The gene *pvaB* encodes oxidized polyvinyl alcohol hydrolase of *Pseudomonas* sp. strain VM15C and forms an operon with the polyvinyl alcohol dehydrogenase gene *pvaA*

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A 5.7 kbp *SphI* fragment containing the polyvinyl alcohol (PVA) dehydrogenase gene *pvaA* and its 1.9 kbp 5'-flanking region was cloned from the PVA-degrading bacterium *Pseudomonas* sp. VM15C. The *pvaB* gene, encoding oxidized PVA hydrolase, was found in the region upstream of *pvaA*. Sequence data and expression studies indicated that *pvaA* and *B* constitute an operon in the order *pvaBA*. The *pvaB* gene encoded a protein of 379 amino acid residues (40610 Da), and a lipoprotein signal sequence and the lipase consensus sequence, Gly-X-Ser-X-Gly, characteristic of the active-site serine region in serine hydrolases, were detected in the deduced amino acid sequence. The *pvaB* product with the *pvaA* product constituted an enzyme system for the cleavage of PVA molecules. The *pvaA* product introduced β -diketone groups into the PVA molecule, and the *pvaB* product hydrolysed these β -diketone groups in oxidized PVA. The *pvaB* product also hydrolysed 4,6-nonanedione at a low rate, but not acetylacetone or 5-nonanone. It was completely inhibited by PMSF and was concluded to be a serine hydrolase. There were no proteins showing high similarity to the *pvaB* product in the databases, but minor similarity to a number of serine hydrolases including polyhydroxyalkanoate depolymerases was apparent.

Keywords: oxidized polyvinyl alcohol hydrolase, PQQ-dependent polyvinyl alcohol dehydrogenase, *pva* operon, *Pseudomonas* sp. VM15C

INTRODUCTION

Polyvinyl alcohol (PVA) is a water-soluble synthetic polymer and is used widely in industry. We previously found that PVA was degraded by a mixed culture composed of two bacterial symbionts (Sakazawa *et al.*, 1981). One symbiont of the mixed culture was the PVA-degrading bacterium *Pseudomonas* sp. strain VM15C, while the other supplied pyrroloquinoline quinone

(PQQ) as an essential growth factor for the PVA-degrading bacterium (Shimao *et al.*, 1984). The novel PVA-oxidizing enzyme, PQQ-dependent PVA dehydrogenase (PVADH, EC 1.1.99.23), catalysing the oxidation of PVA, was found in the membrane fraction of the PVA-degrading bacterium (Shimao *et al.*, 1986). This enzyme was involved in the first step in PVA degradation. We have since cloned the structural gene for this enzyme, *pvaA* (formerly described as *pdh*), from strain VM15C (Shimao *et al.*, 1996).

In this work, we cloned the gene (*pvaB*) encoding oxidized PVA hydrolase (OPH) involved in the second step of PVA degradation from the same strain, and determined the complete nucleotide sequence of the structural gene. OPH was expressed in *Escherichia coli* clones and characterized. OPH attacked oxidized PVA and cleaved the main chain of the macromolecule.

Abbreviations: DIG, digoxigenin-3-O-succinyl-L-aminocaproyl-[5-(3-aminoallyl)-2'-deoxy-uridine-5'-triphosphate]; KPB, potassium phosphate buffer; OPH, oxidized polyvinyl alcohol hydrolase; PES, phenazine ethosulfate; PVA, polyvinyl alcohol; PVADH, polyvinyl alcohol dehydrogenase; PQQ, pyrroloquinoline quinone.

The DDBJ accession number for the sequence reported in this paper is AB008494.

METHODS

Materials. PQQ and PVAs with degrees of polymerization of 500 (PVA500) and 1500 (PVA1500) have been described previously (Shimao *et al.*, 1996). 4,6-Nonanedione was purchased from Acros Organics, and restriction endonucleases, T4 DNA ligase and *Taq* DNA polymerase were from Takara Shuzo. Unisole F-200 30/60 column packing for gas chromatography was from GL Sciences. All other reagents were commercial products of the highest grade.

Bacterial strains and plasmids. The sources of the bacterial strains and plasmids and their relevant characteristics are shown in Table 1.

