Aldehyde oxidase carrying an unusual subunit structure from *Pseudomonas* sp. MX-058

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Summary

*Pseudomonas* sp. MX-058 produces aldehyde oxidase catalysing glyoxal to glyoxylic acid. Two aldehyde oxidases (F10 and F13) were purified to homogeneity from *Pseudomonas* sp. MX-058. F10 and F13 had subunit structures, a heterotetramer and heteropentamer respectively. The enzymes exhibit significantly low activity toward glyoxylic acid compared with formaldehyde and acetaldehyde. In particular, aldehyde oxidases also have great potential as biocatalysts for the production of glyoxylic acid, a useful chemical building block for pharmaceutical intermediates and various organic syntheses (Bennett and Hamilton, 2000; Mahajani, 2000; Cativiela et al., 2003; Bernd et al., 2004), through the oxidation of glyoxal.

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Aldehyde oxidases using molecular oxygen as an electron acceptor, was recently reported to be produced by microorganisms such as *Methyllobacillus* sp. KY4400, *Pseudomonas* sp. KY4690, *Ps. stutzeri* IFO 12695 and *Streptomyces moderatus* ATCC23443 (Yasuhara et al., 2002), and the enzymes from *Pseudomonas* sp. KY4690 and *Ps. stutzeri* IFO 12695 have been extensively purified and characterized (Uchida et al., 2003; 2005). The purified enzyme from *Pseudomonas* sp. KY4690 has a molecular mass of 132 kDa and a heterotramer structure consisting of about 88 (α), 39 (β) and 18 (γ) kDa subunits.

Introduction

Aldehyde oxidase (aldehyde: oxygen oxidoreductase; EC 1.2.3.1), which oxidizes various aliphatic and aromatic aldehydes using molecular oxygen as an electron acceptor, was recently reported to be produced by microorganisms such as *Methyllobacillus* sp. KY4400, *Pseudomonas* sp. KY4690, *Ps. stutzeri* IFO 12695 and *Streptomyces moderatus* ATCC23443 (Yasuhara et al., 2002), and the enzymes from *Pseudomonas* sp. KY4690 and *Ps. stutzeri* IFO 12695 have been extensively purified and characterized (Uchida et al., 2003; 2005). The purified enzyme from *Pseudomonas* sp. KY4690 has a molecular mass of 132 kDa and a heterotramer structure consisting of about 88 (α), 39 (β) and 18 (γ) kDa subunits.

Aldehyde oxidases previously reported have attracted attention concerning the environment as a means of decomposing environmentally toxic aldehydes such as formaldehyde and acetaldehyde. In particular, aldehyde oxidases also have great potential as biocatalysts for the production of glyoxylic acid, a useful chemical building block for pharmaceutical intermediates and various organic syntheses (Bennett and Hamilton, 2000; Mahajani, 2000; Cativiela et al., 2003; Bernd et al., 2004), through the oxidation of glyoxal.

Glyoxylate is produced chemically through the oxidation of glyoxal involving nitric acid or via a three-step reaction that involves the ozonolysis of dimethyl maleate and hydrogenation of the resulting methylglyoxylate hemiacetal (Seip et al., 1993; Gavagan et al., 1995). Chemical production, therefore, has the major drawback of a high concentration of oxalic acid as a by-product. Recently, the electrochemical synthesis of glyoxylic acid through the electrochemical reduction of oxalic acid has received considerable attention as a commercial process, but the process exhibits low performance efficiency due to cathode fouling, which is mainly due to the oxalic acid feedstock, and the anode material is a bottleneck that needs to be overcome (Scott and Cheng, 2002).

