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Cloning and Expression in *Escherichia coli* of the D-Aspartate Oxidase Gene from the Yeast *Cryptococcus humicola* and Characterization of the Recombinant Enzyme

Shouji Takahashi, Toshiyuki Takahashi, Yoshio Kera, Ryuji Matsunaga, Hiroo Shibuya and Ryo-hei Yamada*

Department of Environmental Systems Engineering, Nagaoka University of Technology, Nagaoka, Niigata 940-2188

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The D-aspartate oxidase (DDO) from the yeast *Cryptococcus humicola* UJ1 (ChDDO) is highly specific to D-aspartate. The gene encoding ChDDO was cloned and expressed in *Escherichia coli*. Sequence analysis of the *ChDDO* gene showed that an open reading frame of 1,110 bp interrupted by two introns encodes a protein of 370 amino acids. The deduced amino acid sequence showed an FAD-binding motif and a peroxisomal targeting signal 1 in the N-terminal region and at the C-terminus, respectively, and also the presence of certain catalytically important amino acid residues corresponding to those catalytically important in D-amino acid oxidase (DAO). The sequence exhibited only a moderate identity to human (27.4%) and bovine (28.0%) DDOs, and a rather higher identity to yeast and fungal DAOs (30.4–33.2%). Similarly, phylogenetic analysis showed that ChDDO is more closely related to yeast and fungal DAOs than to mammalian DDOs. The gene expression was regulated at the transcriptional level and specifically induced by the presence of D-aspartate as the sole nitrogen source. ChDDO was expressed in an active form in *E. coli* to an approximately 5-fold greater extent than in yeast. The purified recombinant enzyme was identical to the native enzyme in physicochemical and catalytic properties.

Key words: *Cryptococcus humicola*, D-aspartate oxidase, flavoenzyme, gene expression, yeast.

Abbreviations: CBB, Coomassie Brilliant Blue; ChDDO, D-aspartate oxidase from *Cryptococcus humicola* UJ1; DAO, D-amino acid oxidase; DDO, D-aspartate oxidase; GABA, γ -aminobutyrate; IPTG, isopropyl-β-D-thiogalactopyranoside; LB, Luria-Bertani; pigDAO, pig kidney DAO; PTS1, peroxisomal targeting signal 1; RACE, rapid amplification of cDNA ends; RgDAO, D-amino acid oxidase from *Rhodotorula gracilis*; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SSC, standard saline citrate; TB, Terrific Broth.

D-Aspartate oxidase (DDO) is an FAD-containing enzyme that catalyzes the oxidative deamination of D-aspartate and other acidic D-amino acids to give the corresponding α-keto acids and ammonia (*1*). On the other hand, D-amino acid oxidase (DAO) catalyzes the same reaction of neutral and basic D-amino acids. Because of their common catalytic properties, it has been suggested that they may have derived from the divergent evolution of a single gene (*2*), although no information is available on microbial DDOs at present. With respect to their physiological functions, the two enzymes are probably involved in the regulation of the levels of D-amino acids and in the detoxification of endogenous and exogenous D-amino acids in certain tissues in mammals (*3*); in yeasts, DAO is likely to be involved in the utilization of D-amino acids for cell growth (*4*, *5*), while little is known about yeast DDOs.

DDOs have been purified and characterized from octopus hepatopancreas (*6*), bovine kidney (*7*) and the yeast *Cryptococcus humicola* UJ1 (previously known as *Cryptococcus humicolus* UJ1) (*8*). In addition, *DDO* genes have been cloned from bovine kidney and human brain, and their recombinants expressed in *Escherichia coli* were characterized (*9*, *10*). Of these characterized DDOs, the *C. humicola* DDO (ChDDO) is the only microbial enzyme and is markedly different from the animal DDOs in many aspects: substrate specificity, catalytic efficiency, quaternary structure, and inhibitor specificity. In particular, ChDDO shows a much higher specificity to D-aspartate than the mammalian DDOs (*8*). This property will allow the enzyme to be a useful tool for the specific and rapid determination of D-aspartate, which will help in studies of the physiological functions of the amino acid. Despite this attractive property, information about the structural features of the enzyme is very limited and, therefore, structural differences between the yeast and mammalian DDOs remain unknown, since the yeast gene has not yet been isolated.

In this paper, we report the cloning of the gene encoding DDO from the yeast *C. humicola* UJ1 and its expression in *E. coli*. Moreover, the recombinant ChDDO was purified and characterized. This is the first report of a *DDO* gene from a microorganism.

*To whom correspondence should be addressed. Tel: +81-258-47-9635, Fax: +81-258-47-9635, E-mail: ryyamada@vos.nagaokaut.ac.jp

MATERIALS AND METHODS

Materials—The yeast nitrogen base without amino acids was from Difco (Detroit, MI, USA). D-Aspartate was a generous gift from Tanabe Pharmaceutical (Osaka, Japan). Other amino acids, *N*-methyl-D-aspartate and γ-aminobutyrate (GABA) were from Nacalai Tesque (Kyoto, Japan). Restriction endonucleases and other DNA-modifying enzymes were from Takara Bio (Kyoto, Japan) or Toyobo (Osaka, Japan). Butyl-Sepharose 4FF, SP-Sepharose and Superdex 200 columns were from Pharmacia (Uppsala, Sweden). All other chemicals were of analytical purity and purchased from commercial sources.