Media and growth conditions. Antibiotic medium 3 (AM3) and the basal medium for cultivating *Pseudomonas* sp. VM15C were as previously described (Sakazawa *et al.*, 1981; Shimao *et al.*, 1996). Ampicillin was used at 100 µg ml⁻¹, chloramphenicol at 50 µg ml⁻¹ and IPTG at 200 µM. To prepare the cell extracts, *Pseudomonas* sp. VM15C was grown in the basal medium containing PVA500 at 5 g l⁻¹ and PQQ at 10 µg ml⁻¹, whereas *E. coli* JM101 carrying pMSVA9KN or pMSVA11 was grown in AM3 medium containing ampicillin and IPTG, and *E. coli* JM101 carrying pMSVA12 or pHSG399 was grown in AM3 medium containing chloramphenicol and IPTG, both for 16 h at 30 °C with shaking.

DNA manipulation. The method for preparing total DNA from *Pseudomonas* sp. strain VM15C was as previously described (Shimao *et al.*, 1996). Plasmid DNA isolation, transformation, restriction endonuclease digestion, ligation and agarose gel electrophoresis were performed as described by Sambrook *et al.* (1989).

Preparation of the probe for cloning the OPH-encoding gene.

A part (1200 bp) of the DNA sequence of the structural gene (Shimao *et al.*, 1996) for PVADH was amplified with synthetic oligonucleotides, 5'-AAGATTCGCCGATCGA-3' and 5'-GATGTTGTCCAGCGAC-3', as primers by PCR. The DNA probe was prepared with digoxigenin-3-O-succinyl-ε-aminocaproyl-[5-(3-aminoallyl)-2'-deoxy-uridine-5'-triphosphate] (DIG) via random-primed labelling by using a DIG labelling kit (Boehringer Mannheim) and the PCR product as the template.

Southern and colony hybridization. Southern blotting was carried out with a semi-dry electroblotter (Owl Scientific). Southern and colony hybridization were accomplished with the DIG-labelled probe.

DNA sequencing. The DNA sequences of both strands of pMSVA10 and its deletion derivatives were determined by using a dye terminator cycle sequencing kit and 373A DNA sequencer (Perkin-Elmer). Primers used were M13 universal primers and synthesized oligonucleotides specific to the sample DNA sequences.

Preparation and fractionation of the cell extract. The cell extract was prepared by sonication and was fractionated by ultracentrifugation into membrane and soluble fractions as described previously (Shimao *et al.*, 1996).

Enzyme assay. PVADH activity was assayed as described previously (Shimao *et al.*, 1996). A decrease in the degree of polymerization of PVA in a solution causes a decrease in the viscosity of the solution. Viscosity-decreasing activity was assayed in a reaction mixture containing 50 mM potassium phosphate buffer (KPB, pH 7.5), 150 mg PVA1500, 1 µM PQQ, 1 mM Ca²⁺, 0.1 mM phenazine ethosulfate (PES) and enzyme preparation, in a total volume of 15 ml. The reaction was carried out while reciprocally shaking (110 r.p.m.) at 30 °C. The viscosity of the reaction mixture was measured with an Ostwald viscometer at 30 °C, and the viscosity decrease (%) reflecting the PVA degradation was calculated by the following equation: Viscosity decrease (%) = 100(η_{sp}⁰ - η_{sp}^t)/(η_{sp}⁰ - η_{sp}^{-E-PVA}), where η_{sp}⁰ and η_{sp}^t are the values for the specific viscosity of the reaction mixture at reaction times 0 and t, respectively, η_{sp}^{-E} is that of the reaction mixture not containing the enzyme preparation, and η_{sp}^{-E-PVA} is that containing neither the enzyme preparation nor PVA.

Oxidized PVA was prepared by incubating a reaction mixture containing 50 mM KPB (pH 7.5), 150 mg PVA1500, 1 µM PQQ, 1 mM Ca²⁺ and 0.1 mM PES with cell extract of *E. coli* JM101(pMSVA9KN) containing 750 mU PVADH in a total volume of 15 ml at 30 °C while reciprocally shaking (110 r.p.m.) for 2 h. The reaction was stopped by inactivating PVADH activity with the addition of EDTA to the reaction mixture at a final concentration of 2 mM. A control solution containing no oxidized PVA (-oxPVA solution) was similarly