The enzymatic production of glyoxylic acid has also been reported, mainly with glycolate oxidase (EC 1.1.3.1, glycolate: oxygen oxidoreductase, now referred to as EC 1.1.3.15, (S)-2-hydroxy-acid oxidase) from spinach leaves and catalase (EC 1.11.1.6), and many attempts have been made to make it economically and industrially feasible, e.g., the addition of ethylenediamine (EDA) to prevent further enzymatic oxidation of glyoxylic acid to oxalic acid (Seip et al., 1993), using immobilized enzymes in the presence of a stoichiometric amount of EDA, which can be recycled (Seip et al., 1994), and the use of...
microbial transformant catalysts (Payne et al., 1995; Seip et al., 1995; Jin et al., 2003). The production of glyoxylic acid with glycerol oxidase of Aspergillus japonica and with cells of Alcaligenes sp. GOX373 has also been reported (Isobe, 1995; Isobe and Nishise, 1999), both catalysing the oxidation of glycolic acid to glyoxylic acid, but the production yields were low due to substrate and product inhibition.

In this paper, we describe the isolation and characterization of two aldehyde oxidases responsible for glyoxal oxidation in Pseudomonas sp. MX-058, a glyoxal-assimilating bacterium. The enzymes have been purified to homogeneity, and characterization clearly showed that the aldehyde oxidases of this microorganism have unique subunit structures. Moreover, the enzymes showed the potential to be applicable to glyoxylic acid production.

Results

Purification of the enzyme

Pseudomonas sp. MX-058 was cultivated in the medium comprising ethylene glycol as an enzyme inducer. About 200 g (wet weight) of the cells possessing high glyoxal oxidase activity were obtained from 60 l medium, and then the enzyme was purified through ammonium sulfate fractionation and several column chromatographies. The purification of the enzyme from Pseudomonas sp. MX-058 is summarized in Table 1. Figure 1 shows the elution profile on hydroxylapatite chromatography on a Bio-Scale CHT5-I column (the last step). A protein comprising several peaks was broadly eluted, and activity was detected in all the fractions containing protein (F9-F16). The total protein and activity of F10-F14 were calculated from the data for individual fractions (Table 1). Through the purification procedures described under Experimental procedures, the enzyme was purified 1060-fold, with a yield of 12.7%, from the cell-free extract.

Molecular mass analysis

The fractions (F9-F16) obtained on Bio-Scale CHT5-I chromatography were subjected to SDS-polyacrylamide gel electrophoresis (PAGE; Fig. 2). All the fractions contained multiple subunit structures, and there seemed to be two different subunit composition patterns. One, a representative sample being F10, consisted of four subunits with apparent molecular masses of 80, 39, 14 and 9 kDa. The other, a typical example being F13, consisted of five subunits with apparent molecular masses of 58, 39, 22, 14 and 9 kDa. While the protein bands corresponding to 39, 14 and 9 kDa were common, that of 80 kDa and those of 58 and 22 kDa were specific to F10 and F13 respectively. Both F10 and F13 gave a quite symmetrical peak on high-performance gel permeation liquid chromatography (data not shown), suggesting that

| Step | Total protein (mg) | Total activity (units) | Specific activity (units mg⁻¹) | Fold | Yield (%) |
|------|-------------------|-----------------------|------------------------------|------|-----------|
| Cell-free extract | 19 500 | 329 | 0.0169 | 1 | 100 |
| Ammonium sulfate fractionation | 15 600 | 305 | 0.0195 | 1.15 | 92.3 |
| DEAE-Sephacel | 3 070 | 171 | 0.0554 | 3.27 | 51.6 |
| HiPrep 16/10-O-XL | 570 | 254 | 0.446 | 26.4 | 77.1 |
| Phenyl-Superose HR10/10 | 94.0 | 159 | 1.69 | 100 | 48.2 |
| MonoQ HR10/10 | 36.5 | 158 | 4.33 | 256 | 47.9 |
| HiPrep Sephacryl 16/60 S-200 | 15.3 | 129 | 8.40 | 497 | 39.0 |
| Hydroxypalate | 3.50 | 50.6 | 16.2 | 957 | 17.2 |
| Bio-Scale CHT5-I (F10-F14) | 2.34 | 42.0 | 17.9 | 1060 | 12.7 |
| F10 | 0.414 | 5.90 | 14.2 | 843 | 1.78 |
| F13 | 0.601 | 12.1 | 20.1 | 1190 | 3.66 |

a. Protein concentrations were determined by the method of Bradford (1976).
b. Enzyme activity was measured as described in the text.