Strains, Media, and Growth Conditions—*Cryptococcus humicola* UJ1 (*8*) was used as the source for genomic DNA and total RNA. The yeast cells were grown in YPD medium or SD minimal medium (*11*) containing 10 mM nitrogen sources at 30°C. *E. coli* DH5α and XL1-Blue MRF' were employed in DNA manipulation as a host, and XL1-Blue MRA (P2) was used as a host for the propagation of the yeast genomic DNA library (*12*). *E. coli* BL21 (DE3) and Rosetta (DE3) were used as hosts for recombinant protein production. *E. coli* cells were grown in Luria-Bertani (LB), 2× YT or Terrific Broth (TB) media (*13*). Where necessary, ampicillin, chloramphenicol, or both were added to the media at a final concentration of 100 μg/ml and 34 μg/ml, respectively.

Isolation of the ChDDO Gene—Total cDNA was synthesized from RNA isolated from yeast cells grown on D-aspartate as the sole nitrogen source as described previously (*12*). Using the cDNA as a template, we obtained a *ChDDO* gene fragment by PCR using ExTaq DNA polymerase (Takara) with the forward (5′-ATGCCCTCGGACCCCATCATYGTCCTYGGYGCSGG-3′) and reverse (5′-GTGGCGGGGTCGAGGACRCGRAAGTCSGGGTTRCC-3′) primers, where Y is C/T, R is A/G, and S is C/G, based on the amino acid sequences, MPSDPIIVLGAG and GNPDFRVLDPAT, from the purified DDO protein from the yeast. For the PCR reaction, 2.5 units of the DNA polymerase and 50 pmol of each primer were used. The PCR conditions were 2 min at 94°C followed by 35 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 50°C, and extension for 1 min at 72°C. A resulting approximately 400-bp PCR product was cloned into pT7BlueT vector (Novagen, Madison, WI, USA) by TA cloning and sequenced. Screening of the entire coding region from the yeast genomic DNA library was performed using the PCR product as a probe as reported previously (*12*). A 1.7-kbp *Sal*I DNA fragment from a positive clone was subcloned into pBluescript II KS (+) (Stratagene, La Jolla, CA, USA) at the same site and sequenced.

The cDNA of the *ChDDO* gene was synthesized by PCR using Platinum Pfx DNA polymerase (Invitrogen, Carlsbad, CA, USA) with the forward (5′-GTCTGTCGACCTCTCCCACAACACACCACACCACC-3′; *Sal*I site is underlined) and reverse (5′-CACGGTCGACCAGCTTGCATCGGCCCTGCTCTACG-3′; *Sal*I site is underlined) primers, which anneal just outside the ORF. The total cDNA prepared as described above was used as a template. The amplified PCR product was digested with *Sal*I and cloned into pBluescript II KS (+) at the same site; the resulting plasmid was named pKSCD2 and its nucleotide sequence was determined.

Identification of Transcription Start Site—The transcription start site of the *ChDDO* gene was determined by RNA-ligase-mediated rapid amplification of cDNA ends (RLM-RACE) method (*14*) using a GeneRacer kit (Invitrogen) following the manufacturer's instructions. The GeneRacer kit ensures that only full-length transcripts are amplified by prior elimination of truncated transcripts. Briefly, 5 μg of total RNA extracted from yeast cells grown on D-aspartate as a sole nitrogen source was dephosphorylated with calf intestinal phosphatase to remove the 5′-terminal phosphate group from all RNA molecules without a 5′-methyl guanosine cap structure. The RNA was then treated with tobacco acid pyrophosphatase to remove all the caps and to generate 5′-phosphate groups, which were ligated to synthetic RNA adaptor molecules provided in the kit. A first-strand cDNA template was synthesized using a ThermoScript RT-PCR system (Invitrogen) and GeneRacer oligo dT primer. The 5′-region of the *ChDDO* cDNA was amplified from the cDNA by nested PCR using Platinum Pfx DNA polymerase with the gene-specific primer (5′-GCCTTCCACTCGTCGTACAG-3′) and GeneRacer 5′ primer in the first amplification round, and the nested gene-specific primer (5′-CCAGTGATCCGCGAGTATG-3′) and GeneRacer 5′ nested primer in the second amplification round. The resulting PCR product, which appeared as a single band on agarose gel electrophoresis, was cloned into pBluescript II SK (+) at the *Eco*RV site, and 5 independent clones were sequenced.

Construction of Expression Plasmid—The *ChDDO* gene was amplified by PCR using Platinum Pfx DNA polymerase with the forward (5′-CACCCAT**ATG**CCCCCCTCCGACCCCATCATCG-3′; *Nde*I site is underlined and initiation codon is shown in bold) and reverse (5′-CGGCGGATCC**CTA**CAGCCGTGCACCCCCAGCCCCC-3′; *Bam*HI site is underlined and termination codon is shown in bold) primers to engineer *Nde*I and *Bam*HI restriction sites at the ends of the gene, respectively. The pKSCD2 was used as a template. The amplified fragment was digested with *Nde*I and *Bam*HI and ligated to pET11b (Novagen) at the same sites, and the resulting plasmid was designated pECD5. The construct was verified by sequencing.

