BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Overproduction and characterization of a recombinant D-amino acid oxidase from *Arthrobacter protophormiae*

Birgit Geueke · Andrea Weckbecker · Werner Hummel

Abstract A screening of soil samples for D-amino acid oxidase (D-AAO) activity led to the isolation and identification of the gram-positive bacterium *Arthrobacter protophormiae*. After purification of the wild-type D-AAO, the gene sequence was determined and designated *dao*. An alignment of the deduced primary structure with eukaryotic D-AAOs and D-aspartate oxidases showed that the D-AAO from *A. protophormiae* contains five of six conserved regions; the C-terminal type 1 peroxisomal targeting signal that is typical for D-AAOs from eukaryotic origin is missing. The *dao* gene was cloned and expressed in *Escherichia coli*. The purified recombinant D-AAO had a specific activity of 180 U mg protein^{-1} for D-methionine and was slightly inhibited in the presence of L-methionine. Mainly, basic and hydrophobic D-amino acids were oxidized by the strictly enantioselective enzyme. After a high cell density fermentation, 2.29×10^6 U of D-AAO were obtained from 15 l of fermentation broth.

Introduction

D-Amino acid oxidases (D-AAOs, EC 1.4.3.3) constitute a well-investigated group of flavoenzymes that are known since Krebs 1935. They catalyze the oxidative deamination

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of D-amino acids to the corresponding keto acids, produce ammonia and hydrogen peroxide as by-products and contain flavin adenine dinucleotide (FAD) as non-covalently bound cofactor. D-AAOs from fungi, fish, birds, reptiles and different mammalian tissues have been discovered during the last decades (Konno and Yasumura 1992). The best explored D-AAOs originate from pig kidney (Curti et al. 1973; Fukui et al. 1987; Watanabe et al. 1989) and the yeasts *Rhodotorula gracilis* (Alonso et al. 1998; Casalin et al. 1991; Harris et al. 1999; Molla et al. 1998; Pilone et al. 1989a; Pilone et al. 1987; Ramon et al. 1998) and *Trigonopsis variabilis* (Berg and Rodden 1976; Gonzalez et al. 1997; Isoai et al. 2002; Szwajcer and Mosbach 1985; Yu et al. 2002). The crystal structures of the D-AAOs from *R. gracilis* and pig kidney were resolved (Mattevi et al. 1996; Miura et al. 1997; Umhau et al. 2000), and different approaches of enzyme engineering were performed to better understand their catalytic, structural and biotechnological properties (Boselli et al. 2004; Caldinelli et al. 2006; Ju et al. 2000; Piubelli et al. 2002; Sacchi et al. 2002).

Most of the characterized D-AAOs oxidize hydrophobic, neutral and basic D-amino acids, whereas D-glutamic acid and D-aspartic acid are not accepted as substrates. These substances were oxidized by a functional related group of enzymes, the D-aspartate oxidases (D-AspO, EC 1.4.3.1), that oxidize solely acidic D-amino acids (Negri et al. 1987; Simonic et al. 1997). D-AAOs from yeasts are involved in the metabolic utilization of D-amino acids, whereas the physiological function of mammalian D-AAOs is still uncertain. Recently, it was demonstrated that D-AAO regulates the *N*-methyl-D-aspartate receptor-mediated neurotransmission in the brain by degrading D-serine (Mothet et al. 2000).

D-AAOs are of special biotechnological interest, as some enzymes of this group catalyze the first step in large-scale processes that lead to 7-amino cephalosporanic acid, a

precursor of semisynthetic cephalosporins (Pilone and Pollegioni 2002; Pollegioni et al. 2004; Tishkov and Khoronenkova 2005). In combination with hydride reducing agents, D-AAOs were also applied for the cyclic deracemization and stereoinversion of α-amino acids (Beard and Turner 2002).

Basic local alignment search tool (BLAST) searches reveal various sequences from bacteria that show similarities to known eukaryotic D-AAOs, but no functional bacterial D-AAO has been described so far. In this paper, we report on the purification, overexpression and biochemical characterization of a highly active D-AAO from the environmental isolate *Arthrobacter protophormiae*.

Materials and methods

Media and growth conditions

Different dilutions of soil suspensions were prepared in 0.9% NaCl solution and plated on mineral agar medium containing 0.2 g l^{-1} of sodium propionate as sole carbon source and 0.01 g l^{-1} of potassium nitrate as sole nitrogen source (Goodfellow and Minnikin 1981). Strains were isolated by transferring colonies to M65 medium (10 g of malt extract, 4 g of glucose and 4 g of yeast extract per liter) that was solidified by the addition of 1.5% (w/v) agar. Single colonies were subsequently inoculated in M65 broth and grown at 30°C. The crude extracts of the cells and the culture supernatants were separately tested for D-AAO activity.