Fig. 1. Elution profile of aldehyde oxidase on Bio-Scale CHT5-I column chromatography. Absorbance at 280 nm (—); activity (●); potassium phosphate buffer concentration (○). Protein was eluted from the column as described in the text and aldehyde oxidase activity was assayed using the standard assay for enzyme activity. Each fraction collected was 1.5 ml.
the enzyme was purified to homogeneity. The relative molecular mass \((M_r)\) of both fractions was estimated to be about 150 kDa on gel filtration on a TSK-gel G3000SW column. The total molecular mass, which was determined by SDS-PAGE analysis, of both fractions well agreed with the native molecular mass determined on gel filtration. Besides this, the intensity of the protein band stained with Coomassie brilliant blue R-250 well corresponded to the activity of aldehyde oxidase in each fraction. This implied that \textit{Pseudomonas} sp. MX-058 produced two different kinds of aldehyde oxidase, a heterotetramer (F10) and a heteropentamer (F13), under the same cultivation conditions.

**Spectral properties**

The absorption spectra of the enzymes (both F10 and F13) obtained on hydroxyapatite chromatography on a Bio-Scale CHT5-I column are quite similar to those of the aldehyde oxidases from \textit{Pseudomonas} sp. KY4690 and \textit{Ps. stutzeri} IFO 12685 (Fig. 3; Uchida et al., 2003; 2005). These results suggested that the enzymes might contain similar cofactors, such as flavin and a [2Fe-2S] cluster, to the aldehyde oxidases from the two \textit{Pseudomonas} species described above.

**N-terminal sequences of the enzymes**

The N-terminal amino acid sequences of the material in each protein band of F10 and F13 were then determined by automated Edman degradation with a pulsed liquid phase sequencer, and then homology to other proteins deposited in the database was searched for. A computer-aided homology search revealed that the N-terminal sequences the material in each protein band of F10 and F13 exhibited high similarity to the amino acid sequences of putative oxidoreductases from \textit{Pseudomonas syringae} DC3000, \textit{Ps. putida} KT2440 and \textit{Ps. aeruginosa} PAO1 (Fig. 4). All of these homologous oxidoreductases have a heterotrimer structure consisting of an about 85–89 kDa \(\alpha\)-subunit, an about 37–39 kDa \(\beta\)-subunit and an about 18–23 kDa \(\gamma\)-subunit. Interestingly, we found that the N-terminal sequences of the 9 kDa protein in F10 and F13, those of the 22 and 58 kDa proteins in F13, and that of the 80 kDa protein in F10 were located in the \(\alpha\)-subunit, while those of the 39 and 14 kDa proteins in both fractions were located in the \(\beta\)- and \(\gamma\)-subunits respectively.

**Substrate specificity of the enzyme**

The oxidizing activity of the two fractions toward various substrates including aliphatic aldehydes, aromatic aldehydes, glyoxylic acid, glycolic acid, xanthine and hypoxanthine was examined (Fig. 5). Neither fraction showed any activity toward xanthine or hypoxanthine (a substrate for xanthine oxidase, E.C 1.1.3.22), or glycolic acid (a substrate for glycolate oxidase, EC 1.1.3.15), indicating that they did not have xanthine oxidase or glycolate oxidase activity respectively. Both F10 and F13 showed high activity toward aliphatic aldehydes and aromatic...
aldehydes, preferentially benzaldehyde, and showed essentially the same substrate specificity profile. An advantageous property for glyoxylic acid production from glyoxal with aldehyde oxidases F10 and F13 was the significantly low activity toward glyoxylic acid compared with glyoxal.