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or phenotype	Reference or source
Strains		
<i>Pseudomonas</i> sp. VM15C	Strain utilizing PVA as sole source of carbon and energy	Sakazawa <i>et al.</i> (1981)
<i>E. coli</i> JM101	<i>supE thi Δ(lac-proAB) F'[traD36 proAB⁺ lacI^a lacZΔM15]</i>	Sambrook <i>et al.</i> (1989)
Plasmids		
pUC18	Amp ^r	Messing (1983)
pHSG399	Cm ^r	Takeshita <i>et al.</i> (1987)
pMSVA1KN	pUC18 derivative containing <i>pvaA</i> and <i>ΔpvaB</i>	Shimao <i>et al.</i> (1996)
pMSVA9KN	pUC18 derivative containing <i>pvaA</i> and <i>ΔpvaB</i>	Shimao <i>et al.</i> (1996)
pMSVA10	pUC18 derivative containing <i>pvaA</i> and <i>pvaB</i>	This study
pMSVA11	pUC18 derivative containing <i>pvaA</i> and <i>pvaB</i>	This study
pMSVA12	pHSG399 derivative containing <i>ΔpvaA</i> and <i>pvaB</i>	This study

prepared except that PVA1500 was omitted from the reaction mixture.

OPH activity was assayed at 30 °C with a reaction mixture containing 100 µl 50 mM KPB (pH 7.5), 800 µl oxidized PVA solution and 100 µl enzyme preparation. The control reaction mixture contained –oxPVA solution in place of oxidized PVA solution. At the start of the reaction and 30 min later, the A_{300} of a solution containing 100 µl 1 M sodium carbonate buffer (pH 10), 800 µl H₂O and 100 µl reaction mixture was measured by using a solution containing 100 µl of the 1 M sodium carbonate buffer (pH 10), 800 µl H₂O and 100 µl of the control reaction mixture as a reference. One unit of OPH activity is defined as the amount of enzyme required to decrease A_{300} by 1 unit min⁻¹ in 1 ml of the reaction mixture.

The assay mixture for 4,6-nonanedione-hydrolysing activity contained 2 µl 4,6-nonanedione, 500 µl enzyme preparation and 500 µl 50 mM KPB (pH 7.5). The amount of 2-pentanone produced was determined by gas chromatography as described below. One unit of activity is defined as the amount of the enzyme needed to produce 1 µmol 2-pentanone min⁻¹.

Analyses. Protein concentration was determined by the method of Bradford (1976) with bovine serum albumin as the standard. SDS-PAGE was performed by the method of Laemmli (1970) with a 12% gel. The amounts of 4,6-nonanedione, 2-pentanone and n-butyric acid were determined by gas chromatography. An aliquot (1 ml) of the reaction mixture was acidified with 100 µl 2 M HCl and extracted with 200 µl ethyl ether. After the ethyl ether layer had been separated by centrifugation at 16 000 g for 1 min, 5 µl of the ethyl ether extract was injected into a gas chromatograph (GC-7A, Shimadzu) equipped with a flame-ionization detector and a glass column (3.2 mm × 2.1 m) packed with Unisole F-200 30/60. Nitrogen was used as the carrier gas at a flow rate of 30 ml min⁻¹, and the column temperature was set initially at 80 °C for 2 min, before being raised to 145 °C at a rate of 16 °C min⁻¹. The injector and detector temperatures were maintained at 170 °C. Retention times were as follows under these conditions: 2-pentanone, 2.49 min; n-butyric acid, 9.45 min; and 4,6-nonanedione, 10.3 min.

RESULTS

Cloning of the upstream region of *pvaA*

In the previous study, the nucleotide sequence of the DNA fragment containing the PVADH gene, *pvaA*, was determined (Shimao *et al.*, 1996). We found a putative terminator sequence immediately downstream of the termination codon of the gene, but did not find any promoter-like sequence upstream of the ORF. An expression study also showed the absence on the DNA fragment of any promoter for *pvaA* functioning in *E. coli*. Furthermore, we found an incomplete ORF upstream of *pvaA*. These observations suggested that *pvaA* may be the last gene of an operon.

A Southern hybridization analysis of total DNA from *Pseudomonas* sp. strain VM15C with the DIG-labelled probe specific to the partial sequence of *pvaA* showed that *SphI* digestion generated a 5.7 kbp DNA fragment that hybridized to the probe. The restriction map for the DNA fragment previously cloned in pMSVA1KN (Fig. 1) and the expected hybridization site of the probe indicated that this 5.7 kbp *SphI* fragment contained the

1.9 kbp 5'-flanking region of the PVADH gene. In order to clone the *SphI* fragment, a subgenomic library was constructed in *E. coli* JM101 by ligating into the pUC18 vector the 4–7 kbp DNA fraction isolated from agarose gel after electrophoresis of *SphI*-digested total DNA. The library was subsequently screened by colony hybridization with the probe. Four positive colonies were obtained among the 300 examined. A plasmid designated as pMSVA10 was isolated from a positive clone and used for further study. A restriction map analysis showed that this plasmid had the 5.7 kbp *SphI* insert and that the cloned fragment had the entire structural gene for PVADH in the opposite orientation to the *lac* promoter (Fig. 1). pMSVA11, which contained the *SphI* fragment in the opposite orientation to that in pMSVA10, was obtained via *SphI* digestion and religation. The PVADH activity was expressed in *E. coli* carrying each of these plasmids with or without IPTG induction (data not shown). This observation indicated the possibility that a native promoter for the PVADH gene was present on the *SphI* fragment.