One soil isolate that exhibited D-AAO activity was assigned to the species *A. protophormiae* by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM 15035). The strain was grown aerobically in M65 broth supplemented with 2.7 g of L-alanine per liter (pH 7.2). After 24–28 h of cultivation at 30°C, the cells were harvested by centrifugation and stored at −20°C.

Purification of D-AAO from *A. protophormiae* and *Escherichia coli* BL21(DE3) carrying pE1D

Fifty grams of *A. protophormiae* cells were suspended in 10 mM potassium phosphate buffer (pH 7.0) and disrupted in a glass bead mill (SCP-Disintegrator, Innomed-Konsult AG, Sweden) as described previously (Geueke and Hummel 2002). The crude extract was loaded onto a Q-Sepharose FF column (2.6 by 10 cm; GE Healthcare) that was equilibrated with 10 mM potassium phosphate buffer (pH 7.0). The flow rate was 2 ml min^{-1}. The column was washed with 10 mM potassium phosphate buffer (pH 7.0) containing 0.2 M NaCl and the D-AAO eluted at a concentration of 0.35 M sodium chloride. Active fractions were pooled and loaded onto a column that was

packed with ceramic MacroPrep hydroxyapatite, type I (1.6 by 8.3 cm; BioRad). The column was equilibrated with 150 mM KCl in 10 mM potassium phosphate buffer (pH 6.8) and operated at a flow rate of 2 ml min^{-1}. The D-AAO activity was detected in the flow through. The active fractions were dialyzed against 50 mM triethanolamine (TEA)–HCl buffer containing 20% PEG 20000 (PEG, polyethylene glycol; pH 7.0). The concentrated sample was loaded onto a preparative, native polyacrylamide gel (10%) using the 491 Prep Cell (BioRad) and eluted with 50 mM TEA–HCl buffer (pH 7.0).

Escherichia coli BL21(DE3) carrying the plasmid pE1D was cultivated aerobically in 100-ml shaking flasks containing 20-ml Luria-Bertani (LB) medium supplemented with 100 μg ampicillin ml^{-1}. The cells were incubated at 37°C and 200 rpm and induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at an optical density of OD$_{550}$ 0.5. After induction, the temperature was reduced to 30°C. *Escherichia coli* BL21(DE3) carrying pE1D was disrupted by ultrasonification. The recombinant D-AAO was purified using anion exchange and hydroxyapatite chromatography as described for the wild-type enzyme.

N-terminal sequencing

The N-terminal sequence of the D-AAO was determined on an Automated Sequencer 477A with a 12A PTH analyser (Applied Biosystems). Partially purified D-AAO was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and digested with the endoproteinase Lys-C. The digest, separation by high-performance liquid chromatography (HPLC) and sequencing of one internal peptide was carried out by Toplab (Martinsried, Germany).

PCR conditions

All PCR reactions were performed using the *Taq* polymerase (Qiagen) with the supplied buffer. Two degenerated primers were constructed for the partial amplification of the *dao* gene from genomic DNA (P8for 5′-cgsatcaccgtsatcggctccggcgtsat-3′ and P10rev 5′-cttgtgytgsagsacytcsaggccytcsag-3′). The amplification procedure consisted of 3 min at 95°C followed by 8 cycles of 40 s at 95°C, 1 min at 72–64°C, 1 min at 72°C and 25 cycles of 40 s at 95°C, 1 min at 68°C and 1 min at 72°C. The reaction was stopped after a final incubation step for 10 min at 72°C. DNA sequencing was carried out by Sequiserve (Vaterstetten, Germany).

Identification and cloning of the *dao* gene

The *dao* gene sequence was isolated with the Universal GenomeWalker™ Kit (BD Clontech; http://www.clontech.com/products/literature/pdf/brochures/GenomeWalker.pdf).

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                                              Region I
A. protophormiae, D-AAO   -------------------MPTAPLR-----ITVIGSGVIGLSAAHEL   24
M. leprae                 --------------------MASNGQ-----QIVVIGAGVSGLTSAVCL  24
S. coelicolor             -------------------METELDDERDGEVVVVGCGVIGLTTAVVL   29
A. gambiae                -------------------VQQKCMGNMKQIVVIGAGVSGLSVAVQL    28
C. elegans                --------------------------MTPKIAIIGEGVIGCSTALQV    21
H. sapiens, D-AspO        MRPARHWETRFGARDFGGFQDCFFRDRLMDTARIAVVGAGVVGLSTAVCI  50
B. taurus, D-AspO         --------------------MDTVRIAVVGAGVMGLSTAVCI         22