Catalytic properties of the enzymes

Normal hyperbolic kinetics were observed with all substrates listed in Table 2, and Lineweaver–Burk treatment of the data yields apparent $K_m$ values for glyoxal of 11.6 mM (F10) and 22.8 mM (F13); and the $V_{\text{max}}$ values were 18.6 units mg$^{-1}$ (F10) and 34.1 units mg$^{-1}$ (F13). F10 thus showed higher affinity for glyoxal than F13, although it showed lower affinity for the other three substrates. The affinity for benzaldehyde of F13 was about four times higher than that of F10, while the $V_{\text{max}}$ for benzaldehyde of the two fractions were almost the same. A reduction in enzyme activity was observed when the concentrations of acetaldehyde and benzaldehyde were higher than 32 and 40 mM respectively (data not shown).

Effects of temperature and pH on enzyme activity

The effect of temperature on the enzyme activity was investigated by conducting the assay over the temperature range of 25–75°C. The apparent optimum temperatures were determined to be 65°C for F10 and 60°C for F13, and both were stable at high temperature although their activities slightly decreased at 75°C. The optimum pH was found to be approximately 3.5–7.0 for both F10 and F13, which is a considerably broad pH range, when the enzymes were assayed in acetate, phosphate, Tris and glycine buffers spanning the pH range of 2.5–10.5.

Discussion

An oxygen-dependent aldehyde oxidase of microbial origin was first reported by Crawford and his coworkers (Crawford et al., 1982) in Streptomyces viridosporus, however, its enzymatic properties have not been fully investigated. The partially purified enzyme of S. viridosporus preferentially oxidizes vanillin and other aromatic aldehydes to the corresponding aromatic acids, but exhibits no preference for aliphatic aldehydes, and whole cells of S. viridosporus have been used for the oxidation of vanillin to vanillic acid (Pometto and Crawford, 1983). Recently, Yasuhara and colleagues (2002) reported the presence of oxygen-dependent aldehyde oxidases in some microorganisms. Among them, Pseudomonas sp. KY4690 exhibited strong aldehyde oxidase activity, and the enzyme has then been extensively purified and characterized (Uchida et al., 2003). The aldehyde oxidase of
Pseudomonas sp. KY4690 exhibits a molecular mass of 132 kDa and a heterotrimer structure consisting of about 88 (α), 39 (β) and 18 (γ) kDa subunits, respectively, and its Mr and subunit structure have been reported to be different from those of enzymes of animal and plant origin, which are similar in Mr (300 kDa) and subunit structure, α2, the molecular mass being about 147 kDa for each subunit (Calzi et al., 1995; Sekimoto et al., 1997). The aldehyde oxidase of Pseudomonas sp. MX-058 examined in the present study has unique protein structures, α1α2βγ for F10 and α1α2α1α1α1α2βγ for F13, a heterotetramer and heteropentamer, respectively; however, its Mr is similar to those of microbial aldehyde oxidases previously reported. It is strongly suggested to be a novel aldehyde oxidase.

The most compelling evidence that the aldehyde oxidase of Pseudomonas sp. MX-058 purified by us is a novel one is the results of a computer-aided homology search for the N-terminal sequences of F10 and F13, as described in the text. Further evidence, which supports this conclusion, is the clear distribution of different protein bands when the enzymes were subjected to SDS-PAGE (Fig. 2). The results strongly suggested that the 89 kDa α-subunits of F10 and F13 might be decomposed into two [80 (α1) and 9 (α2) kDa] and three [58 (α1), 22 (α1) and 9 (α2) kDa] subunits, respectively, while the β- and γ-subunits remain intact, resulting in the heterotetramer structure of F10 and the heteropentamer structure of F13. We propose here three possibilities for the different subunit structure formation of the aldehyde oxidase produced by Pseudomonas sp. MX-058: first, the possession of the αβγ structure, which later changed into the α1α2βγ structure of F10 and the α1α1α1α1α1α2βγ structure of F13 during the purification; second, the possession of the F10 structure due to modification in MX-058 cells, some of which was later modified into the F13 structure during the purification steps; and third, the possession of the F10 and F13 structures, which remained intact throughout the purification steps. Further investigation is needed to clarify the subunit structure formation. While F10 and F13 have different subunit

