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Detection and characterisation of catechol 2,3-dioxygenase in an indigenous soil *Pseudomonad* by MALDI-TOF MS using a column separation

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Abstract

The key enzyme catalyzing the second step in the phenol degradation *meta*-cleavage pathway (C23O) has been purified to homogeneity from a new bacterial strain, which belongs to genus Pseudomonas. The species was growing on phenol as carbon source. The C23O was detected and identified by absorption spectroscopy. The protein was isolated using sucrose density centrifugation and anion exchange chromatography. The purified protein showed a molecular mass of 32 kDa to sodium dodecyl sulfate polyacrylamid gel electrophoresis and an isoelectric point of 5 estimated by analytical isoelectrical focusing. Matrix-assisted laser desorption ionization-time of flight mass spectrometry and peptide mapping was attempted for the identification of the isolated protein and proteins involved in the metabolic pathway.

Abbreviations: C12O – catechol 1,2-dioxygenase; C23O – catechol 2,3-dioxygenase; MALDI-TOF – matrix assisted laser desorption ionisation-time of flight; MS – mass spectrometry; SDS PAGE – sodium dodecyl sulfate polyacrylamid gel electrophoresis

Introduction

The aerobic catabolism of aromatic compounds has been extensively investigated for a variety of microorganisms and for different natural and xenobiotic compounds (Bouwer & Zehnder 1993; Williams & Sayers 1994). Three of the most well known key dihydroxyaromatic intermediates resulting from aromatic compounds biodegradation are catechol, protocatechuic acid and gentisic acid. These intermediates further undergo ring fission to yield metabolites, such as pyruvic acid, acetic acid, succinic acid, and acetyl-CoA, for the Krebs cycle (Houghton & Shanley 1994; Johnson & Stanier 1971).

Of the dihydroxyaromatic intermediates, the most frequently encountered metabolite before ring cleavage is catechol. The formation of catechol proceeds *via* the incorporation of molecular oxygen into its aromatic precursor (Hammer et al. 1996), and the primary substrates that can be funnelled into catechol range from single ring benzene to three-ring phenanthrene (Harwood & Parales 1996). Once catechol is formed, it can be degraded through either the *meta* or the *ortho* ring cleavage pathway by enzymes catechol 2,3-dioxygenase (C23O) and catechol 1,2-dioxygenase (C12O), yielding 2-hydroxymuconic semialdehyde and *cis*, *cis*-muconate, respectively. C23Os have been purified from a variety of organisms

comprising *Pseudomonas*, *Alcaligenes*, and *Bacillus* (Chen et al. 1998; Kang et al. 1998; Kataeva & Golovleva 1990; Ornston & Stanier 1966). The molecular weight of the enzymes is about 34 kDa and showing a quaternary structure composed of four subunits (Kita et al. 1999; Winkler et al. 1995).

Mass spectrometry has become a powerful tool in recent years for the characterization of proteins by direct analysis of proteolytic peptide fragment mixtures (peptide mapping) (Quadroni & James 1999; Yates et al. 1995). The ability to identify the gene products expressed by a particular cell, or entire organism and to quantify changes in their expression levels resulting from external (e.g., environmental) is essential to the functional analysis of biological systems.

The phenol degrader subject of the present investigation was isolated from enriched mixed culture from samples of petroleum-contaminated soil in Denmark. The strain referred as *Pseudomonas sp*. strain phDV1 was isolated and identified by phenol utilization and 16S rRNA sequence analysis. 16S-rRNA sequence analysis of phDV showed 99.8% DNA sequence identity to *Pseudomonas pseudoalcaligenes* (Polimenakou & Stephanou, unpublished results).

In the present study we report the isolation of a C23O from a novel strain of *Pseudomonas sp*. grown on phenol as sole carbon and energy source. The C23O was detected and identified by absorption spectroscopy and mass spectrometric peptide mapping.

Material and methods

Microorganism and culture conditions

The bacterium was cultured in a modified minimal medium as described by (Kunz & Chapman 1981). The carbon source (phenol) was added to a final concentration of 200 mg l^{-1}. The bacterium was grown at 30 °C. Measuring the optical density at 600 nm monitored the growth.