A. protophormiae, D-AAO   AAAGHQVT---VAYDQELAECVSSVAAAIWFPYHSEN--SPAADKLLADS  69
M. leprae                 AEAGWPVQ---LWAAAMPQQTTSAVAGAVWAPPRPEER-ASTSLAWTGHS  70
S. coelicolor             AERGRRVR---LWTREPAERTTSVVAGGLWWPYRIEP--VALAQAWALRS  74
A. gambiae                AEHYYNVANVTLISENVSPNTTGDGSAGLWGPYYCGNTPDHKIVKWSSET  78
C. elegans                AQAVPDAR-VTVLSDRPFEQTCSFGPAGLFRIDDIAN------REFGKST  64
H. sapiens, D-AspO        SKLVPRCS-VTIISDKFTPDTTSDVAAGMLIPHTYPDTPIHTQKQWFRET  99
B. taurus, D-AspO         SKMVPGCS-ITVISDKFTPETTSDVAAGMLIPPTYPDTPIQKQKQWFKET  71

A. protophormiae, D-AAO   LARFEQLSEHP---ETGIDLRRGLNVDHLPG--ADRS--WTRIVAGTEEA 112
M. leprae                 LQIFRELANDP---STGVRMAPALTVGDLPG--ANAMGLAAKLIPDLRPA 115
S. coelicolor             LDVYEELAARPG--QTGVRMLEG-VLGETGL--DEVDGWAAARLPGLRAA 119
A. gambiae                HTFFHQLWKNGLGCKIGVCLQPCMRLTTDPN--GYPEPAWKEIVFGCQKI 126
C. elegans                FDWFAHLHRTEKGDKTGVKLLSGHIQSDSKERLEQQQKAYGDIVYNFRFL 114
H. sapiens, D-AspO        FNHLFAIANSAEAGDAGWHLVSGWQIFQSTP--TEEVPFWADVLGFRKM  147
B. taurus, D-AspO         FDHLFAIVNSAEAKDAGWILVSGWQIFQSIP--TEEVPYWADVLGFRKM  119

A. protophormiae, D-AAO   SPADLPDGA-------HAGVWATVPIITMSTYLGWRGRVEELCADFAKG  155
M. leprae                 DLADLPDGF-------GAGFFGTMPMIDMPQYLDWYTARLAAACCELETR 158
S. coelicolor             SAAEYAG----------TGLWARLPLIDMSTHLPWIRERLLAACGTVEDR 159
A. gambiae                QEPELKRLSNEHGRSYTGGYHFATFTCEPSGLLPYIFNRFIAVGGKFVAS 176
C. elegans                EKREILDLFPN---PSEHCIHYTAFASEGWKYVPYLKFQCQARCVEFLHR 161
H. sapiens, D-AspO        TEAELKKFP-----QYVFGQAFTTLKCECPAYLPWLEKRIKGSGGWTLTR 192
B. taurus, D-AspO         TKDELKKFP-----QHVFGHAFTTLKCEGPAYLPWLQKRVKGNGGLILTR 164

                                              Region II
A. protophormiae, D-AAO   TVTDLAQLKGG--ADLVVLAAGLRGGELLGDDDTVYPIRGQVVRLANTKN 203
M. leprae                 SVRSLAEAADT--APIIVVNCTGLGAAALANDD-TVRPLFGQHVVLTNPGL 205
S. coelicolor             AVTDLAEAD----APVVVNCTGLGARELVPD-PAVRPVRGQLVVVENPGI 204
A. gambiae                KVRNFDEIILNRPVDLIVNCSGLGSLELLASDKA-VLPIRGQVARVSAPWI 225
C. elegans                KVRDLEELANEG-YDVIVNCAGLSGGTLAGDDDSVYPIRGVVLDVEAHWH 210
H. sapiens, D-AspO        RIEDIWELHPS--FDIVVNCSGLGSRQLAGDSK-IFPVRGQVLQVQAPWV 239
B. taurus, D-AspO         RIEDLWELHPS--FDIVVNCSGLGSRQLAGDSK-IFPVRGQVLKVQAPWV 211

                                              Region III
A. protophormiae, D-AAO   LTQWLCDDNYPDGVSYIIPRREDIIVGGTDTANDWNREVEPQTSIDILER 253
M. leprae                 QQLFLEVNNGS-EWICYFPHPLRVVCGGISIPGRWDTTPDPAVTDRILQR 254
S. coelicolor             HNWLVAADADSGETTYFLPQPGRLLLGGTAEEDAWSTEPDPEVAAAIVRR 254
A. gambiae                YEVILD---DSDDGNYIIPNCETVILGGTHQMNDFNRNVKRDDSRFIRDG 272
C. elegans                KHFN-----YKDFITFTIPKENSVVIGSVKQENRWDLEITDVDRKDILER 255
H. sapiens, D-AspO        EHFIR----DGSGLTYIYPGTSHVTLGGTRQKGDWNLSPDAENSREILSR 285
B. taurus, D-AspO         KHFIR----DSSGLTYIYPGVSNVTLGGTRQKGDWNLSPDAEISKEILSR 257