Southern and Northern Blot Analysis—Southern blot analysis was carried out using the DNA fragment used for the library screening as a probe as reported previously (*12*). Northern blot analysis was carried out as follows. Yeast cells were grown on SD minimal medium containing 10 mM sole nitrogen source at 30°C until they reached an early exponential phase, OD_{600} of about 1.0. Total RNA was extracted from the yeast cells by the acid-phenol extraction method (*15*). Ten micrograms of total RNA was size-fractionated on a 1.3% (w/v) agarose/2.2 M formaldehyde denaturing gel and transferred onto a Hybond-N^+ membrane (Amersham Bioscience) by capillary action using 20× SSC. RNA was cross-linked to the membrane using a UV cross linker (UVP, Upland, CA, USA). Hybridization and detection were performed using an Alkphos Direct Labelling and Detection System with CDP-star with the *ChDDO* cDNA from pKSCD2 as a probe according to the instructions of the manufacturer.

The signal was obtained by exposing the filter to X-ray film (Fuji Film, Tokyo, Japan). To normalize for RNA loading, the membrane was detected with *C. humicola* UJ1 18S ribosomal DNA (DDBJ/EMBL/GenBank databases accession no. AB118907) as a probe after the bound *ChDDO* probe was stripped.

Expression and Purification of Recombinant ChDDO—*E. coli* BL21 (DE3) transformant cells were grown in LB medium containing 100 μg/ml ampicillin. *E. coli* Rosetta (DE3) transformant cells were grown in TB medium containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. The cells were cultivated at 30°C with shaking. When the OD_{660} reached 0.5, IPTG was added to the medium at a final concentration of 1.0 mM, and the cells were cultivated further at 30°C for 6 h with shaking. The recombinant enzyme was purified from the *E. coli* Rosetta (DE3) transformant cells as described previously (*16*). The homogeneity of the purified enzyme was confirmed by SDS-PAGE. D-Aspartate oxidase activity was assayed by colorimetric measurement of keto acids produced from D-aspartate (*8*). The various enzymatic properties of the purified recombinant enzyme were determined as described previously (*8*).

Western Blot Analysis—The proteins separated by SDS-PAGE were transferred onto a PVDF membrane (Bio-Rad, Hercules, CA, USA) by an electroblotting method, and the ChDDO protein was detected using an Immuno-Blot Kit (Bio-Rad) with rabbit anti-ChDDO polyclonal antiserum raised in our laboratory as the primary antibody and alkaline phophatase-conjugated anti-rabbit IgG (Bio-Rad) as the secondary antibody.

Other Methods—All DNA sequencing was carried out at least twice on both strands with an ALF express II DNA sequencer (Amersham Bioscience) using a Thermo Sequenase cycle sequencing kit with 7-deaza-dGTP as described previously (*12*). Protein concentration was determined by the method of Lowry *et al.* (*17*) using bovine serum albumin as a standard. SDS-PAGE was performed according to Laemmli (*18*). The native molecular mass of the purified recombinant ChDDO was estimated by gel filtration with a Superdex 200 column (1.6 × 60 cm) at 4°C. Absorption spectra were measured with a Shimadzu UV-3100PC spectrophotometer as described previously (*8*).

RESULTS AND DISCUSSION

Isolation and Characterization of the ChDDO Gene—To obtain a *ChDDO* gene fragment as a probe, we carried out PCR with total cDNA from the yeast cells grown on D-aspartate as the sole nitrogen source as a template and primers based on partial amino acid sequences of the purified ChDDO from the yeast. As a result, we obtained an approximately 400-bp DNA fragment encoding a polypeptide that was moderately homologous to yeast DAO. To isolate the entire coding region, we screened a yeast genomic DNA library using the PCR product as a probe. Screening of 20,000 plaques from the library yielded four positive clones. A 1.7-kbp *Sal*I DNA fragment containing the entire coding region of the *ChDDO* gene was subcloned from one clone, and its nucleotide sequence was determined (Fig. 1). Because the nucleotide

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gtcgacacgatcgcgtccaagccgcccgcgagcggcatcggcatcgtgcgcgacgaccag  -395
gccggcccgggcgagggcggcacaaagtggtaccgcgacattattgcgggctggccgctg  -335
gggcaggcggcgtaggcaggcgcgttaggggtatagatcatcttggttgtatagacgcat  -275
catgcatggcgagggtgtcgggattgcaacttgcgtcggctcggtgtcggacgagggcgg  -215
cgtgcctgacgtcactggttggcgataatctcgccgcatcgccgtttctgcgacgacatc  -155
gccgttcgcgtgtcaacatcgccgcttgctgctcaacatcgccgattctggccagcaacg   -95
acgaacgcgcgggcgagcatcgggcgcagcacggagcgtacttaagcgcgccgcgagacg   -35