Purification of the catechol 2,3 dioxygenase

The purification procedure was carried out in 50 mM Tris–SO_4 buffer, pH 8.0, at 4 °C unless otherwise stated. Cell suspensions were diluted in 50 mM Tris–SO4, pH 8 in a ratio 1 to 2. Cells were broken by sonication with a Broxon ultrasonic processor at the maximum power (20 times, 15 sec with 45 s intervals) with a freezing bath. Unbroken cells and cell debris were removed from the resulting suspension by centrifugation at 10,000 g for 15 min at 4 °C. The supernatant was then centrifuged at 100,000 g for 1 h at 4 °C to pellet the membrane fraction. The supernatant obtained after centrifugation was loaded on a sucrose density gradient. The steps were 30% sucrose (1 ml), 20% sucrose (4 ml) and 10% sucrose (4 ml). The sucrose solution was in 50 mM Tris–SO_4 buffer, pH 8 containing 10 mM acetone. After centrifugation in a Sorvall TH-641 rotor (210,000 g, 10 h) the yellow band located in 10% sucrose step, which show C23O activity was collected. The sample was applied to a MonoQ column (Pharmacia) attached at a Dionex HPLC system previously equilibrated with 50 mM Tris–SO_4, pH 8.0 containing 10 mM acetone and 25 mM NaCl. The column was washed with at least 80 ml of the same buffer and then the bound proteins were eluted with a linear gradient of 25–500 mM Na_2SO_4 in the same buffer at 1 ml min^{-1}. The fractions containing the highest C23O activities were collected and dialysed against 20 mM Tris–SO_4, pH 8.0 containing 10 mM acetone, overnight. The dialysed protein solution were loaded again on to a MonoQ (Pharmacia) attached on a Dionex HPLC System previously equilibrated with 20 mM Tris–SO_4, pH 8 containing 10 mM acetone. After protein injection the bound proteins were eluted with a linear gradient of 0–500 mM Na_2SO_4 in the same buffer at 1 ml min^{-1}.

Enzyme assay

Optical spectra were recorded on an Aminco dual wavelength DW 2000 UV-Vis spectrophotometer (SLM Aminco). C23O and C12O activity was measured following the formation of 2-hydroxymuconate semialdehyde and *cis*,*cis*-muconic acid at 375 nm and 260 nm and 25 °C ($\varepsilon_{375} = 4.4 \times 10^4$ $mole^{-1}$ cm^{-1}, $\varepsilon_{260} = 1.6 \times 10^4$ $mole^{-1}$ cm^{-1}), respectively by the procedure of (Kojoma et al. 1961). Spectroscopic measurements were performed in 3 ml quartz vials with 1 cm path length. C12O activity was monitored in the presence of 1 mM EDTA and 33 mM Tris/HCl at pH 8 and 10 μl enzyme extract with the total

volume being 2 ml. The reaction was started by catechol addition (0.1 mM end concentration). A unit of enzymatic activity is defined as the amount of enzyme producing 1 μmol of *cis*, *cis*-muconate or 2′ hydroxymuconate semialdehyde per minute at 25 °C.

Analytical methods

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) was performed using a self-built system with 12% acryl amide gels ($26 \times 24 \times 0.1$ cm) according to (Schaegger & von Jagow 1987). Staining was carried out with 0.02% Coomassie Brilliant Blue G-250 in 10% acetic acid. Analytical isoelectric focusing was run as described by (Tsirogianni & Tsiotis 2002).

In situ *gel digestion and sample preparation for MALDI-MS*

Coomassie blue stained protein spots were excised from gels. Gel slices were distained with a solution of 50% v/v acetonitrile and 25 mM ammonium bicarbonate, pH 8.0. After dehydration with acetonitrile and dried in a Speed Vac, proteins were reduced with 10 mM DTT in 100 mM ammonium bicarbonate for 1 h in 56 °C and then alkylated using 55 mM iodoacetamide in 100 mM ammonium bicarbonate for 45 min at room temperature in the dark. The gel pieces washed with 100 mM ammonium bicarbonate and dehydrated with acetonitrile. After the drying of the gel pieces as above, protein was digested using 50 mM ammonium bicarbonate, 5 mM $CaCl_2$, 125–300 ng Bovine Trypsin (Sequence Grade from Boehringer/Roche) for 45 min on ice. After centrifugation the supernatant was removed and replaced with digestion buffer without trypsin and digestion occurred at 37 °C overnight. The peptides from the gel pieces were extracted using 50% acetonitrile and 5% formic acid, concentrated to dryness in a Speed Vac, and resuspended in 5 μl 50% acetonitrile and 1% trifluoroacetic acid. 0.4 μl of each sample was loaded onto a MALDI plate (PerSeptive Biosystems) with an equal volume of 10 mg ml^{-1} w/v α-cyano-4-hydroxy-cinnamic acid solution in 50% acetonitrile and 1% trifluoroacetic acid. MALDI-TOF MS was performed on a Voyager-DE STR instrument (PerSeptive Biosystems) run in delayed extraction reflector mode using an accelerating voltage of 20 kV. Spectra were externally calibrated with a five peptide mixture using Data Explorer (PE Biosystems). The resulting peptide mass fingerprints were searched using the programs MS-Fit, PROWL and Mascot.