                                              Region IV
A. protophormiae, D-AAO   AAKLVPELEGLEVLEHKVGLRPARETIRLEH-VACHPL------------ 290
M. leprae                 CRRIEPRLGEAAVIETITGLRPDRPSPRVEV-ELSGSV------------ 291
S. coelicolor             CAALRPEIAGARVLAHLVGLRPARDAVRLERGTLPDGR------------ 292
A. gambiae                CERHLPSLRNASVTNEWVGLRPGRDSVRLEMEHYRSDD---------QKG 313
C. elegans                YVALHPAHREPKILGEWSGLRPARKTIRIEKVEKKSEKSGKKYTVSSKKK 305
H. sapiens, D-AspO        CCALRPSLHGACNIREKVGLRPYRPGVRLQTELLARDG-----------Q 324
B. taurus, D-AspO         CCALRPSLRGAYDLREKVGLRPTRPSVRLEKELLAQDS-----------R 296

                                 Region V
A. protophormiae, D-AAO   --PVIAAYGHGGAGVTLSWGTAQRVAELAAQLAGEPAS-------     326
M. leprae                 --RYIHNYGHSSSGVTLSWGCARDVVRLVSD--------------     320
S. coelicolor             --RLVHNYGHGGAGVTVAWGCAQEAARLAS---------------     320
A. gambiae                SVPIIHNYGHGGCGVTLCWGCAKEVVEISSTLGWSKSST---[SKL]    355
C. elegans                GKNVVHYGHGGNCFTLGWGTAVEATKLVKSALNS-------[SKL]    343
H. sapiens, D-AspO        RLPVVHHYGHGSGGISVHWGTALEAAARLVSECVHALRTPIPKSNL    369
B. taurus, D-AspO         RLPVVHHYGHGSGGIAMHWGTALEATRLVNECVQVLRTPAPK[SKL]   341
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Genomic DNA was cut with *NruI*, *EcoRV*, *PvuII*, *StuI*, *NaeI* and *FspI* (BD Clontech or New England Biolabs). The GenomeWalker adaptors were ligated to the genomic DNA fragments, and these libraries were used as templates in nested PCR reactions with the following primers: Apfor3 5′-gcaccaggtcaccgtcgcctacgacca-3′, Aprev3 5′-caccagcttgg cagcgcgttccagga-3′, Apfor4 5′-cgccgccgccatctggttcccg tacca-3′, Aprev4 5′-ctgcggttcgacttcgcggttccagtcgtt-3′. The amplification procedures consisted of 3 min at 94°C followed by 4 cycles of 1 min at 94°C, 30 s at 65°C, 3 min at 72°C, 30 cycles of 1 min at 94°C, 30 s at 62°C and 3 min at 72°C. The reaction was stopped after a final incubation step for 10 min at 72°C.

The restriction sites *NdeI* and *BamHI* were attached to the *dao* gene by PCR to allow cloning into the expression plasmid pET21a (EMD Biosciences). Genomic DNA from *A. protophormiae* was used as a template, and the following primers were applied: ApNdeIfor 5′-ggaattccatatgcccacag caccgttgag-3′ and ApBamHIrev 5′-cgggatccc tagctggccggctcgcca-3′. The PCR product and pET21a were digested with *NdeI* and *BamHI*, purified, ligated and transformed into *E. coli* BL21(DE3). Clones were tested by restriction digest and sequencing of the newly obtained plasmid pE1D.

High cell density fermentation of *E. coli* BL21(DE3) carrying pE1D

A fed-batch fermentation was carried out using the following medium for the batch (per liter): 0.2 g of NH_4Cl, 2 g of $(NH_4)_2SO_4$, 13 g of KH_2PO_4, 10 g of K_2HPO_4, 6 g of $NaH_2PO_4 \cdot H_2O$, 3 g of yeast extract, 2 g of glucose, 0.1 g of ampicillin, 9.3 mg of $Na_2EDTA \cdot 2H_2O$, 0.1 g of thiamine, 0.3 ml of antifoam, 5 ml of vitamin and 4 ml of trace element solution. The feed medium consisted of 0.2 g of NH_4Cl, 2 g of $(NH_4)_2SO_4$, 13 g of KH_2PO_4, 10 g of K_2HPO_4, 6 g of NaH_2PO_4, 21 g of yeast extract, 600 g of glucose, 10 g of $MgSO_4 \cdot 7H_2O$, 0.4 ml of antifoam, 5 ml of vitamin and 2 ml of trace element solution per liter. The vitamin solution contained (per liter): 0.1 g of riboflavin, 10 g of thiamine/HCl, 0.5 g of nicotinic acid, 0.5 g of

