Acta Biotechnol. **23** (2003) 1, 3–17

Purification and Characterisation of the Enantiospecific Dioxygenases from *Delftia acidovorans* MC1 Initiating the Degradation of Phenoxypropionate and Phenoxyacetate Herbicides

WESTENDORF*, A., MÜLLER, R. H., BABEL, W.

UFZ – Umweltforschungszentrum Leipzig – Halle GmbH
Sektion Umweltmikrobiologie
Permoserstraße 15
04318 Leipzig, Germany

* Corresponding author
Phone: + 49 341 235 2418
Fax: + 49 341 235 2247
E-mail: annewest@umb.ufz.de

Summary

After cultivation on (*R,S*)-2-(2,4-dichlorophenoxy)propionate, two α-ketoglutarate-dependent dioxygenases were isolated and purified from *Delftia acidovorans* MC1, catalysing the cleavage of the ether bond of various phenoxyalkanoate herbicides. One of these enzymes showed high specificity for the cleavage of the *R*-enantiomer of substituted phenoxypropionate derivatives: the K_m values were 55 μM and 30 μM, the k_{cat} values 55 min^{-1} and 34 min^{-1} with (*R*)-2-(2,4-dichlorophenoxy)propionate [(*R*)-2,4-DP] and (*R*)-2-(4-chloro-2-methylphenoxy)propionate, respectively. The other enzyme predominantly utilised the *S*-enantiomers with K_m values of 49 μM and 22 μM, and k_{cat} values of 50 min^{-1} and 46 min^{-1} with (*S*)-2-(2,4-dichlorophenoxy)propionate [(*S*)-2,4-DP] and (*S*)-2-(4-chloro-2-methylphenoxy)propionate, respectively. In addition, it cleaved phenoxyacetate herbicides (i.e. 2,4-dichlorophenoxyacetate: K_m = 123 μM, k_{cat} = 36 min^{-1}) with significant activity. As the second substrate, only α-ketoglutarate served as an oxygen acceptor for both enzymes. The enzymes were characterised by excess substrate inhibition kinetics with apparent K_i values of 3 mM with (*R*)-2,4-DP and 1.5 mM with (*S*)-2,4-DP. The reaction was strictly dependent on the presence of Fe^{2+} and ascorbate; other divalent cations showed inhibitory effects to different extents. Activity was completely extinguished within 2 min in the presence of 100 μM diethylpyrocarbonate (DEPC).

Introduction

The degradation of chlorinated/methylated phenoxyalkanoate herbicides is usually initiated by the cleavage of the ether bond of these compounds leading to the formation of respective phenolic moieties and the oxidised alkanoates. The prevailing reaction is catalysed by an α-ketoglutarate-dependent dioxygenase as studied with the purified enzyme from *Ralstonia eutropha* JMP134; α-ketoglutarate is oxidatively decarboxylated

 0138-4988/03/0101-0003 $ 17.50+.50/0

to succinate in this step [1, 2]. The enzyme from the above strain was determined to be highly specific for the phenoxyacetate structure. Genes encoding this type of enzyme, i.e. *tfdA,* were found to be widespread in microbial communities [3–9]. By contrast, there are only a few examples of axenic strains that are able to productively utilise phenoxypropionate herbicides: *Sphingomonas herbicidovorans* (*Flavobacterium* sp.) MH [10, 11], *Rhodoferax* sp. P230 [12] and *Delftia* (*Comamonas*) *acidovorans* MC1 [13]. Remarkably, these strains utilise a broader spectrum of phenoxy herbicides; in addition to their ability of degrading the racemic phenoxypropionates, they similarly attack phenoxyacetate derivatives. Again, this reaction proved to be catalysed by an α-ketoglutarate-dependent dioxygenase [14–16].

Preliminary investigations revealed enantioselective properties of the enzymes catalysing the cleavage of the ether bond of the racemic phenoxypropionates as holds for *S. herbicidovorans* MH [14, 17] and *D. acidovorans* MC1 [15]. The enantioselectivity of this reaction is also supported by the fact that a strain of *Alcaligenes denitrificans* is only able to utilise the *R*-enantiomer of 2-(4-chloro-2-methylphenoxy)propionate [18]. These enzymes have, however, not been studied in detail.