Nucleotide sequence of the upstream region of PVADH

The nucleotide sequence of the upstream region of *pvaA* in the *SphI* fragment was determined. The sequence revealed an ORF of 1140 bp, encoding a protein of 379 amino acids with a molecular mass of 40 610 Da, immediately upstream of *pvaA*, and a potential Shine–Dalgarno sequence (Shine & Dalgarno, 1975), AGGGGG, of the ORF. In accordance with the molecular mass of the protein deduced from the ORF, a 40 kDa polypeptide was expressed from pMSVA11 as described below and from pMSVA12 (data not shown). Furthermore, as was expected from the expression study, the sequences, TTGCCA and TATACT, that are homologous to the –35 and –10 regions of the *E. coli* consensus promoters (Harley & Reynolds, 1987), respectively, were found upstream of the Shine–Dalgarno sequence. The stop codon of the ORF was followed by the Shine–Dalgarno sequence of *pvaA* after a spacing of only 7 bp. The sequence data and results of the expression study on PVADH indicated that the newly found gene, *pvaB*, and the PVADH gene, *pvaA*, constitute an operon.

Some putative functional sites, the lipoprotein signal sequence and the lipase consensus sequence, were recognized in the deduced amino acid sequence of *pvaB*. Gly-Ile-Ser-Ser-Gly from positions 201 to 205 in the deduced amino acid sequence coincides with the lipase consensus sequence, Gly-X-Ser-X-Gly (Jendrossek *et al.*, 1993). The amino-terminal sequence was a possible lipoprotein signal sequence. This sequence had positively charged Arg9 and Arg12 residues followed by a hydrophobic core from Val14 to Ala20, and Ala16-Leu-Ala-Leu-Ala-Ser-Val-Ala-Ser-Gly-Cys26, which represents the consensus sequence of a prokaryotic membrane lipoprotein lipid attachment site (Bairoch & Bucher, 1994).

Fig. 1. Restriction maps of the plasmids. pMSVA10 was obtained as described in the text; pMSVA11 and pMSVA12 were subsequently derived from it. pMSVA1KN and its derivative, pMSVA9KN, were obtained in previous work (Shimao *et al.*, 1996). The published maps of pMSVA1KN and pMSVA9KN indicated a *Bam*HI site on the left ends of their inserts. However, the *Bam*HI recognition sequence was absent in this work. The sequence of the *Bam*HI site in these plasmids was produced by digesting the CGATCC sequence with *Sau*3AI and ligating to *Bam*HI-digested pUC18. *pvaA*, PVADH gene; *pvaB*, OPH gene; B, *Bam*HI; E, *Eco*RI; K, *Kpn*I; S, *Sal*I; Sc, *Sac*I; Sm, *Sma*I; Sp, *Sph*I; arrow, direction of the *lac* promoter of pUC18 or pHSG399.

Function of the *pvaB* product

To investigate the function of the *pvaB* product, the viscosity-decreasing (PVA-degrading) activity was investigated by using cell extracts of *E. coli* JM101 carrying cloned genes (Fig. 2). The extract of cells carrying pMSVA12 (*pvaA*⁻ *pvaB*⁺) did not show any viscosity-decreasing activity, and the activity of the extract of cells carrying pMSVA9KN (*pvaA*⁺ *pvaB*⁻) was also low. On the other hand, the activity expressed by pMSVA11 (*pvaA*⁺ *pvaB*⁺) was much greater than that of pMSVA9KN. The mixed cell extract of *E. coli* strains carrying pMSVA9KN and pMSVA12 also showed synergistic activity, being much greater than that of each cell extract and similar to that of the pMSVA11 cell extract. The activities of the cell extract from *E. coli* JM101(pMSVA11) and the mixed cell extract were also similar to that of the cell extract from *Pseudomonas* sp. strain VM15C. Furthermore, as described below, the JM101(pMSVA12) cell extract decreased the viscosity of PVA after treatment with PVADH. These findings indicated that the *pvaB* product is an enzyme which degrades oxidized PVA formed by PVADH.