◄ **Fig. 1** Alignment of D-AAO from *A. protophormiae* (AAP70489), four hypothetical D-AspOs and two mammalian D-AspO. The sequence identities of the D-AAO from *A. protophormiae* to the putative D-AspOs as well as the mammalian D-AspOs and the corresponding accession numbers are as follows: 33%—*Mycobacterium leprae* (CAC30966), 37%—*Streptomyces coelicolor* (T35265), 35%—*Anopheles gambiae (*EAA01013), 32%—*Caenorhabditis elegans* (T19979), 29%—human D-AspO (AAH32786), 31%—bovine D-AspO (P31228). The alignment was generated with the ClustalW algorithm. Identical amino acids are marked *black*, residues with high similarity *dark gray* and with low similarity *light gray*. Regions *I* and *III* and regions *II, IV* and *V* are involved in coenzyme and substrate binding, respectively. All eukaryotic proteins have the C-terminal type 1 peroxisomal targeting signal S(K/N)L (*marked by a frame*)

pyridoxine/HCl, 0.5 g of Ca-panthotenate, 1 mg of biotin, 2 mg of folic acid and 10 mg of cyanocobalamin. The trace element solution contained (per liter): 10 g of $CaCl_2 \cdot H_2O$, 0.5 g of $ZnSO_4 \cdot 7H_2O$, 0.25 g of $CuCl_2 \cdot 2H_2O$, 2.5 g of $MnSO_4 \cdot H_2O$, 1.75 g of $CoCl_2 \cdot 6H_2O$, 0.125 g of H_3BO_3, 2.5 g of $AlCl_3 \cdot 6H_2O$, 0.5 g of $Na_2MoO_4 \cdot 2H_2O$, 10 g of $FeSO_4 \cdot 7H_2O$.

The fermenter was inoculated by adding 1% (v/v) of preculture grown in LB medium containing 100 μg of ampicillin ml^{-1}. The cultivation was carried out in a Techfors fermenter using the IRIS software (Infors, Bottmingen, Switzerland). The batch volume was 10 l and the feed volume 5 l. For the fed-batch fermentation, an exponential feeding strategy according to Korz et al. (1995) was slightly modified and applied according to the following equation:

$$m_S(t) = \left(\frac{\mu_{set}}{y_{X/S}} + m \right) - V(t_0) - X(t_0) - e^{\mu_{set}(t-t_0)}$$

m_S is the mass flow of substrate (g h^{-1}), $y_{X/S}$ is the biomass/ substrate yield coefficient (g g^{-1}), m is the specific maintenance coefficient (g g^{-1} h^{-1}), X is the biomass concentration (g l^{-1}) and V is the cultivation volume (l). The constant specific growth rate μ_{set} was set to 0.12 h^{-1}. The cells were induced with 0.5 mM IPTG after 28 h of cultivation. The pO_2 was adjusted to 30% by adapting the stirrer speed, the pressure and the aeration rate. The pH was maintained at 7 by adding NH_4OH, and the temperature was kept at 30°C. The cells were harvested with a separator KA06 (Westfalia, Oelde, Germany) after a total fermentation time of 39 h.

Analytical methods

The D-AAO activity was measured spectrophotometrically at 30°C and 436 nm following the oxidation of *o*-dianisidine by hydrogen peroxide that was catalyzed by an excess of peroxidase (ε=8.1 mM^{-1} cm^{-1}). Unless otherwise stated, 1 ml of assay mixture contained 10 mM D-methionine, 0.2 M TEA–HCl buffer (pH 7.6), 0.2 mg of *o*-dianisidine, 100 U peroxidase and D-AAO in limiting amounts. One U was defined as the amount of enzyme that catalyzes the degradation of 1 μmol D-amino acid per minute. The K_m for the D-AAO was determined at 30°C with D-methionine concentrations in the range of 0.2– 10 mM. The influence of L-methionine on the D-AAO activity was measured in the presence of 90 mM L-methionine and 1 to 20 mM D-methionine. The kinetic data were fitted with the MicroMath Scientist software (Saint Louis, USA).