tctcgacgcctctcccacaacacaccacaccaccATGCCCCCCTCCGACCCCATCATCGT    26
                                  M  P  P  S  D  P  I  I  V      9
CCTCGGCGCCGGCGTGATAGGCCTCACCACGGCCGTGCGGCTGCTCGAGGCGCACCTTGG   86
 L  G  A  G  V  I  G  L  T  T  A  V  R  L  L  E  A  H  L  G     29
CGCGAACGTCCACATACTCGCGGATCACTGGCCTTCGGACGCGCTGGACGCGCAGTACGC   146
 A  N  V  H  I  L  A  D  H  W  P  S  D  A  L  D  A  Q  Y  A     49
CAGTACGATCGCCGGCGCACACCACCTCAGCTTTGCGGATGACGGCGACGCGCGCCAGCG   206
 S  T  I  A  G  A  H  H  L  S  F  A  D  D  G  D  A  R  Q  R     69
CCGCTGGGACATGCGGAgtgggtcgtgtgtcgcgcactagagctgacggcagCGTTTGAC   266
 R  W  D  M  R  T                Intron 1                F  D     77
GTGCTGTACGACGAGTGGAAGGCCGTCGGCGAGAGGACCGGGCTTATGGCGCTCACGCAG   326
V  L  Y  D  E  W  K  A  V  G  E  R  T  G  L  M  A  L  T  Q      97
ACCGAGATGTGGGAGGGCGCGACGTCGCATCTCGCCGTGTACGAGGGGAACCCAGATgta   386
T  E  M  W  E  G  A  T  S  H  L  A  V  Y  E  G  N  P  D       116
agttgggcgaggaggccacggactgatagtagTTCCGCGTGCTCGACCCGCGTACCGCCC   446
          Intron 2                F  R  V  L  D  P  R  T  A  P   126
CGTGCAGCAACATCACCCACATGGTGTCCTTCACGAGCCTGACGATCGCGCCGACGGTGT   506
  C  S  N  I  T  H  M  V  S  F  T  S  L  T  I  A  P  T  V  Y   146
ACCTCGCCGCGCTGGAAGCCCGCGTGCGCGACCTCGGCGCGAAGCTGCACCGTGCCCACG   566
  L  A  A  L  E  A  R  V  R  D  L  G  A  K  L  H  R  A  H  V   166
TCCCATCGCTGGGCGCGcTGCGCACCGACCCGGCCCTGCTGGCGCTGTACACTCGCCCCC   626
  P  S  L  G  A  L  R  T  D  P  A  L  L  A  L  Y  T  R  P  P   186
CGGCCGCGGTGTTCGTCTGCGCCGGCCTCGGCGCGCGCCACCTCGTGCCCGCGCCTGAGG   686
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  A  A  L  F  P  T  R  G  Q  V  V  V  V  R  A  P  W  M  R  A   226
CAGGGTTCACGCGCCAGGTCGGCTCGCTCGGCGGCGGCGAGGGCGGCACGCGCACGTACA   806
  G  F  T  R  Q  V  G  S  L  G  G  G  E  G  G  T  R  T  Y  I   246
TTATCCCGCGGTGTAACGGCGAGGTCGTGCTTGGCGGCACGATGGAGCAGGGCGACTGGA   866
  I  P  R  C  N  G  E  V  V  L  G  G  T  M  E  Q  G  D  W  T   266
CGCCGTACCCCCGCGACGAGACAGTCACGGACATCCTCACGCGCGCGCTGCAGATCTGCC   926
  P  Y  P  R  D  E  T  V  T  D  I  L  T  R  A  L  Q  I  C  P   286
CGGACATCGCGCCACCGTACGCGCGCTCCTGGCCCAAGGACGACCAGGTGGCCGCGCTGC   986
  D  I  A  P  P  Y  A  R  S  W  P  K  D  D  Q  V  A  A  L  R   306
GTTCCATTGTCGTGCGCGATGCGGTCGGCTTTAGGCCTAGCCGCGCCGGCGGCGCCCGGG  1046
  S  I  V  V  R  D  A  V  G  F  R  P  S  R  A  G  G  A  R  V   326
TCGCGCTCGCCTCGGCGGCCGGCATGCGCGTCGTGTATAACTATGGCCATGGCGGCGCGG  1106
  A  L  A  S  A  A  G  M  R  V  V  Y  N  Y  G  H  G  G  A  G   346
GGTGGCAAAGCTGCTGGGGCTGTGCAGAGGACGCGGTGGCGCTGTGGGCCGGGGGGGCTG  1166
  W  Q  S  C  W  G  C  A  E  D  A  V  A  L  W  A  G  G  A  G   366
GGGGTGCACGGCTGtagtcgtagagcagggccgatgcaagctgcattgtcgtgctgggtg  1226
  G  A  R  L  *                                                370
ccgcgcaagcagagggcgggtggtgacaatgggggtgtggtggtgcctgttgcagccagc  1286
cgccattgtggcgaacaacactgtggcggaggtggccgtcgac                   1329
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Fig. 1. **Nucleotide sequence of the genomic *ChDDO* gene and its deduced amino acid sequence.** The numbers on the right are the nucleotide and amino acid positions from the initiation codon and first amino acid, respectively. Non-coding regions are shown in lowercase letters. An A+C-rich sequence is shown in italics. The 5′- and 3′-splicing signals and branch sequence in the introns are underlined with solid lines. The transcription start site is indicated by a bent arrow. The asterisk below the nucleotide sequence indicates the termination codon. The deduced amino acid sequence is shown in single letter amino acid abbreviations. An FAD-binding motif is underlined with a dashed line. Peroxisomal targeting signal 1 (PTS1) is boxed.

sequence suggested the presence of introns in the coding region, the cDNA was also synthesized and sequenced.