### Table 2. $K_m$ and $V_{max}$ values of aldehyde oxidases from Pseudomonas sp. MX-058.

| Substrate                  | $K_m$ (mM) | $V_{max}$ (Unit mg$^{-1}$) | $V_{max}/K_m$ |
|----------------------------|------------|----------------------------|---------------|
|                            | F10        | F13                       | F10           | F13           | F10 | F13 |
| Glyoxal                    | 11.6       | 22.8                      | 18.6          | 34.1          | 1.6 | 1.5 |
| Glycolaldehyde             | 29.8       | 20.0                      | 10.6          | 7.77          | 0.35| 0.38|
| Acetaldehyde               | 12.3       | 4.94                      | 11.6          | 22.9          | 0.94| 4.6 |
| Benzaldehyde               | 38.6       | 8.71                      | 38.9          | 29.9          | 1.0 | 3.4 |

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structures, they had essentially the same specific activity and substrate specificity.

In this study, we investigated the inducing effects of various substrates such as ethylene glycol, propylene glycol, glyoxal and glycolic acid (data not shown). Ethylene glycol caused either elevated microbial growth or satisfactory enzyme production, while the others had no effect. Thus, ethylene glycol was used as an inducer for enzyme production by *Pseudomonas* sp. **MX-058.** The relatively low activity toward glyoxylic acid together with the high apparent optimum temperature and broad pH optimum range indicate the aldehyde oxidases from *Pseudomonas* sp. **MX-058** as very potential biocatalysts for glyoxylic acid production. As the glyoxal-oxidizing activity of *Pseudomonas* sp. **MX-058** might be not sufficient for industrial production of glyoxylic acid (specific activity ratio of the purified enzyme and cell-free extract is about 1000; Table 1) even if ethylene glycol is added to the medium as an enzyme inducer, further studies, such as cloning of the full-length aldehyde oxidase gene and DNA sequence analysis, must be performed to obtain a recombinant strain that overexpresses the aldehyde oxidase gene. Using the recombinant microorganisms overproducing the aldehyde oxidase as a catalyst, glyoxylic acid production through glyoxal oxidation might be possible; however, improvement of the enzyme stability by directed evolution method must be also required for high-concentration reaction because substrate and product are toxic compounds for enzymes.

**Experimental procedures**

**Materials**

All chemicals used in this study were of analytical grade, commercially available, and used without further purification. Ethylene glycol, a 40% glyoxal solution, and glyoxylic acid monohydrate were purchased from Wako Pure Chemicals (Osaka, Japan). All chromatographic columns were purchased from Amersham Bioscience (UK) and Bio-Rad (USA). The molecular mass standard protein kits for SDS-PAGE and high-performance gel permeation liquid chromatography were purchased from Daiichi Pure Chemicals (Tokyo, Japan) and Amersham Bioscience respectively.

**Microorganism and cultivation**

*Pseudomonas* sp. **MX-058,** which was isolated from soil sample as a glyoxal-assimilating microorganism, was used as the enzyme source. The microorganism was cultivated in a 100 l jar fermentor containing 60 l of medium comprising 1% (w/v) ethylene glycol, 0.8% (w/v) Nutrient broth (Difco, USA), 0.7% (w/v) *K*$_2$HPO$_4$ and 0.3% (w/v) *KH*$_2$PO$_4$, pH 7.0. Cultivation was carried out at 28°C, with 1 vvm air supply and agitation at 200 rev min$^{-1}$ to achieve the best enzyme production. Cells were harvested after 40 h of cultivation by continuous centrifugation and then washed with a 0.85% NaCl solution. The washed cells (213 g wet weight) were stored at −20°C until used for enzyme extraction.