Protein concentration was determined according to Bradford with bovine serum albumin as a standard. The purity and expression rates of D-AAO samples were

monitored by polyacrylamide gel electrophoresis using Bis/Tris-gels (4–12%) and the NuPAGE-System (Invitrogen). The native molecular weight of the purified enzyme was determined by gel filtration with a Superdex 200 PG column as published previously (Geueke and Hummel 2002). Isoelectric focussing was performed using precast Serva polyacrylamide gels with a narrow pH range (pH 4–6).

Accession number

The nucleotide sequence of the *dao* gene was submitted to the GenBank under accession no. AY306197.

Results

Screening for D-AAO activity

A selective screening of 99 soil samples was performed to isolate bacteria that belong to the genera *Rhodococcus* and *Nocardia* and have L-amino acid oxidase activity (Geueke and Hummel 2002). Four of the isolated strains exhibited D-AAO activity, whereas only one isolate oxidized L-amino acids. The strain with the highest activity toward the D-amino acids D-phenylalanine, D-leucine and D-methionine was a gram-positive, non-motile and strict aerobic bacterium. Analysis of the 16S rDNA gene sequence showed that the organism belongs to the species *A. protophormiae* (DSM15035). Under the reported growth conditions, a specific activity of 0.003 U mg protein^{-1} was reached in the crude extract of the wild-type strain with D-methionine as substrate. Different D- and L-amino acids were added to the medium and tested as possible inducers, but no increase in the activity could be observed (data not shown). As the attempt to raise the D-AAO expression rate in the wild type to a biotechnologically interesting level did not seem to be promising, we decided to identify the *dao* gene.

Purification of the D-AAO and identification of the *dao* gene sequence

The D-AAO was purified from 50 g of *A. protophormiae* cells using two chromatographical steps followed by dialysis and preparative native gel electrophoresis. The enzyme was partially inactivated during the purification procedure. The N-terminal sequence of the D-AAO was determined by automated Edman degradation (PTALPRITVIGSGVIGL SAA). The N-terminal methionine seemed to be processed. After proteolytic digestion with endoproteinase Lys-C and separation of the peptides by HPLC, one internal peptide was also sequenced (LVPELEGLEVLEHK). Degenerated primers were designed on the basis of this sequence information and used for PCR. A fragment of 700 bp was amplified and

identified as part of the *dao* gene. The application of the Universal GenomeWalker™ Kit led to a DNA fragment of 1,933 bp that was completely sequenced by primer walking. The *dao* open reading frame is coding for a protein of 326 amino acids corresponding to a theoretical molecular mass of 34.6 kDa per monomer. The start codon ATG is preceded by a potential ribosome binding site (aaagga).

A BLAST search revealed that the D-AAO from *A. protophormiae* showed high similarities to hypothetical proteins from *Mycobacterium leprae* (CAC30966), *Streptomyces coelicolor* (T35265), *Anopheles gambiae* (EAA01013) and *Caenorhabditis elegans* (T19979) and to D-AspO from human (AAH32786) or bovine (P31228) origin (Fig. 1).

Heterologous expression of the dao gene

The *dao* gene was cloned into the multiple cloning site of the expression plasmid pET21a yielding pE1D that was subsequently transformed into *E. coli* BL21(DE3). Experiments in shaking flasks were performed to optimize the expression conditions by varying the induction temperature (25, 30, 37°C), the IPTG concentration (0.01–1 mM) and the induction time (2–6 h). Best results were obtained when the cells were induced with 0.1 mM IPTG at an optical density of 0.5 followed by 4 h of incubation at 30°C. Under these conditions, the maximal specific D-AAO activity in the crude extract reached 30 U mg protein^{-1}.

Different concentrations of FAD (0–15 μM) were added to the assay mixture to measure if the enzyme was partially expressed as apoenzyme and if it could be reconstituted by the addition of cofactor. The activity in the crude extracts was not increased when external FAD was added.

Production and purification of recombinant D-AAO

After the high cell density fermentation, 1.9 kg of cells (wet weight) that had an activity of 1,200 U g cells^{-1} (wet weight) was harvested. The specific activity in the crude extract was 15 U mg protein^{-1}. We obtained 2.29×10^6 U of D-AAO from one 15-l fermentation.

The recombinant D-AAO was isolated to homogeneity using a two-step procedure (Table 1). The specific activity of 30 U mg protein^{-1} in the crude extract could be increased sixfold to 180 U mg protein^{-1} after the second chromatography. We obtained 1,434 U of D-AAO from 3 g of cells (wet weight) that corresponded to a yield of 56%. The purification process was monitored by polyacrylamide gel electrophoresis (Fig. 2).