Figure 1 shows the nucleotide sequence of the genomic *ChDDO* gene and its deduced amino acid sequence. Alignment of the cDNA and genomic DNA sequences shows an open reading frame consisting of 1,110 nucleotides interrupted by two introns, with a high G+C con-

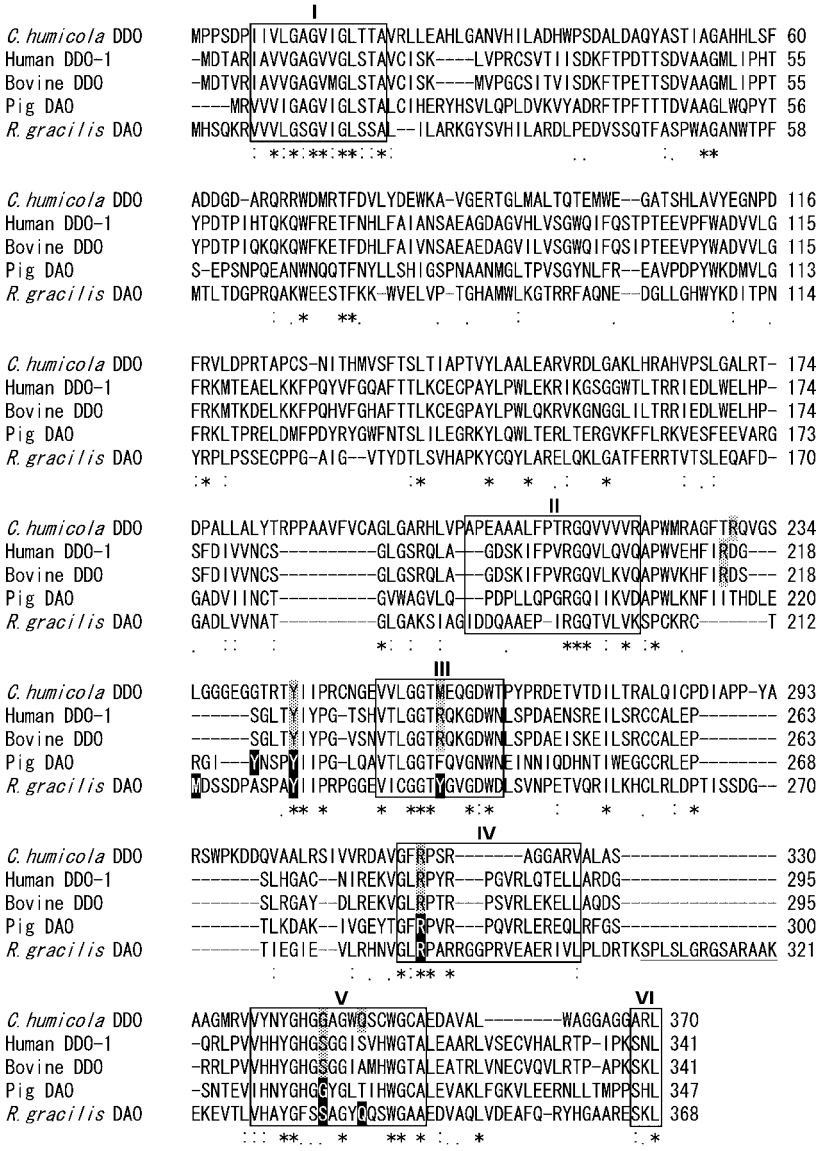

Fig. 2. **Multiple alignment of the amino acid sequences of DDOs and DAOs.** The sequences of *C. humicola* DDO**,** human DDO-1 (Swiss-Prot accession no: Q99489), bovine DDO (P31228), pig DAO (P00371), and *R. gracilis* DAO (P80324) are aligned. The alignment was generated by the Clustal X 1.81 program. Six highly conserved regions (*3*), labeled I-IV, are boxed. Identical residues and amino acid substitutions with low and high similarity are indicated by asterisks, dots, and double dots, respectively. Catalytically important residues in DAOs are shown in reverse letters. Putative catalytically important residues in DDOs are shown in gray boxes. The residues involved in the dimerization of *R. gracilis* DAO (*20*) are underlined with a solid line. Gaps are introduced to optimize alignment.

tent of 71.9% (Fig. 1). Both introns span 35 nucleotides and have a typical 5′-GTNNGY sequence, where N is any nucleotide and Y is C/T, and a typical YAG-3′ sequence at their splice junctions. In addition, a strictly conserved branch point in fungal systems, CTRAY, where R is A/G, is present. The 5′-flanking region shows no apparent TATA or CAAT boxes. However, an A+C-rich sequence is present just upstream of the putative initiation codon (Fig. 1) as found in the *URA3* gene from yeast (*12*), and the transcription start site, which was determined by 5′-RACE PCR, lies 16 bp upstream of the initiation codon, indicating that the ATG is the translation start site. The 3′-flanking region shows no canonical polyadenylation sequence, AATAAA, and no typical terminator consensus sequence, TAATTTTAGT, as are also not found in the *URA3* gene (*12*). Southern blot analysis of the genomic DNA digested with several restriction endonucleases suggested the existence of a single genomic copy of the *ChDDO* gene (data not shown).

Analysis of the Deduced Amino Acid Sequence—The deduced amino acid sequence from the *ChDDO* gene shows the enzyme to contain 370 amino acid residues (Fig. 1) with a calculated molecular mass of 39,459 Da, which agrees well with the subunit molecular mass (40 kDa) of ChDDO purified from the yeast as judged by SDS-PAGE (*8*). The N-terminal amino acid sequence (residues 1–20), including the central part of the dinucleotide-binding motif (Fig. 1), GXGXXG, is identical to that previously reported (*8*), with the exception of an additional proline residue. The C-terminal tripeptide is ARL, in agreement with the consensus sequence of peroxisomal targeting signal 1 (PTS1) (*19*), -S/A/C-K/R/H-L/M, suggesting that it is a peroxisomal protein as reported previously (*20*).