The present investigation is aimed at elucidating the enzymatic basis of the broad herbicide consumption profile including the phenoxyacetate derivatives in *D. acidovorans* MC1. Enzymes carrying ether-cleaving activity with respect to the various phenoxyalkanoate herbicides were purified and characterised with regard to their catalytic properties.

Materials and Methods

Cultivation

Strain MC1 was inoculated from individual colonies grown on (*R,S*)-2,4-DP into PYE medium (3 g/l of each peptone and yeast extract and 1.8 g/l of fructose) and propagated in an overnight culture; then it was incubated for a further day in the presence of 100 mg/l of (*R,S*)-2,4-DP. One litre of this culture was used to inoculate a fermenter (INFORS, ALGU 503) operating at a working volume of 4 l. Cultivation proceeded under aerobic conditions at pH 8.0 and 30 °C. The medium was composed of [mg/l]: NH_4Cl, 1520; KH_2PO_4, 680.5; K_2HPO_4, 870.9; $CaCl_2 \times 6\ H_2O$, 11; $MgSO_4 \times 7\ H_2O$, 142; $FeSO_4 \times 7\ H_2O$, 10; $CuSO_4 \times 5\ H_2O$, 1.57; $MnSO_4 \times 4\ H_2O$, 1.23; $ZnSO_4 \times 7\ H_2O$, 0.88; $Na_2MoO_4 \times 2\ H_2O$, 0.5. (*R,S*)-2,4-DP was supplied as the sole carbon and energy source in a fed batch regime by adapting the feed rate to the substrate consumption capacity of the culture. Excess substrate in the medium did not exceed 0.5 g/l. In addition, a trace element solution containing the respective salts in the concentration specified above was continuously fed at a rate of 0.77 ml/h. Biomass increase (OD_{700}) and substrate consumption were monitored off-line. After the cell density had reached an $OD_{700} = 2$, about 50% of the suspension was harvested. The remaining culture in the fermenter was supplied with an adequate portion of the mineral salt solution and cultivation continued.

Cells were harvested by centrifugation at 4 °C at 8875 × g. The pellet was washed and re-suspended in 10 mM Tris/HCl buffer, pH 7.5 (buffer A). The suspension was stored at –20 °C pending further use.

Purification

Purification was performed by application of a GradiFrac System including pump P-1 and monitor UV-1 (AMERSHAM PHARMACIA BIOTECH). Unless otherwise indicated, all steps were carried out at 4 °C.

Step 1: Cells were disintegrated using a French Pressure Cell (AMINCO, Silver Spring, USA) by three passages at 140 MPa. Particle-free supernatants were obtained by centrifugation at 4 °C for 25 min at 20,000 × g.
Step 2: Protamine sulphate (1%, pH 7.0) was added to the supernatant to give a final concentration of 0.15%. The solution was stirred for 20 min and separated from the precipitate by centrifugation for 20 min at 20,000 × g.
Step 3: Ammonium sulphate was added by stirring the supernatant from step 2 to give a final concentration of 1.25 M. After stirring for 20 min, the precipitate was removed (20 min centrifugation at 20,000 × g). A further quantity of ammonium sulphate was added to the supernatant to give a final concentration of 2.5 M. After stirring for a further 20 min, the pellet was collected by centrifugation and used for further steps.
Step 4: The pellet was solubilised in a small quantity of buffer A and applied to a Hi Prep 26/10 desalting column (PHARMACIA, Sweden) filled with Sephadex G-25. The column was equilibrated with buffer A. Application of the sample and elution proceeded at a rate of 5 ml/min. The eluate was sampled in fractions of 10 ml, which were monitored for conductivity and protein content.
Step 5: Ion exchange chromatography (IEX) was carried out on a Source30Q (PHARMACIA, Sweden) column (25/14). The column was equilibrated with buffer A. The sample was applied with a rate of 1 ml/min and further treated with buffer A to remove non-bound proteins. The proper elution was started at the same rate by linearly increasing NaCl gradients of varying steepness. The total volume of the gradient solutions amounted to 120–180 ml. Eluates were collected in fractions of 6 ml.
Step 6: Hydrophobic interaction chromatography (HIC) was performed on a Butyl-Sepharose (PHARMACIA, Sweden) column with a bed volume of 20 ml. The column was equilibrated with buffer A containing different initial concentrations of ammonium sulphate. The protein samples derived from each purification step were supplied with ammonium sulphate to reach the desired initial concentration corresponding to the column equilibrium concentration. Desorption was performed by using a linearly decreasing ammonium sulphate gradient at a rate of 1 ml/min. The total volume of the gradient solutions amounted to 180 ml. Eluates were collected in fractions of 6 ml.
Step 7: Gel filtration was performed on a Superdex200 prep grade (PHARMACIA, Sweden) column (100/10). It was equilibrated with buffer A containing 0.15 M NaCl. Before application, the protein solutions were concentrated by precipitation with 3.4 M ammonium sulphate, re-solubilised in a small volume and applied and eluted at a rate of 0.5 ml/min. The eluates were collected in fractions of 10 ml.