Structural and biochemical characterization

The D-AAO had a native molecular mass of 69 kDa as measured by gel filtration. In combination with the

Table 1 Purification scheme of D-AAO from the recombinant *E. coli* BL21(DE3)/pE1D (3 g cells, wet weight)

	Total activity (U)	Total protein (mg)	Volume (ml)	Specific activity (U mg^{-1})	Purification (-fold)	Yield (%)
Crude extract	2,556	85	10	30	1.0	100
Q-Sepharose	1,909	24	9.7	80	2.7	75
Hydroxy-apatite	1,434	8	12	180	6.0	56

Table 2 Substrate preferences of the recombinant D-AAO

Substrate	Relative activity (%)
D-Methionine	100
D-Lysine	99
4-Fluoro-DL-phenylalanine	81
D-Arginine	80
D-Phenylalanine	54
DL-Homophenylalanine[a]	31
D-Ornithine	15
D-Leucine	12
D-Histidine	5.9
D-Tyrosine[a]	4.7
N-Methyl-DL-leucine	2.1
D-allo-Isoleucine	1.0
D-Valine	0.35
O-Methyl-DL-serine	0.33
D-Alanine	0.26
D-Proline	0.25
DL-*tert.*-Leucine	0.12
D-Serine	–
D-Asparagine	–
D-Aspartic acid	–
D-Glutamic acid	–
D-Threonine	–
D-Tryptophane	–
Glycine	–
Cephalosporin C[a]	–

The activities were measured at a final substrate concentration of 10 mM. In case DL-amino acids were used, the concentration was increased to 20 mM
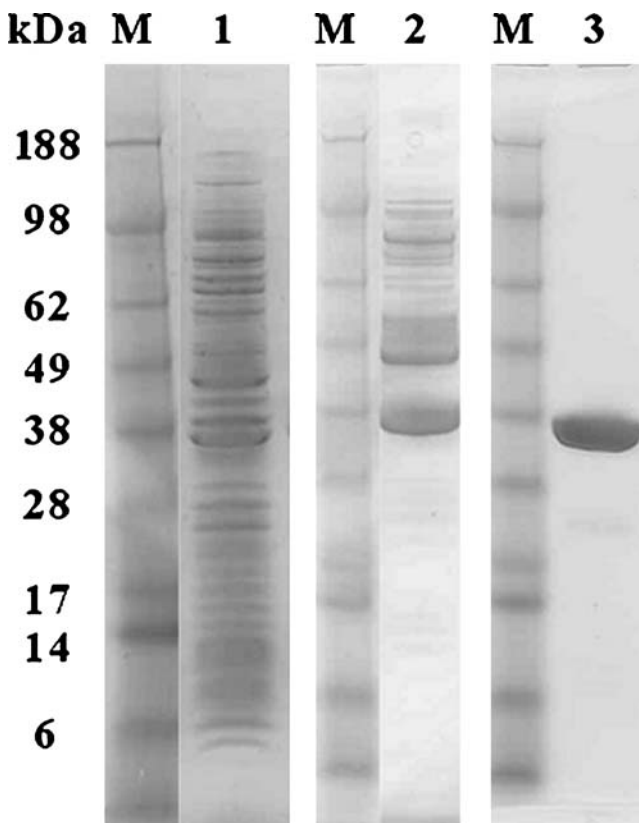
experimentally and theoretically determined molecular mass of the monomer, this result clearly indicated that the enzyme formed a homodimer. The isoelectric point was 4.4 as determined by isoelectric focussing.

The D-AAO showed activity mainly against basic and hydrophobic D-amino acids (Table 2). Small uncharged and acidic D-amino acids as well as cephalosporin C were not, or only weakly, oxidized. The enzyme exhibited an absolute stereoselectivity for the tested D-enantiomers. The K_m for D-methionine was 1.0 ± 0.1 mM, whereas L-methionine was a weak, noncompetitive inhibitor for the D-AAO ($K_i=315\pm39$ mM) when D-methionine was used as sub-

Fig. 2 Polyacrylamide gel electrophoresis of D-AAO containing samples at different stages of purification from *E. coli* BL21(DE3)/pE1D, corresponding to Table 1. *Lane M* marker; *lane 1* crude cell extract; *lane 2* pool after anion exchange chromatography; *lane 3* pool after hydroxyapatite chromatography

strate. No further L-amino acids were tested as inhibitors. The purified enzyme had a specific activity of 180 U mg protein^{-1} for D-methionine. The D-AAO had a pH optimum between 6.5 and 8.5, and it was stable at pH values between 6 and 9. The enzyme did not loose activity at temperatures up to 30°C for at least 2 h; it had a half life of 21 min at 40°C and of 2 min at 50°C when it was incubated in 100 mM TEA–HCl buffer (pH 7.6).