The amino acid sequence was aligned with those of DDOs and DAOs from various organisms (Fig. 2). Six conserved regions in DAOs have been reported (*3*): regions I and III are involved in coenzyme binding; regions II, IV and V contain the highly conserved residues of the active site; and region VI is the C-terminal tripeptide sequence representing PTS1. These regions are also conserved in ChDDO (Fig. 2). However, two long

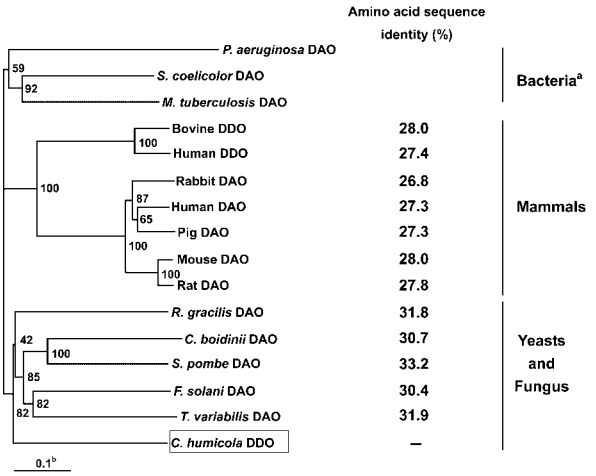

Fig. 3. **Phylogenetic relationship of DDOs and DAOs from various organisms.** The phylogenetic tree date was calculated using the Clustal X 1.81 program with default setting and the tree was generated using the TreeView program. Bootstrap values obtained from 1,000 resamplings are given in percentages and are indicated at the corresponding branch nodes. [a]The bacterial DAOs are putative proteins and are used as an out group. [b]The scale bar represents a distance of 0.1 substitutions per site. Accession numbers: *P. aeruginosa*. *Pseudomonas aeruginosa* DAO (PIR, B83078); *S. coelicolor*, *Streptomyces coelicolor* DAO (PIR, T35265); *M. tuberculosis*, *Mycobacterium tuberculosis* DAO (PIR, F70518); Human DAO (PIR, S01340) and DDO (Swiss-Prot, Q99489); Bovine DDO (Swiss-Prot, P31228); Pig DAO (Swiss-Prot, P00371); Rat DAO (Swiss-Prot, O35078); Mouse DAO (PIR, JH0185); Rabbit DAO (Swiss-Prot, P22942); *R. gracilis* (Swiss-Prot, P80324); *C. boidinii*, *Candida boidinii* (DAD, AB042032–1); *S. pombe*. *Schizosaccharomyces pombe* (PIR, T40989); *F. solani*, *Fusarium solani* (Swiss-Prot, P24552); *T. variabilis*, *Trigonopsis variabilis* (Swiss-Prot, Q99042).

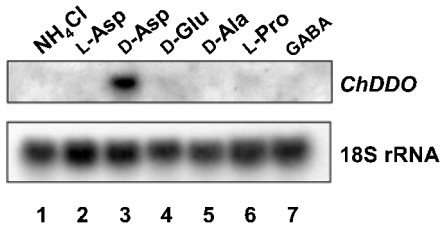

Fig. 4. **Northern blot analysis of ChDDO expression in *C. humicola* cells grown on several sole nitrogen sources.** Ten micrograms of total RNA from yeast cells grown on 10 mM of a sole nitrogen source was applied to each lane. The *ChDDO* mRNA was detected using the full length *ChDDO* cDNA as a probe (upper panel). To normalize for RNA loading, 18S rRNA was detected using an 18S rDNA fragment as a probe (lower panel).

stretches, from Arg184 to Ala193 and from Tyr292 to Asp300, were found in the sequence (Fig. 2). In the DAO from the yeast *Rhodotorula gracilis* (RgDAO), the stretch from Ser308 to Lys312 has been reported to contribute to its dimerization (*21*), implying that the stretches in ChDDO are involved in the tetramerization of the enzyme. The ChDDO showed only a moderate degree of identity to human DDO-1 (27.4%) and bovine DDO (28.0%), or to mammalian DAOs (26.8–28.0%) (Fig. 3). The identities to yeast and fungal DAOs (30.4–33.2%) are slightly higher than to mammalian DDOs and DAOs. Similarly, phylogenetic analysis showed that ChDDO forms clusters with yeast and fungal DAOs but not with mammalian DDOs, which are grouped with mammalian DAOs (Fig. 3). These findings raise the possibility that ChDDO may have separated from yeast and fungal DAOs during the course of evolution.

The three-dimensional structures of RgDAO and pig kidney (pigDAO) have been determined, and the forms of their complexes with substrate analogues suggest their modes of substrate binding (*22*): Tyr223 (RgDAO) and Tyr224 (pigDAO), Tyr238 (RgDAO) and Tyr228 (pigDAO), and Arg285 (RgDAO) and Arg283 (pigDAO) interact with an α-COO^- of the ligand; carbonyl group of Ser335 (RgDAO) and Gly313 (pigDAO) and a water molecule held in Gln339 (RgDAO) interact with an α-NH_3^+ (Fig. 2). These residues are considered to fix their substrate in the correct position and orientation. In ChDDO, the respective corresponding residues are conserved at Tyr245, Arg317, Gly344, and Gln348, except that Met260 seems to be placed at the structurally equivalent position to Tyr238 (RgDAO) and Tyr228 (pigDAO) (Fig. 2), suggesting that these residues in ChDDO may play the same role as their counterparts in the DAOs. Recently, RgDAO was converted so as to be catalytically active to D-aspartate by rational design (*23*): Met213Arg and Met213Arg/Tyr238Arg mutants of RgDAO were constructed because these residues were presumed to be placed at the equivalent positions to Arg216 and Arg237, respectively, in the active-site model of bovine DDO. The Met213Arg mutant was able to oxidize D-aspartate, but the double mutation (Met213Arg/Tyr238Arg) caused a decrease in the catalytic efficiency, indicating that Arg216 in bovine DDO may play an important role in substrate specificity to acidic D-amino acids. In ChDDO, Arg230 seems to be equivalent to Arg216 in bovine DDO (Fig. 2), and thus may play the same role. On the other hand, Met260 seems to be placed at the equivalent position to Arg237 in bovine DDO (Fig. 2). Although the function of these residues in DDO is unknown, the difference may contribute to the different catalytic properties of yeast and mammalian DDOs. To reveal the details, however, it is necessary to determine the three-dimensional structure or to perform mutation and deletion analyses of the enzyme.