When the cell extract of *E. coli* JM101(pMSVA12) was reacted with 4,6-nonanedione, two compounds were detected by gas chromatography as degradation products and were identified as butyric acid and 2-pentanone by their retention times and co-chromatography with authentic samples. The degradation pro-

Fig. 2. PVA degradation by various cell extract systems of *E. coli* JM101 carrying *pvaA* and *B*. The reaction mixtures contained a cell extract of *E. coli* JM101 carrying the following plasmid(s), or of *Pseudomonas* sp. VM15C, at the concentration shown in parentheses (mg protein per ml reaction mixture): □, pMSVA9KN (2.05); ▲, pMSVA12 (1.70); ○, pMSVA11 (1.69); ■, pMSVA9KN (2.05)+pMSVA12 (0.482); △, pMSVA9KN (2.05)+pHSG399 (0.499); ●, *Pseudomonas* sp. VM15C (0.423). PVADH activity in the reaction mixture containing the cell extract of *E. coli* JM101 carrying pMSVA9KN or pMSVA11, or of *Pseudomonas* sp. VM15C, was 50 mU ml⁻¹.

ducts were also identified from their mass spectra (data not shown). Equimolar amounts of butyric acid and 2-pentanone were produced and the 4,6-nonanedione was

Table 2. Stoichiometric relationship between 4,6-nonanedione degradation and the formation of 2-pentanone and butyric acid

A reaction mixture containing 40.0 mg protein from the cell extract of *E. coli* JM101(pMSVA12), 50 mM KPB (pH 7.5) and 1 mM 4,6-nonanedione in a total volume of 12 ml was incubated in a sealed tube at 30 °C. Ethyl ether extract of the reaction mixture was analysed by gas chromatography as described in the text.

Reaction period (h)	4,6-Nonanedione degraded (mM)	2-Pentanone formed (mM)	Butyric acid formed (mM)
4	0.105	0.107	0.107
8	0.210	0.199	0.206
12	0.280	0.281	0.290

consumed during the reaction (Table 2). 4,6-Nonanedione was not degraded by the cell extract of *E. coli* carrying pHSG399, which was used as the vector of pMSVA12. Thus, the *pvaB* product hydrolysed 4,6-nonanedione and was concluded to be a hydrolase.

Cleavage of the PVA molecule by PVADH and OPH

Oxidation of PVA by PVADH. PVA was oxidized with a cell extract of *E. coli* JM101(pMSVA9KN) (*pvaA*⁺ *pvaB*⁻), and the UV absorption of the reaction mixture was examined by difference spectroscopy at pH 7.5 and 10 with a control reaction mixture that contained no PVA (–oxPVA) as the reference (Fig. 3). The difference spectra indicated that a UV-absorbing substance was produced by PVADH reaction. The product showed much more intense absorption at pH 10 than at pH 7.5 and shifted its absorption maximum from 280 nm at pH 7.5 to 300 nm at pH 10. Such a spectral property is typical of a β -diketone compound (Blout *et al.*, 1946; Silverstein *et al.*, 1991). The oxidized PVA was isolated from the reaction mixture by addition of trichloroacetic acid at a final concentration of 1%, followed by standing for 5 min on ice, centrifugation at 30000 g for 5 min at 4 °C, dialysis of the supernatant against water, and freeze-drying. The ¹H-NMR spectrum (500 MHz) of the oxidized PVA dissolved in D₂O was recorded on a JEOL JNM-ECP500 spectrometer. In the spectrum, a small signal that was not found before oxidation by PVADH was apparent at 2.79 p.p.m. The signal was the only difference between the spectra of intact PVA and oxidized PVA. The signal was assigned as external α -methylene protons of β -diketone. The internal methylene groups of the β -diketone were assumed to give no signals because of rapid proton exchange with D₂O. These results suggested that β -diketone groups were formed in the PVA polymer during oxidation by PVADH.