**Standard assay for enzyme activity**

Aldehyde oxidase activity was assayed by measuring the amount of hydrogen peroxide generated on the oxidation of glyoxal. The standard reaction mixture comprised, in 2.5 ml, 2.4 mM glyoxal, 100 mM potassium phosphate buffer (pH 7.0), 0.64 mM 4-aminoantipyrine, 1.04 mM N-ethyl-N-(2-hydroxy-3-sulphopropyl)-3-methylalanine (TOOS), 10 units ml$^{-1}$ peroxidase (from *Arthromyces ramosus*; Suntory, Japan), and an appropriate amount of enzyme. After 2 min incubation without a substrate at 30°C, the reaction was started by addition of the substrate, and then the increase in absorbance at 555 nm due to the formation of quinoneimine dye was determined. One unit was defined as the amount of enzyme that produced 1 μmol of hydrogen peroxide per minute. Specific activity was expressed as units of enzyme activity per milligram of protein.

**Protein determination**

Protein concentrations were determined with a Bio-Rad Protein assay kit with bovine serum albumin as a standard (Bradford, 1976).

**Purification of aldehyde oxidase**

All purification procedures were carried out at 0–4°C. Potassium phosphate buffer (pH 7.0) and centrifugation at 14 000 g for 20 min were usually used and performed, respectively, throughout the enzyme purification procedure.

**Step 1. Preparation of a cell-free extract.** Wet cells (213 g) of *Pseudomonas* sp. **MX-058** were suspended in 426 ml of 20 mM buffer and then disrupted with an ultrasonicator (Kubota Insonator 201M) for 1 h at 0°C. Cell debris was removed by centrifugation and the supernatant was used as the cell-free extract.

**Step 2. Ammonium sulfate fractionation.** The cell-free extract was fractionated with solid ammonium sulfate. The precipitate obtained at 20–60% saturation dissolved in 20 mM buffer exhibiting high enzyme activity was dialysed against 40 l of 20 mM buffer.

**Step 3. Batch-wise DEAE-Sepharcell chromatography.** The volume of the dialysate (506 ml containing 15.6 g protein) was adjusted to an appropriate volume (600 ml) for easy handling, followed by application to 1560 ml of DEAE-Sepharcell resin (Amersham Bioscience) previously equilibrated with 20 mM buffer. The slurry was stirred at 4°C for 1 h and then filtered through Whatman No. 2 filter paper (filtrate F1). The resin was washed with 600 ml of 20 mM buffer consecutively for another two elutions (filtrates F2 and F3), and then the enzyme was eluted from the resin by three consecutive elutions with 600 ml of 20 mM buffer containing 1 M NaCl (filtrates F4, F5 and F6). The active fractions (filtrates F4, F5 and F6) were combined, dialysed against...
20 mM buffer and then concentrated to about 85 ml by ultrafiltration with an Amicon membrane filter apparatus (Amicon, USA) equipped with a YM-10 membrane.

Step 4. HiPrep 16/10-Q-XL column chromatography. The enzyme was applied to a HiPrep 16/10-Q-XL column (Amersham Bioscience) equilibrated with 20 mM buffer, and then eluted with a linear gradient of 0–1 M NaCl (400 ml). The fractions containing enzyme activity (74 ml) were combined and dialysed against 20 mM buffer.

Step 5. Phenyl-Superose HR10/10 column chromatography. After adjusting the ammonium sulfate concentration to 1.2 M with solid ammonium sulfate, the enzyme solution was placed on a Phenyl-Superose HR10/10 column (Amersham Bioscience) equilibrated with 20 mM buffer containing 1.2 M ammonium sulfate. The enzyme was eluted by lowering the ionic strength of ammonium sulfate linearly from 1.2 to 0 M in 20 mM buffer (160 ml). The active fractions were combined (40 ml), dialysed against 20 mM buffer, and then concentrated to 10 ml with an Amicon YM-10 membrane.