Results and discussion

C23O purification

Several aromatic substrates (benzene, toluene, phenol, m-cresol, nitrobenzene, and bromobenzene) were investigated, but only phenol was utilized as the sole source of carbon and energy at concentration varies from 100 to 1000 mg l^{-1}. C23O was purified to homogeneity from *Pseudomonas sp*. strain phDV1 after grown at 16–20 h, were maximum activity of the enzyme was detected. The purification procedure involved a sucrose density fractionation of the crude cell extract which has been demonstrated to be useful to reduce the complexity of the total protein mixture as well as to collect and concentrate the cell fraction exhibiting C23O activity. The active fraction that was found to have C23O activity by spectrometric analysis was collected. A two-step anion exchange chromatography using MonoQ column was used for the purification of the C23O. As shown in Figure 1a, the protein peak appeared at 100 mM $(NH_4)_2SO_4$ on the second MonoQ corresponding to C23O as determined by activity measurements. The pure C23O with specific activity of 20 U mg^{-1} is obtained after a 25-fold enrichment, and a yield of 49% similar to isolation protocols for the C12O from *Acinetobacter radioresistens* (Briganti et al. 2000; Briganti et al. 1997). In contrast a 29% yield has been reported for the isolation of C23O from *Pseudomonas putida* mt-2 (Nakai et al. 1983).

Molecular and spectroscopic properties

Electrophoretic analysis of the enzyme with a Tricine SDS-PAGE stained with Coomassie blue gave a single sharp band corresponding to a molecular mass of 32,000 Da (Figure 1b, lane 2) like to other C23Os (Kitayama et al. 1996; Nordlund & Shingler 1990). When the purified C23O was analysed by analytical isoelectric focussing it was found to have an isoelectrical point of 5, similar to that reported for

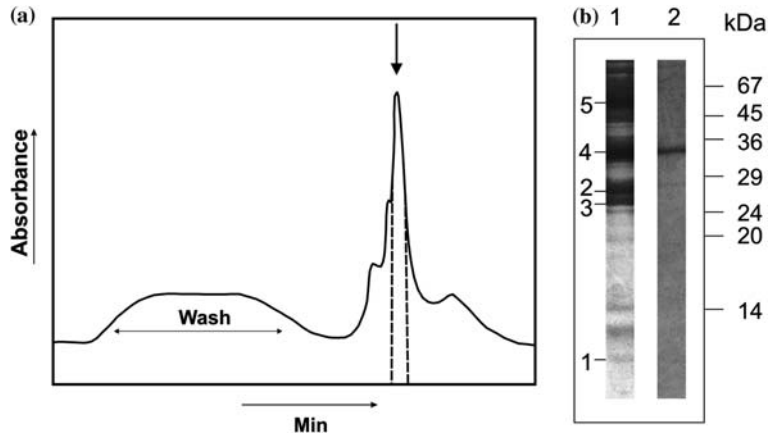

Figure 1. (a) The elution profile of the second MonoQ. The arrow shows the peak, which shows C23O activity. (b) Tricine PAGE of protein fraction loaded on the column with C23O activity after the first anion exchange column (lane 1), and of the protein fraction which shows C23O activity (the dotted lanes indicate the peak) (lane 2).

Pseudomonas CF600 and *Pseudomonas aeruginosa* JI104 (Kitayama et al. 1996; Nordlund & Shingler 1990; You et al. 1991).

C23O are nearly colourless, unlike Fe(III) intradiol dioxygenase (Kobayashi et al. 1995). Figure 2 shows UV/visible spectrum of the purified C23O. The insert in Figure 2 show the absorption spectrum of the purified protein in the presence of catechol in the range of 230–450 nm (continuous line). The increase of the peak at 375 nm (dotted lines) indicates the formation 2-hydroxymuconate semialdehyde. That could be clearly identified the isolated protein as a C23O and the strain metabolise phenol *via* the *meta* pathway. The purified enzyme was unstable losing the activity completely at 8 °C within 3 days. The activity could be stabilized by the addition of 10 mM acetone which is weak competitive inhibitor and also the most effective stabilizer (Kobayashi et al. 1995).

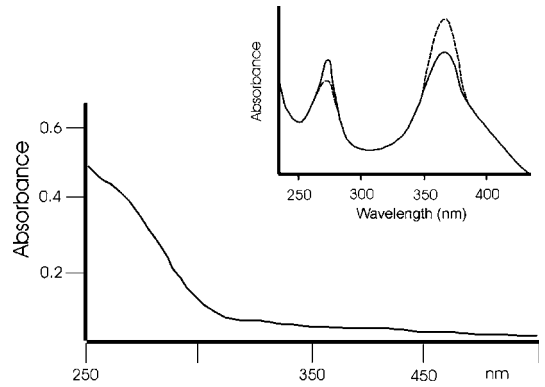

Figure 2. Absorption spectrum of the purified protein. Insert absorption spectrum in the presence of catechol (dotted line) and after 4 min (continuous line). The spectrum shows that the peak at 274 nm decreases and of 375 nm increases.