The increase in A_{300} , which was measured by the difference spectroscopy at pH 10 and reflected the

Fig. 3. Difference spectra of the reaction mixture containing PVA and the cell extract of *E. coli* JM101(pMSVA9KN). The complete reaction mixture contained 1.25 mg cell extract protein ml⁻¹ (50 mU PVADH activity ml⁻¹), 10 mg PVA1500 ml⁻¹, 1 mM Ca²⁺, 1 μ M PQQ and 0.1 mM PES in a total volume of 7.5 ml. Difference spectra were measured with the control reaction mixtures containing no PVA as the references.

increase in β -diketone groups in the PVA molecules, was linear for 3 h, its rate being proportional to the PVADH activity (data not shown). The rate of increase in A_{300} measured at pH 10 was 3.14 units min⁻¹ in the reaction mixture containing *E. coli* JM101(pMSVA9KN) cell extract at 1 U PVADH ml⁻¹. It was also noted that the increase of A_{300} reached saturation of about 6 at 1 g PVA l⁻¹; thus, about 0.26 for 1 mM of vinyl alcohol monomer unit. 4,6-Nonanedione revealed an absorption band with its maximum at 300 nm and pH 10, and the absorption coefficient at 300 nm was 14.6 mM⁻¹ cm⁻¹. If it is assumed that the absorption of the oxidized product is similar to that of 4,6-nonanedione, then the saturation level of β -diketone groups introduced into PVA was estimated to be only about 3.5% of the total vinyl alcohol units.

Degradation of oxidized PVA by OPH. OPH hydrolysed 4,6-nonanedione, a β -diketone compound. To confirm

Fig. 4. PVA degradation by successive attack of cell extracts of *E. coli* JM101 carrying pMSVA9KN and pMSVA12. The reaction mixture contained 1.11 mg ml⁻¹ protein of the cell extract of *E. coli* JM101(pMSVA9KN) (50 mU PVADH activity ml⁻¹), 10 mg PVA1500 ml⁻¹, 1 mM Ca²⁺, 1 μ M PQQ and 0.1 mM PES in a total volume of 15 ml, and was incubated for 120 min at 30 °C. The PVADH reaction in the mixture was stopped by incubation with 2 mM EDTA at 30 °C for 10 min, then 1 ml 50 mM KPB containing 1.49 mg protein of the cell extract of *E. coli* JM101 carrying pMSVA12 (+OPH) or not (-OPH) was added to the reaction mixture, followed by further incubation at 30 °C for 120 min. The viscosity decrease in the +OPH (■) and -OPH (□) reactions and the changes in A_{300} in the +OPH (●) and -OPH (○) reactions were measured. At 120 min, values for the viscosity decrease and A_{300} at the end of the PVADH reaction and those at the start of the OPH reaction are shown.

that OPH would degrade the putative β -diketone structure formed in PVA by the PVADH reaction, a two-step procedure in which PVADH was active in the first step, while OPH attacked oxidized PVA in the second step, was carried out. PVADH requires Ca²⁺ for PQQ binding and can be completely inactivated by EDTA (Shimao *et al.*, 1996), but OPH was not inhibited by EDTA, as described below. First, PVA was oxidized with the cell extract of *E. coli* JM101(pMSVA9KN) (*pvaA*⁺ *pvaB*⁻), then PVADH in the reaction mixture was inactivated with EDTA and, after this inactivation, the JM101(pMSVA12) (*pvaA*⁻ *pvaB*⁺) cell extract was added to the reaction mixture and the whole was incubated (Fig. 4). In the control reaction without the JM101(pMSVA12) cell extract, the rates of decrease of A_{300} measured at pH 10 and of viscosity were low. In the reaction with the JM101(pMSVA12) cell extract, however, both the parameters decreased rapidly, indicating that cleavage of the molecular chain of oxidized PVA was caused by the disruption of a β -diketone group. The rate of decrease of A_{300} was proportional to the amount of the JM101(pMSVA12) cell extract added to the reaction mixture (data not shown). On the other hand, the oxidized PVA prepared as described above was degraded with cell extract of JM101(pMSVA12). The degraded PVA was isolated by the same procedure as

described for the oxidized PVA. IR spectra of the degraded PVA showed a new but slight absorption at 1580 cm, which was not observed for untreated PVA and the oxidized PVA, suggesting the occurrence of a small amount of carboxylate groups in the degraded PVA (data not shown).

Substrate specificity of OPH

5-Nonanone was not degraded by the *E. coli* JM101(pMSVA12) cell extract, indicating that OPH is not active toward the monoketone structure. This enzyme was also inactive toward the lower-molecular-mass β -diketone compound acetylacetone.

The activity of OPH for oxidized PVA and 4,6-nonanedione was compared. The JM101(pMSVA12) cell extract had OPH activity causing a decrease in A_{300} of 1.65 units min⁻¹ (mg protein)⁻¹. On the other hand, the 4,6-nonanedione-hydrolysing activity of the cell extract was 0.326 mU (mg protein)⁻¹. Again, based on the assumption that the absorbance of the oxidized PVA and that of 4,6-nonanedione are similar, OPH can be estimated to be about 350 times more active toward oxidized PVA than 4,6-nonanedione.