Step 6. MonoQ HR10/10 column chromatography. The enzyme solution was applied to a MonoQ HR10/10 column equilibrated with 20 mM buffer, and then eluted with a linear gradient of 0–1 M NaCl (160 ml). The active fractions were combined (10 ml), dialysed against 20 mM buffer and then concentrated to 1 ml by ultrafiltration with a Centricon-10 concentrator (Amicon).

Step 7. HiPrep Sephacryl 16/60 S-200 column chromatography. The enzyme solution was applied to a HiPrep Sephacryl 16/60 S-200 column (Amersham Bioscience) equilibrated with 20 mM buffer containing 0.2 M NaCl. The rates of sample loading and column elution were both maintained at 0.125 ml min⁻¹. The active fractions were combined (12 ml) and dialysed against 5 mM buffer.

Step 8. Hydroxyapatite chromatography. The enzyme was then applied to a HR10/10 column (Amersham Bioscience) packed with hydroxyapatite resin (Seikagaku-Kogyo, Tokyo, Japan) and equilibrated with 5 mM buffer prior to enzyme application. The enzyme was eluted by increasing the ionic strength of the buffer linearly from 5–500 mM (160 ml). The active fractions were then combined, dialysed with 5 mM buffer and then concentrated to about 3 ml using a Centricon centrifugal filter device (Amicon) equipped with a YM-10 membrane.

Step 9. Bio-Scale CHT5-I column chromatography. The concentrate from Step 8 was placed on a Bio-Scale CHT5-I column (Bio-Rad) equilibrated with 5 mM buffer. The enzyme was eluted from the column with 100 ml of the buffer as performed in step 8. The active fractions were then used for characterization of the enzyme.

High-performance gel permeation chromatography
The enzyme solution (50 µl, 13.8 µg protein for F10 and 20.1 µg protein for F13) obtained on hydroxyapatite chromatography on a Bio-Scale CHT5-I column (the last step of the purification) was subjected to high-performance gel permeation chromatography (Shimadzu LC-6A) on a column of TSK G-3000SW (0.75 x 30 cm; Tosoh, Japan) at 0.5 ml min⁻¹ with 20 mM potassium phosphate buffer (pH 7.0) containing 0.2 M NaCl as the mobile phase, at room temperature. The absorbance of the effluent was monitored at 280 nm with a data module (Shimadzu C-R6A) automatically. The relative molecular mass of the enzyme was determined from its mobility relative to those of the standard proteins.

Characterization of the enzyme
The native and subunit molecular masses of the enzyme were determined as described previously by SDS-PAGE on a 15% polyacrylamide slab gel with a Tris/glycine buffer system (King and Laemmli, 1971) and high-performance gel permeation chromatography respectively. N-terminal amino acid sequence analysis of the enzyme was performed as described previously (Kataoka et al., 2000). The N-terminal sequence of the enzyme was compared with those of other proteins deposited in a database (SwissProt, TrEMBL, PRF).

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References
Bennett, D.J., and Hamilton, N.M. (2000) A facile synthesis of N-benzyl allylglycine. Tetrahedron Lett 41: 7961–7964.
Bernd, N., Gerald, B., Thomas, K., Thomas, B., Peter, E., Jergen, E., et al. (2004) Indolyl-3-glyoxylic acid derivatives having therapeutically valuable properties. U.S. Patent 0171668.
Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein-dye binding. Anal Biochem 72: 248–254.
Calzi, M.L., Raviolo, C., Ghiaudi, E., De Gioia, L., Salamina, M., Cazzaniga, G., et al (1995) Purification, cDNA cloning, and tissue distribution of bovine liver aldehyde oxidase. J Biol Chem 270: 31037–31045.
Cattiviela, C., Fraile, J.M., García, I.J., Lázaro, B., Mayoral, J.A., and Pallarés, A. (2003) Heterogeneous catalysis in the synthesis and reactivity of allantoin. Green Chem 5: 275–277.
Crawford, D.L., Sutherland, J.B., Pometto, A.L., Ill, and Miller, J.M. (1982) Production of an aromatic aldehyde oxidase by Streptomyces viridosporus. Arch Microbiol 131: 351–355.