Electrophoresis

One-dimensional electrophoresis was carried out with a PowerEase 500 system (NOVEX, San Diego, USA) on 12% Tris glycine precast acrylamide gels (NOVEX). The samples contained 1–2 μg of protein. The running buffer was composed of 2.9 g/l Tris base, 14.4 g/l glycine and 1.0 g/l SDS. The sampling buffer contained 2 ml glycerol, 4 ml 10% SDS [w/v], 0.5 ml 1% bromophenol blue and 2.5 ml 0.5 M Tris/HCl, pH 6.8, in 10 ml distilled water. The gels were treated by 10 mA. Mark 12^{TM} Wide Range Standard (NOVEX) was applied as a reference.
Gels were stained by using the SilverXpress kit (NOVEX) following the instructions of the supplier, or by using Coomassie brilliant blue.

Enzyme Measurement

Ether-cleaving dioxygenase activity was routinely measured according to FUKUMORI and HAUSINGER [1] by determining the phenolic intermediates liberated in this enzymatic reaction after their reaction with 4-aminoantipyrine. The assay for the enzyme reaction contained 1 mM herbicide (sodium salt), 1 mM α-ketoglutarate, 200 μM ascorbic acid, 200 μM ammonium iron(II) sulphate in 10 mM imidazole buffer (pH 6.75). The buffer was gassed with air at 30 °C for 30 min before adding the individual components. The reaction was performed at 30 °C, started by adding the enzyme at a

concentration of 0.05–0.3 μM (with respect to the monomer). After respective times, usually within a period of 10 min, samples (up to 5) were taken and the enzyme reaction was stopped by adding 50 μl of 20 mM EDTA to 1 ml of the reaction mixture. The phenolic products were determined by adding 100 μl of borate buffer, pH 10 (3.09 g H_3BO_4; 3.73 g KCl; 44 ml 1 N NaOH ad 1 l), 10 μl 2% 4-aminoantipyrin and 10 μl 8% potassium hexacyanoferrate III. After 5 min of incubation at 30 °C, the extinction of the samples was measured at 510 nm (U-2000, HITACHI, Tokyo, Japan). The progress curves proved almost linear under these conditions as followed from linear regression giving a measure of confidence of > 0.98 with the substrate concentrations tested. The standard deviation of the individual points derived from triplicates amounted to ≤ 8 %. A comparison of the colorimetric assay with a direct measurement of the reaction by following substrate disappearance and product accumulation via HPLC gave identical rates.

Analytical Methods

Phenoxyalkanoates were determined by HPLC according to [19]. The biomass concentration was measured on the basis of the optical density at 700 nm. An $OD_{700} = 1$ corresponds to a biomass concentration of about 0.5 g/l dry mass.