Inhibitors

The effects of various enzyme inhibitors on the 4,6-nonanedione-hydrolysing activity and OPH activity in the cell extract of *E. coli* JM101(pMSVA12) were examined. The cell extract was treated with the inhibitors at 30 °C for 5 min, and the OPH and 4,6-nonanedione-hydrolysing activities were assayed. Each inhibitor was used at a final concentration of 1 mM. PMSF, a serine hydrolase inhibitor, completely inhibited both activities. This supports the hypothesis that the site having the lipase consensus sequence is the catalytic centre in OPH. On the other hand, semicarbazide hydrochloride, NaF, NaN₃, KCN, the chelators 1,10-phenanthroline, iminodiacetic acid, 2,2'-bipyridine and EDTA, and the thiol reagents *p*-chloromercuribenzoic acid, iodoacetic acid and *N*-ethylmaleimide, did not significantly inhibit either activity.

Amino acid sequence similarity

The deduced amino sequence of OPH was compared with others in the SWISS-PROT database by using the FASTA program (Pearson & Lipman, 1988). The search did not indicate any protein showing high similarity to OPH, but showed that poly(hydroxyalkanoate) depolymerase (SWISS-PROT accession PHA_PSELE) of *Pseudomonas lemoignei* (Jendrosseck *et al.*, 1993) had low homology to OPH (22% identity in a 205 amino acid overlap). Poly(hydroxyalkanoate) depolymerases have the lipase consensus sequence at their active sites. A BLAST search in a non-redundant sequence database at the National Center for Biotechnology Information, Bethesda, MD, USA, by using the BLASTP program (Altschul *et al.*, 1990), showed that the domain incor-

Fig. 5. SDS-PAGE of *E. coli* JM101(pMSVA11). *E. coli* JM101 carrying pMSVA11 or pUC18 was grown in AM3 medium containing ampicillin and IPTG at 30 °C for 16 h. Lanes 1 and 10, molecular mass standards (top to bottom: 94, 67, 43, 30, 20.1 and 14.4 kDa); lane 2, JM101(pMSVA11) whole cells (0.103 mg dry weight); 3, JM101(pUC18) whole cells (0.121 mg dry weight); 4, JM101(pMSVA11) cell extract (50 µg protein); 5, JM101(pUC18) cell extract (48 µg protein); 6, JM101(pMSVA11) soluble fraction; 7, JM101(pUC18) soluble fraction; 8, JM101(pMSVA11) membrane fraction; 9, JM101(pUC18) membrane fraction. The analysed amounts of the membrane and soluble fractions are equivalent to those prepared from 50 µg protein of the cell extracts. The arrow indicates the 40 kDa protein of the *pvaB* product.

porating the lipase consensus sequence in OPH was similar to those of several poly(hydroxyalkanoate) depolymerases (GenBank accessions U68039, U68170, U10470) from *Pseudomonas* spp. (Briese *et al.*, 1994; Schirmer & Jendrossek, 1994), esterase D (PIR locus S36781) (Ferreira *et al.*, 1993) from *P. fluorescens*, and acetylsterase (DDBJ accession D87681) from *Aspergillus awamorii*.

***pvaB* product and its subcellular localization**

Cells, cell extract, and soluble and membrane fractions of *E. coli* JM101(pMSVA11) were analysed by SDS-PAGE (Fig. 5). The *pvaB* product, a 40 kDa polypeptide, was detected in the cells carrying pMSVA11, but not in the cells carrying pUC18. This molecular mass agrees with that calculated from the deduced amino acid sequence of the *pvaB* product. Although the product was seen in lanes of both the soluble and membrane fractions, the membrane fraction contained more of the product than the soluble fraction. Furthermore, the specific activity of OPH in the membrane fraction was several times higher than that in the soluble fraction (data not shown). These facts suggested that OPH may be a membrane-bound enzyme. SDS-PAGE could not detect the *pvaA* product as a distinguishable protein band. The *pvaB* product may have been expressed in a larger amount than the *pvaA* product.

DISCUSSION

This is the first report on the cloning and characterization of the gene for OPH. This gene, *pvaB*, encodes a protein of 379 amino acids with a molecular mass of 40610 Da. The deduced amino acid sequence has the lipoprotein signal sequence and the lipase consensus sequence.