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Gavagan, J.E., Fager, S.K., Seip, J.E., Payne, M.S., Anton, D.L., and DiCosimo, R. (1995) Glyoxylic acid production using microbial transformant catalysts. *J Org Chem* **60**: 3957–3963.

Isobe, K. (1995) Oxidation of ethylene glycol and glycolic acid by glycerol oxidase. *Biosci Biotech Biochem* **59**: 576–581.

Isobe, K., and Nishise, H. (1999) A method for glyoxylic acid production using cells of *Alcaligenes* sp. GOX373. *J Biootechnol* **75**: 265–271.

Jin, J., Tan, T., Wang, H., and Su, G. (2003) The expression of spinach glycolate oxidase (GO) in *E. coli* and the application of GO in the production of glyoxylic acid. *Mol Biotechnol* **25**: 207–214.

Kataoka, M., Honda, K., and Shimizu, S. (2000) 3,4-Dihydrocoumarin hydrolase with haloperoxidase activity from *Acinetobacter calcoaceticus* F46. *Eur J Biochem* **267**: 3–10.

King, J., and Laemmli, U.K. (1971) Polypeptides of the tail fibers of bacteriophage T4. *J Mol Biol* **62**: 465–477.

Mahajani, S.M. (2000) Reaction of glyoxylic acid with aliphatic alcohols using cationic exchange resin as catalysts. *React Func Polym* **43**: 253–268.

Payne, M.S., Petrillo, K.L., Gavagan, J.E., Wagner, L.W., DiCosimo, R., and Anton, D.L. (1995) High-level production of spinach glycolate oxidase in the methylotrophic yeast *Pichia pastoris*: engineering a biocatalyst. *Gene* **167**: 215–219.

Pometto, A.L., III, and Crawford, D.L. (1983) Whole-cell bioconversion of vanillin to vanillic acid by *Streptomyces viridosporus*. *Appl Environ Microbiol* **45**: 1582–1585.

Scott, K., and Cheng, H. (2002) The anode behaviour of Ebonex® in oxalic acid solution. *J Appl Electrochem* **32**: 583–589.

Seip, J.E., Fager, S.K., Gavagan, J.E., Gosser, L.W., Anton, D.L., and DiCosimo, R. (1993) Biocatalytic production of glyoxylic acid. *J Org Chem* **58**: 2253–2259.

Seip, J.E., Fager, S.K., Gavagan, J.E., Anton, D.L., and DiCosimo, R. (1994) Glyoxylic acid production using immobilized glycolate oxidase and catalase. *Bioorg Medic Chem* **2**: 371–378.

Seip, J.E., Fager, S.K., Gavagan, J.E., Payne, M.S., Anton, D.L., and DiCosimo, R. (1995) Glyoxylic acid production using microbial transformant catalysts. *J Org Chem* **60**: 3957–3963.

Sekimoto, H., Seo, M., Dohmae, N., Takio, K., Kamiya, Y., and Koshiba, T. (1997) Cloning and molecular characterization of plant aldehyde oxidase. *J Biol Chem* **272**: 15280–15285.

Uchida, H., Kondo, D., Yamashita, A., Nagaosa, Y., Sakurai, T., Fuji, Y., *et al* (2003) Purification and characterization of an aldehyde oxidase from *Pseudomonas* sp. KY 4690. *FEBS Microbiol Lett* **229**: 31–36.

Uchida, H., Fukuda, T., Satoh, Y., Okamura, Y., Toriyama, A., Yamashita, A., *et al* (2005) Characterization and potential application of purified aldehyde oxidase from *Pseudomonas stutzeri* IFO 12695. *Appl Microbiol Biotechnol* **68**: 53–56.

Yasuhara, A., Akiba-Goto, M., Fujishiro, K., Uchida, H., Uwajima, T., and Aisaka, K. (2002) Production of aldehyde oxidases by microorganisms and their enzymatic properties. *J Biosci Bioeng* **94**: 124–129.