Discussion

The D-AAO from *A. protophormiae* is, to our knowledge, the first characterized enzyme of this class that originates from a bacterial source. The discovery of this prokaryotic D-AAO was facilitated by screening microbial isolates from soil applying a coupled *o*-dianisidine/peroxidase assay. The high stability and sensibility of oxidized *o*-dianisidine (Gabler et al. 2000) allowed the detection of very low enzyme amounts in the crude extract of *A. protophormiae*. One reason for the, hitherto, lack of information on prokaryotic D-AAOs might be the low expression rate in the wild-type strains as we observed in this study. A

comparison of the specific D-AAO activities in the crude extract of *A. protophormiae* (0.003 U mg protein^{-1}) with recombinant cells (15–30 U mg protein^{-1}) showed that the enzyme was expressed at extremely low amounts in the wild type. The expression rates of the fungal D-AAO-producing strains *R. gracilis* and *T. variabilis* were 0.6 U mg protein^{-1} (Pilone et al. 1989b) and up to 233 U g cell dry weight^{-1} (Gabler and Fischer 1999; Hörner et al. 1996; Umhau et al. 2000), respectively. These D-AAO activities were induced in the presence of *N*-carbamoyl-D-alanine, D-alanine or D-3-aminobutyric acid.

After the high cell density fermentation of *E. coli* BL21 (DE3)/pE1D, we obtained 153,000 U l fermentation broth^{-1}. This value is at least 20-fold higher than all activities that were published for the expression of recombinant and wild-type D-AAOs (Yu et al. 2002; Zheng et al. 2006). Also the specific activity in the crude extracts of recombinant cells (15–30 U mg protein^{-1}) exceeds the published data for yeast D-AAOs by factors between 3.4 and 60 (Yu et al. 2002; Zheng et al. 2006).

Addition of FAD to the crude extract of *E. coli* BL21 (DE3)/pE1D and to the purified D-AAO did not increase the activity of the enzyme, indicating that it might be completely expressed as holoenzyme with a very low dissociation constant of FAD. It is also conceivable that it was partially expressed as apoenzyme, but could not bind the added FAD. This behavior differs from other D-AAOs that were expressed in *E. coli*. Up to 62% of the *R. gracilis* D-AAO were expressed as apoenzyme in *E. coli* (Alonso et al. 1998), and the activity of recombinant *T. variabilis* D-AAO could be increased by 33% when FAD was added to the enzyme assay (Lin et al. 2000).

Under the specified conditions, purified D-AAO from *A. protophormiae* exhibits a specific activity of 180 U mg protein^{-1} for D-methionine. Comparing the activities of D-AAOs from different sources shows that the bacterial enzyme belongs to the highly active D-AAOs. The *T. variabilis* D-AAO had a specific activity of 86 U mg protein^{-1} for D-alanine (30°C, 50 mM sodium pyrophosphate, pH 8.5; Pollegioni et al. 1993). Depending on the purification scheme, the *R. gracilis* D-AAO reached specific activities of 58–175 U mg protein^{-1} for D-alanine, when the activity was measured at 37°C in 75 mM sodium pyrophosphate, pH 8.5 (Pilone et al. 1989a; Pilone et al. 1987). The D-AAOs from the yeasts *T. variabilis*, *R. gracilis* and *C. boidinii*, as well as the fungal and mammalian enzymes from *Fusarium oxysporum* and pig kidney, respectively, also oxidize D-methionine with high activities.

The purified recombinant D-AAO has a dimeric structure like the enzymes from the well-known yeast strains and an isoelectric point of 4.4, which is lower than the pIs measured for the D-AAOs from *R. gracilis* (7.8/7.4/7.2), porcine kidney (7.0/7.2) and *T. variabilis* (5.1) (Pilone et al.

1989a; Pollegioni et al. 1993). The *A. protophormiae* D-AAO has high sequence similarities to putative D-AAos from different sources and two mammalian D-AspOs (Fig. 1). Several conserved stretches were identified according to Faotto et al. (1995) (Fig. 1). Regions I and III are part of the coenzyme binding site; regions II, IV and V contain residues that are involved in substrate binding and form the active site. The published primary structures of eukaryotic D-AAOs and D-AspO contain a C-terminal signal sequence (SKL) that directs the protein to the peroxisomes (organelles that contain enzymes mainly catalyzing oxidative reactions) and is missing in the shown prokaryotic sequences. The localization of mammalian and yeast D-AAOs in the peroxisomes was proven for several species.

The results described here show that the spectrum of biotechnologically useful D-AAOs was broadened by this highly active and biochemically interesting enzyme from *A. protophormiae*.

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