate-utilizing (Tabata et al., 1999) and polyalginate-utilizing strains (Momma et al., 2000). Since both PVADH and OPH from strain 113P3 exist in the periplasm of the organism, and those of strain VM15C are membrane-associated (Shimao et al., 2000), PVA (M<sub>n</sub> 75 000) has to be incorporated into the periplasm to be degraded by metabolic enzymes.

The OPH from *Sphingomonas* sp. strain 113P3 was purified for the first time and characterized. PVADH and OPH are constitutively produced, as is already known to be the case for other PVADHs (Matsumura et al., 1998, 1999). The inductivity of OPHs from strain VM15C and *Pseudomonas vesicularis* PD (Sakai et al., 1985, 1986) has not been documented. The molecular masses of the native enzyme and a monomer protein were estimated to be 70 kDa and 35 kDa, respectively, indicating that the enzyme is a homodimer. The gene for the OPH of *Pseudomonas* sp. strain VM15C was cloned, but since the enzyme was not purified, its enzymic characteristics are not clear (Shimao et al., 2000). The enzyme of strain 113P3 was inhibited by PMSF in a manner similar to that of strain VM15C, but did not confirm whether the polymer was depolymerized. In this paper, the M<sub>n</sub> (11 000) of oxidized PVA was clearly shifted to lower M<sub>n</sub> values (7500–3700) and a peak at M<sub>n</sub> 1400 increased, showing that the polymer was actually depolymerized. M<sub>n</sub> values of oxidized PVA (11 000 and 1400) formed from PVA 500 (M<sub>n</sub> 22 000) might be due to non-enzymic cleavage during enzymic oxidation and concentration after reaction or contaminating esterase activity.

We cloned the gene for OPH and found that the open reading frame consists of 1095 bp, corresponding to 364 deduced amino acid residues, encoding a signal peptide and a mature protein of 34 and 330 residues, respectively. By BLAST analysis, OPH exhibited a high homology (63 % identity) to the OPH from *Pseudomonas* sp. VM15C, and to PHB depolymerases from various sources, but no significant homology to other enzymes including lipases/esterases. The amino acid sequences of the OPHs from strain 113P3 and strain VM15C were aligned by CLUSTAL W (Thompson et al., 1994) with the hypothetical PHB depolymerases from *Mesorhizobium loti*, *Rhizobium* sp. and *Sinorhizobium melliloti* strain 1021, to which the OPH from strain 113P3 showed homology (29–32 % identity) and the well-characterized PHB depolymerases from *Pseudomonas lemoignei* (PhaZ1 to PhaZ5; Jendrossek et al., 1995b) (Fig. 3). PHB depolymerases share common structural domains conserved in the group as a whole: in the N-terminus, the signal peptide, the catalytic domain including the lipase box, the threonine-rich region or the type III module of fibronectin, and the substrate-binding site (Jendrossek et al., 1995a, b). The primary structure of the enzyme hydrolysed PNPA and oxidized PVA, but did not hydrolyse any mono- or diketones. Shimao et al. (2000) reported that the cell extract of *E. coli* harbouring oph has an activity toward oxidized PVA about 350 times greater than that toward 4,6-nonanediol (diketone). Thus, the OPH from strain 113P3 exhibits a different specificity. Shimao et al. (2000) measured OPH activity toward oxidized PVA only by A<sub>300</sub>, but did not confirm whether the polymer was depolymerized. The OPH from strain 113P3 has to be confirmed whether the polymer was depolymerized.

**Fig. 3.** Alignment of the deduced amino acid sequences of OPHs from *Sphingomonas* sp. strain 113P3 and *Pseudomonas* sp. strain VM15C with PHB depolymerases. OPH-113P3, OPH from strain 113P3; OPH-VM15C, OPH from strain VM15C; PHB-Mloti, PHB depolymerase from *Mesorhizobium loti* (accession no. 2705259CCD); PHB-Rhizo, PHB depolymerase from *Rhizobium* sp. (3003308KT); PHB-Sinor, PHB depolymerase from *Sinorhizobium melliloti* strain 1021 (C95396); PhaZ1ple to PhaZ5ple, PHB depolymerases from *Pseudomonas lemoignei*. The regions surrounding the putative active sites are aligned. The amino acids serine (S), aspartate (D), and histidine (H) of the catalytic triad and histidine of the putative oxynion hole are shaded.
catalytic domain of these depolymerases contains certain conserved structures such as an oxyanion hole (histidine) and a triad of three amino acid residues (serine, aspartate and histidine) that is conserved among the serine proteases (Brenner, 1988; Kim et al., 2004; Lassy & Miller, 2000). Their consensus sequences are L* ***HGC-QtAs, ID-n-vY-V-GLS-G+--t, vw-G-sDyTv, and GM-H-P---G, respectively (* indicates hydrophobic, + a small side chain, and the corresponding residues are underlined). These structures are putatively conserved in the OPHs as well; an oxyanion hole and a catalytic triad were found at positions shifted downstream by about 50 amino acid residues from the corresponding positions in the PHB depolymerases. However, further work, including site-directed mutagenesis, is needed to determine the catalytic residues in OPHs.

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