In accordance with the presence of the lipoprotein signal sequence, the *pvaB* product expressed in cells of *E. coli* JM101(pMSVA11) is suggested to be membrane-bound. The rather large amount of product detected in the soluble fraction was probably released from the membrane during sample preparation. The deduced amino acid sequence of the *pvaA* product, PVADH, also had a signal sequence and was a membrane-bound enzyme (Shimao *et al.*, 1986). The presence of both enzymes at the bacterial cell surface may be necessary for the degradation of the macromolecule.

The lipase consensus sequence indicated that OPH may be a serine hydrolase and that Ser203 may be an active centre. This possibility was supported by the complete inhibition of activity by PMSF. Serine hydrolases generally have a charge-relay system, a catalytic triad consisting of serine, histidine and aspartate, and an oxyanion hole (Blow, 1976). In addition to the putative serine active centre, the charge-relay system may also be present in OPH, but the amino acids involved remain to be identified.

We have also found for the first time evidence indicating that the genes for PVADH and OPH, *pvaA* and *B*, constitute an operon, *pvaBA*. Its putative promoter was found upstream of *pvaB*, and its putative terminator downstream of *pvaA*.

The *pvaB* product, OPH, together with the *pvaA* product, PVADH, constitute an enzyme system for cleavage of the molecular chain of PVA. OPH degrades oxidized PVA formed by the action of PVADH, but does not degrade unoxidized PVA. As to the mechanism for PVA degradation, PVADH appears to introduce β -diketone groups into the PVA molecule which are then attacked by OPH. Thus, cleavage of the PVA molecules proceeds through the formation of β -diketone groups by the action of PVADH and then by hydrolysis catalysed by OPH (Fig. 6). PVA is a polyhydroxyl compound, but PVADH can also oxidize monohydroxyl secondary alcohols and give monoketone compounds (Shimao *et al.*, 1986). This raises the question of whether PVADH would form β -diketones in PVA molecules fortuitously as a result of the random oxidation of hydroxyl groups, or preferentially as a result of the selective oxidation of vicinal pairs of hydroxyl groups. The linear increase in A_{300} , and thus the linear increase in the β -diketone structure during the oxidation of PVA by PVADH, indicates that PVADH carries out selective oxidation of vicinal pairs of hydroxyl groups in the PVA molecule. On the other hand, PVADH oxidized 4,6-nonanediol to 4,6-nonanedione via 4-hydroxy-6-oxononane (data not shown). Thus PVADH oxidized the vicinal hydroxyl

Fig. 6. Proposed mechanism for cleavage of the main chain of PVA molecules by the *pvaA* and *B* products.

groups of the 4,6-nonanediol step by step. For the assumed selective oxidation of vicinal pairs of hydroxyl groups in PVA, an interesting mechanism may function but this remains to be studied in future work.

In relation to the PVA degradation mechanism, it was also noted that PVA oxidized by PVADH was spontaneously degraded, this being shown by the slow decrease in viscosity during oxidation by PVADH and after the inactivation of PVADH with EDTA (Fig. 4). Degradation also accompanied the spontaneous hydrolysis of the β -diketone structure, as apparent from the decrease in A_{300} . Furthermore, the oxidized PVA isolated from the reaction mixture was found to be degraded slowly at 30 °C in 50 mM KPB (pH 7.5) and even in distilled water. The degradation rate in the buffer was slightly faster than in distilled water (data not shown). These facts indicate that the β -diketone structure formed in oxidized PVA was unstable and spontaneously hydrolysed, this hydrolysis being enzymically accelerated by OPH.

OPH also hydrolysed the low-molecular-mass β -diketone compound 4,6-nonanedione, producing *n*-butyric acid and 2-pentanone. Since OPH was not active toward 4-nonanone, OPH seems to be specific for β -diketone compounds. A comparison of the β -diketone-degrading activity of OPH towards oxidized PVA and low-molecular-mass β -diketone compounds suggests that OPH is more specific for oxidized PVA than for the low-molecular-mass β -diketone compounds.

In accordance with such specificity of OPH, no proteins similar to OPH have yet been reported. It was, however, found that OPH has slight similarity to some poly(hydroxyalkanoate) depolymerases that are also serine

hydrolases having the lipase consensus sequence. Interestingly, both are enzymes that act on high-molecular-mass polymers. The slight but interesting similarity of OPH to poly(hydroxyalkanoate) depolymerases may have bearing on this common property. However, the substrate specificity and dissimilarity of OPH to other types of protein may indicate that *pvaB* is evolutionarily unique.

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