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*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. 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-nonanedione (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 
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 characteriza-
tion 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 mole-
cular weight', $M_n$) 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, $M_n$ 22 000) was used (or omitted as a 
control). The oxidation rate of hydroxyl groups was calculated to be 
approximately 4-0 % from $\varepsilon = 14-6$ mM$^{-1}$ 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 through-
out. 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 pre-
viously (Hatanaka et al., 1995a). The glucose medium contained the 
same components as PVA medium except that glucose was added 
instead of PVA117. The bacterium was also grown on nutrient broth 
(Eiken Chemical Co.). The cells were harvested by centrifugation, 
washed twice with 0-85 % NaCl and kept at $-80 \degree C$ until use. *E. coli* 
DH5x and *E. coli* transformants were grown at 37 \degree C in LB medium, 
supplemented with 50 $\mu$g ampicillin ml$^{-1}$ when necessary.

**Taxonomic identification.** As genetic evidence for identification, 
the partial 16S rDNA (tDNA) sequence of strain 113P3 was ana-
lysed, 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 con-
tained 1 mM PNPA and 50 mM potassium citrate buffer (pH 6.0), 
which was preincubated at 37 $\degree C$ for 1 min. The reaction was started 
by the addition of enzyme solution and carried out at 37 $\degree C$ for 
20 min. The enzyme activity was assayed in duplicate by measuring 
the $A_{415}$ (due to $p$-nitrophenol liberated; $\varepsilon = 2591$ M$^{-1}$ cm$^{-1}$) with 
a Shimadzu UV-160 spectrophotometer (1 cm light path). A reac-
tion 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 $\mu$mol PNPA min$^{-1}$ under the assay 
conditions. Activity toward oxidized PVA was measured by the 
decrease of $A_{300}$ in carbonate buffer (pH 10-0: $\varepsilon = 14-6$ mM$^{-1}$ 
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 
hydrolysis of 1 mol diketone corresponded to 0-1 mol oxidized 
PVA. Based on this calculation, specific activity and $K_m$ and $V_{max}$ 
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 hydrxamate 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/HCl buffer (pH 8.0) and sonicated with a UD-200 
ultrasonic disruptor (Tomiy Seiko Co.) at 20 kHz for 10 min below 
4 $\degree C$, 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 (wt weight 190 g obtained 
from 5 l culture on PVA medium) was suspended in an appropriate 
amount of 30 mM Tris/HCl 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 $\degree C$ to obtain the plasmolysed cells. 
The supernatant was used as the sucrose-EDTA fraction. The plasmo-
lysed cells were suspended in 10 vols cold water containing 1 mM 
MgCl$_2$ and the suspension was gently stirred for 30 min on ice. 
The mixture was centrifuged at 16 000 g for 30 min at 4 $\degree C$ 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 osmo-
lysed cells were sonicated with a UD-200 ultrasonic disruptor at 20 kHz 
for 10 min below 4 $\degree C$ and centrifuged at 68 000 g for 90 min at 
4 $\degree C$. 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/HCl buffer (pH 8.5) at 4 $\degree C$ overnight, with several 
changes of the buffer. The pellets formed during dialysis were dis-
carded by centrifugation. The supernatant was applied to a DEAE-
Sepharose column (2.5 x 10 cm) pre-equilibrated with 20 mM Tris/ 
HCl buffer (pH 8.5). The column was washed with two bed volumes 
of the same buffer and the elution was performed with a linear gra-
dient 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 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 x 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 x 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 Prosite 491 protein sequencer (Applied Biosystems). Internal amino acid sequences were analysed by the method of Aebersold et al. (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 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 (Olivk et al., 1991) to amplify the specific fragment. In the first PCR, the primers used were Nt1 [5'-GA(G/A)/TGGGGGC(G/A)(C/T)/GC(C/G)CGA(G/A)GG-3'] and In1 [5'-CC(C/T)/TT-(G/A)TA(G/A)/TGG(G/A)/TC(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)GC(G/G)/CTGCCGC(G/A)(C/T)/TAGA(G/A)GG(A/C)(G/G)/GC(G/G)ATGC(G/A)TC-3'] and In2 [5'-GG(G/A/C)(C/T/G)GT(G/A/C/T)TAGA(T/G)GC(G/G)ATGC(G/A)TC-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 region surrounding oph. Nael-digested and self-ligated chromosomal DNA was used as a template and amplified with the primer pair OPH-Inv-F (5'-GACCATCGGAAACCCACACGGG-3') and OPH-Inv-R (5'-GCCGCTGGAATGCGATCTC-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. 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'-GAGCTTTAAAGGAGTTTATTATATGAAGGCGAATGGGCCTGCCCG-3') and ExOPH-C (5'-AAGCTTTTCATTGTAATGATC-3'), which contained SacI and HindIII sites (underlined) and the Shine–Dalgarno sequence (italicized). 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 transformants 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 95-9-93.0% identity with Sphingomonas species (S. chlorophenolica, S. yanoikuyae, S. maccrogolabidus, S. terrae, S. adhaesiva, S. parapaucimobilis, S. sanguis [= S. sanguinis] and S. paucimobilis); 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 PQP was studied as well. The organism could not grow on PVA in the absence of PQP. 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 PQP 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
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>
</tr>
</tbody>
</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.

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 35 kDa monomers. The specific activity of the enzyme was 0·1 and 2·6 units mg⁻¹ toward oxidized PVA and PNPA, respectively.

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 $M_n$ 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 hydroxyacetone), diketones (2,3-butanedione, 2,4-pentanediione, 2,4-hexanediione, 2,5-hexanediione, 2,4-nonanediione, 5,5′-dimethyl-1,3-cyclohexadione, 1,3-diphenyl-1,3-propanediione) and esters (methyl and ethyl butyric acid and ethyl propionic acid, methyl 2-butenoate, 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-TPKAG. Two internal amino acid sequences, (In1) IGRF-TDKY 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 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>
</tr>
</thead>
<tbody>
<tr>
<td>Periplasmic extract</td>
<td>87</td>
<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 (PHB) 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.
**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 periplasm of the organism, and those of strain VM15C are membrane-associated (Shimao et al., 2000). Since both PVADH and OPH from strain 113P3 exist in the periplasm of the organism, the polymer was actually depolymerized. In this paper, the *M*ₐ values of oxidized PVA (11 000 and 1400) formed from PVA 500 (*M*ₐ 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 melloti* 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; Lendrossek 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 (Lendrossek 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-nonanedione (diketone). Thus, the OPH from strain 113P3 exhibits a different specificity. Shimao et al. (2000) measured OPH activity toward oxidized PVA only by *A*₅₈₀, but did not confirm whether the polymer was depolymerized. In this paper, the *M*ₐ values of oxidized PVA were clearly shifted to lower *M*ₐ values (7500–3700) and a peak at *M*ₐ 1400 increased, showing that the polymer was actually depolymerized. *M*ₐ values of oxidized PVA (11 000 and 1400) formed from PVA 500 (*M*ₐ 22 000) might be due to non-enzymic cleavage during enzymic oxidation and concentration after reaction or contaminating esterase activity.

![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. (3003309KT); PHB-Sinor, PHB depolymerase from *Sinorhizobium melloti* strain 1021 (C96396); 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.](image-url)
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***I-HC-GtAs, ID-n-vYV-GtLS-G++-t, wv-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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