Transcription of the ChDDO Gene in Yeast—It has been reported that DDO activity in yeast cells is induced when the cells are grown on D-aspartate as the sole nitrogen source, but not when grown on other D,L-amino acids, including L-aspartate or D-glutamate (*8*). Since there has, however, been little information on the induction, we analyzed the transcription of the *ChDDO* gene in yeast cells grown on various sole nitrogen sources by Northern blot analysis. As shown in Fig. 4, the positive band specific to a *ChDDO* probe was shown in the RNA from the cells grown on D-aspartate as the sole nitrogen source (Fig. 4, lane 3), whereas its enantiomer, L-aspartate, and another acidic D-amino acid, D-glutamate, were ineffective in activating its transcription (Fig. 4, lanes 2 and 4). Moreover, D-alanine, which induces *DAO* gene expression in the yeasts *Candida boidinii* (*24*) and *R. gracilis* (*25*), was also ineffective (Fig. 4, lane 5). It has been reported that the expressions of many genes involved in nitrogen assimilation in *S. cerevisiae* are under nitrogen catabolite repression (*26*), in which they are repressed on rich nitro-

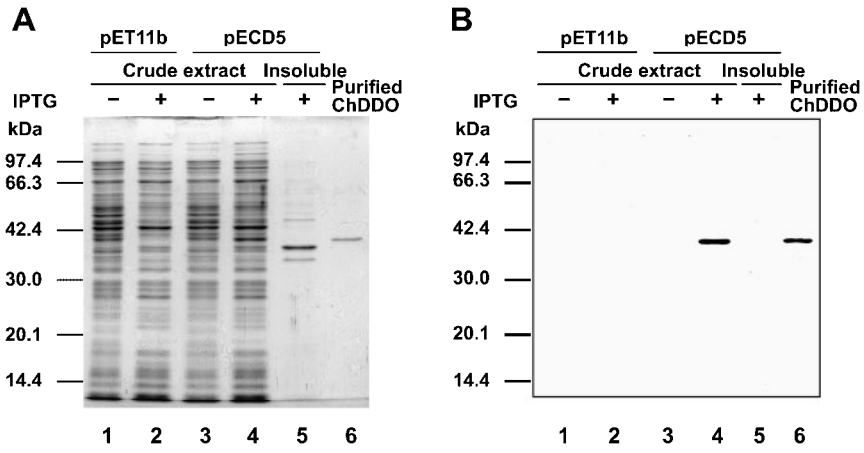

Fig. 5. **SDS-PAGE and Western blot analysis of the ChDDO protein expressed in *E. coli*.** (A) SDS-PAGE analysis of extracts from *E. coli* BL21 (DE3) transformant cells. Proteins were separated by 12% SDS-PAGE and stained with Coomassie Brilliant Blue. Lane 1, the crude extract from uninduced cells with pET11b; lane 2, the crude extract from IPTG-induced cells with pET11b, lane 3, the crude extract from uninduced cells with pECD5; lane 4, the crude extract from IPTG-induced cells with pECD5; lane 5, the insoluble fraction from IPTG-induced cells with pECD5; lane 6, purified DDO from *C. humicola* UJ1. Ten micrograms of protein in the extracts and 0.2 μg of purified ChDDO from yeast as a positive control protein were analyzed. The volume of the insoluble fraction analyzed was equivalent to the volume of the crude extract applied. (B) Western blot analysis of extracts from *E. coli* BL21 (DE3) transformant cells. The proteins in the samples described above were separated by 12% SDS-PAGE and transferred onto a PVDF membrane. The ChDDO protein was detected using rabbit anti-ChDDO polyclonal antiserum raised in our laboratory as the primary antibody, and alkaline phosphatase-conjugated anti-rabbit IgG as the secondary antibody.

gen sources, such as ammonia and L-glutamine, but are derepressed on poor nitrogen sources, such as L-proline and γ-aminobutyrate (GABA), thereby resulting in gene expression. Transcripts of the *ChDDO* gene were not detected in cells grown on either poor nitrogen sources (Fig. 4, lanes 6 and 7), suggesting that the expression is not subject to nitrogen catabolite repression. These results demonstrate that *ChDDO* gene expression is regulated at the transcriptional level and induced specifically by the presence of D-aspartate as the sole nitrogen source. In mouse liver, similarly, DDO activity is induced by the administration of D-aspartate (*27*), whereas no induction of enzyme activity was found in carp (*28*). Although its regulatory mechanism in mouse tissue is entirely unknown, it is possible that there is a common regulatory mechanism for *DDO* gene expression between yeast and mammals.