Protein identification by mass spectrometric peptide mapping

Identification of the isolated protein was achieved by mass spectrometric peptide mapping. The protein band (Figure 2b, lane 2) was excised, in-gel digested with trypsin, and analysed by MALDI-TOF MS. The MALDI mass spectrum (data not shown) of the digestion mixture of the protein showed peptides with a sequence coverage of 71% (218/307 amino acids) of the C23O from *Pseudomonas aeruginosa* (Q8KQ37, Swiss-Prot accession number) (Chen et al. 2002). Figure 3 shows the MALDI-MS peptide mapping obtained peptide sequences (in bold) in the sequences of the C23O from *Pseudomonas aeruginosa*. In contrast attempts using degenerate primers for C23O and C12O was not successful to detect the key enzyme of the metabolic pathway (Okuta et al. 1998). The mass spectrometric peptide mapping data of proteins after *in situ* gel digestion generally provided increased sensitivity compared with genetic studies, leading to a larger number of peptides identified.

In addition we applied MS peptide mapping for the identification of the proteins present in the fraction which has been loaded in the second anion exchange chromatography. Proteins were

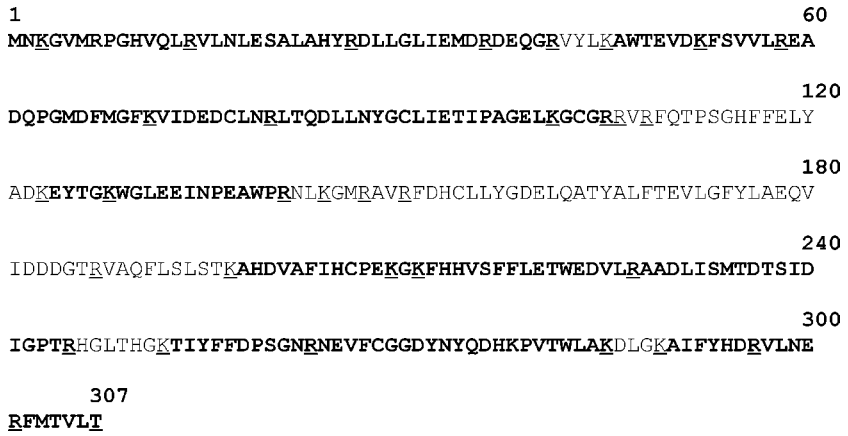

Figure 3. The obtained peptide sequences (in bold) which fit with the sequences of C23O of *Pseudomonas aeruginosa*. The underlined amino acids indicate the sites of the tryptic cleavage.

separated by 1-DE Tricine PAGE and stained with Coomassie dye (Figure 2b, lane 1). Bands were excised, in-gel digested with trypsin, and analysed by MALDI-TOF MS peptide mapping. The proteins are listed in Table 1. This included C23O (Q8KQ37; DmpB), 4-oxalocrotonate decarboxylase (Q9Z431; NahH), 2-hydroxypent-2,4-dienoate hydratase (Q52059; DmpE), benzene monoxygenase oxygenase subunit (P95408; BmoA) and BmoB protein (P95409; BmoB). In agreement with the spectroscopic data, all the proteins analysed were proteins involved in the *meta* phenol degradation pathway.

Conclusions

In conclusion, *Pseudomonas sp*. strain phDV1 is a degrader of phenol. Proteomic approach combined with column purification was very useful for screening the proteins for biodegradation in soil bacteria. On the basis of the results obtained in this study, the degradation of phenol by *Pseudomonas sp*. strain phDV1 occurs *via* the *meta* pathway. Future investigation will be addressed in the inducible proteins but also towards the complete set of proteins necessary to metabolize aromatic molecules.

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Table 1. Identification of phenol metabolic proteins by MALDI-TOF MS

Spot No.	Accession No./ Organism	Protein definition (Gene name)	Predicted MW/pI	Peptides matched
1	P95409/*Pseudomonas aeruginosa*	BmoB protein (BmoB)	9767/6.4	9
2	P49156/*Pseudomonas putida*	4-oxalocrotonate decarboxylase (DmpH)	28427/5.1	14
3	Q52059/*Pseudomonas putida*	2-hydroxypent-2,4-dienoate hydratase (DmpE)	27867/5.2	15
4	P17262/*Pseudomonas sp*.	catechol 2,3 dioxygenase (DmpB)	35575/5.55	9
5	P95408/*Pseudomonas aeruginosa*	benzene monoxygenase oxygenase subunit (BmoA)	58012/4.89	26

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