Results

Enzyme Purification

Treatment of crude extract in the presence of 0.15% protamine sulphate and precipitation of proteins in the range of 1.25–2.5 M ammonium sulphate kept almost all of the ether-cleaving enzymes of the crude extract detected by measuring activities in the presence of the individual enantiomers of 2,4-DP (Tab. 1) and of 2,4-D (not shown). Application of IEX chromatography separated two protein fractions from each other, which exhibited distinct activity to the various substrates. The fraction eluted at low salt concentrations of around 0.12 M exhibited pronounced activity toward (*S*)-2,4-DP and, in addition, activity with respect to 2,4-D which coincided with the peak at low NaCl concentration. A second fraction found at around 0.35 M NaCl exhibited selective activity by only converting the *R*-enantiomer. Pooled fractions carrying the respective activities were further treated by HIC chromatography. This resulted in a significant purification of the two enzymes (Tab. 1), which were consecutively named the *R*-specific RdpA and the *S*-specific SdpA enzyme, respectively. Again, activity with respect to 2,4-D cleavage coincided with the activity profile for (*S*)-2,4-DP. Characterisation by SDS-PAGE revealed that this purification protocol resulted in a rather pure product with the *S*-specific enzyme (Fig. 1). In contrast, significant impurities were still observed with the *R*-specific enzyme (Fig. 2). Densidometric analysis revealed an enrichment of 80% of the *R*-specific enzyme at this preparation step. Use of gel filtration to remove these impurities was less successful as the activity was drastically diminished (Tab. 1). This step was therefore omitted in further preparations. These impurities did, however, not disturb consecutive kinetic investigations as it was proven that any reaction using the phenolic intermediate as the substrate (chlorophenol hydroxylase) was absent after IEX chromatography. This was verified by keeping product, i.e. dichlorophenol, unutilised within 12 h of incubation in the presence of the respective enzyme

fractions. From the electrophoretic patterns (electropherograms), molecular weights of the (subunits of the) *S*-specific and the *R*-specific enzyme of about 32 kD and 36 kD, respectively, were derived.

Tab. 1. Purification of 2,4-DP/α-KG dioxygenases from *D. acidovorans* MC1

	(*R*)-specific enzyme			(*S*)-specific enzyme		
Purification step	Specific activity [mU/mg]	Purification [-fold]	Total activity [%]	Specific activity [mU/mg]	Purification [-fold]	Total activity [%]
Crude extract	7.2	1	100	6.8	1	100
PS-P	6.7	0.93	93	5.8	0.85	95
AS-P	13.8	1.9	92	11.3	1.7	86
Source 30Q	25.4	3.5	66	101	14.9	65
Butyl-sepharose	172.8	24	45	259	38	8
Gel filtration	154	21.4	1	205	30.2	0.8

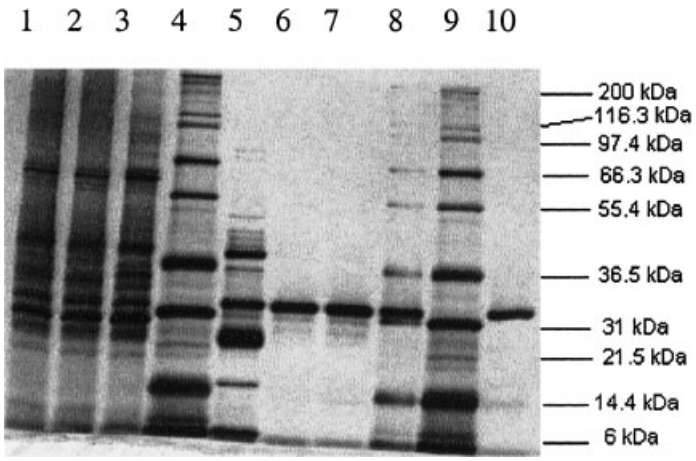

Fig. 1. Purification of *(S)*-2,4-DP/α-KG dioxygenase from *D. acidovorans* MC1
Protein samples were analysed by gel electrophoresis on a 12% polyacrylamide gel and visualised by silver staining.
1: Crude extract, 2: Supernatant after the treatment with 0.15% protamine sulphate, 3: Fraction precipitated between 1.25–2.5 M ammonium sulphate, 5: Pooled fractions with (*S*)-2,4-DP-cleavage activity after IEX, 6+7: Fractions with (*S*)-2,4-DP-cleavage activity after HIC, 8+10: Pooled fractions with (*S*)-2,4-DP-cleavage activity after GF, 4+9: Molecular weight marker.

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS
Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS
Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS
Sync your system to PACER to automate legal marketing.