Expression of the ChDDO Gene in E. coli—To confirm that the gene encodes functional DDO, we constructed an expression system for the *ChDDO* gene in *E. coli*. The cDNA was inserted into pET11b to generate an expression plasmid, pECD5, and expressed under the control of the T7 promoter in *E. coli* BL21 (DE3) with 1.0 mM of IPTG. Significant DDO activity (1.66 U/mg of protein) was detected in the crude extract of IPTG-induced cells harboring pECD5, whereas no activity was detected in crude extracts of uninduced cells or in the extract of cells harboring the control vector, pET11b. To analyze the gene product, we carried out SDS-PAGE and Western blot analysis. On SDS-PAGE (Fig. 5), a slightly intense band is apparent in the extract of induced cells harboring pECD5 in comparison with other lanes, and this band has the same mobility as the purified native enzyme (Fig. 5A, lanes 4 and 6); no band is observed at the same mobility in the insoluble fraction (Fig. 5A, lane 5). In Western blotting, a single band with the same mobility as the native enzyme was detected only in the extract of induced cells harboring pECD5 (Fig. 5B, lanes 4 and 6); no band was detected in the other extracts or in the insoluble fraction (Fig. 5B, lane 5). These results clearly demonstrate that the cloned gene encodes ChDDO and that the recombinant ChDDO is expressed as a soluble form with activity in *E. coli*. To produce more enzyme, we examined several expression conditions. The best conditions involved gene expression in *E. coli* Rosetta (DE3) cells carrying tRNA genes for rare *E. coli* codons as a host, in the presence of 1.0 mM of IPTG for 6 h at 30°C in TB medium. Under these conditions, the recombinant protein was estimated to account for about 1.7% of the total bacterial protein assuming that the recombinant protein exhibits the same specific activity as the purified native enzyme (150 U/mg protein) (*16*). The production of the recombinant enzyme reached approximately 9.0 mg/liter culture, which is about 5-fold higher than that in yeast. In addition, the specific activity in the crude extract, 1.21 U/mg protein (Table 1), was 1.64-fold higher than that in yeast.

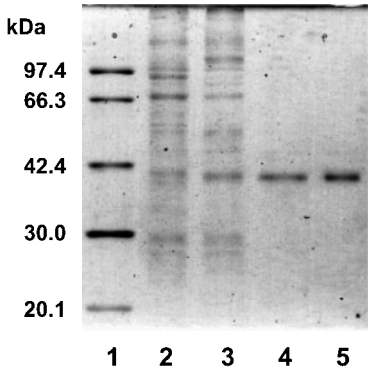

Fig. 6. **SDS-PAGE analysis of samples at the purification stages of recombinant *C. humicola* D-aspartate oxidase.** Proteins were separated by 12% SDS-PAGE and stained with Coomassie Brilliant Blue. Lane 1, marker proteins; lane 2, crude extract (10 μg); lane 3, 10–35% ammonium sulfate precipitate (7.5 μg); lane 4, Butyl-Sepharose (0.6 μg); lane 5, SP-Sepharose (0.5 μg).

Purification and Characterization of Recombinant ChDDO—Table 1 summarizes the results of the purification procedure. The recombinant enzyme was purified 101-fold from the crude extract and, after the final step, had a specific activity of 123 U/mg, which is comparable to that of the native enzyme, 76.1 U/mg (*8*) or 150 U/mg (*16*). On SDS-PAGE, the final preparation migrated as a

Table 1. **Purification of recombinant *C. humicola* D-aspartate oxidase from *E. coli* Rosetta (DE3) cells harboring pECD5.**

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	1210	997	1.21	1	100
10–35% $(NH_4)_2SO_4$ precipitate	706	116	6.09	5.03	58.3
Butyl-Sepharose	464	7.57	61.2	50.5	38.3
SP-Sepharose	161	1.32	123	101	13.4

The activity was measured in 50 mM potassium phosphate buffer (pH 7.5) at 37°C. Starting material was 14.1 g of *E. coli* cell paste.

single protein band with a molecular mass of 40 kDa (Fig. 6, lane 5). The native molecular mass was estimated to be 164 kDa by gel filtration, showing that the recombinant enzyme is in the form a homotetramer, the same as the native enzyme.

To confirm that the recombinant has the same enzymatic properties as the native enzyme, we determined several properties of the purified recombinant enzyme. Malonate and D-malate inhibited the activity by 60.4 and 33.2%, respectively, whereas *meso*-tartrate, a potent inhibitor of mammalian DDO, and benzoate, an inhibitor of mammalian DAO, inhibited the enzyme activity by only 4.7 and 6.1%, respectively, as observed for the native enzyme. The absorption spectra of the recombinant enzyme, with or without the addition of malonate or D-aspartate (data not shown), are identical to those of the native enzyme (*8*), demonstrating that the recombinant also contains FAD as a cofactor and that the inhibitor and D-aspartate bind to the active site. The optimum temperature and pH of the recombinant enzyme are 37°C and 7.5, respectively, and the recombinant is relatively stable up to 40°C and its activity is completely lost at 60°C. The K_m, V_{max} and k_{cat} values of the recombinant enzyme are: 2.92 mM, 124 U/mg protein and 82.6 s^{-1} for D-aspartate; 62.6 mM, 2.30 U/mg protein and 2.00 s^{-1} for D-glutamate; and 85.3 mM, 57.2 U/mg protein and 38.1 s^{-1} for *N*-methyl-D-aspartate, yielding a markedly high k_{cat}/K_m value for D-aspartate (28,300 $s^{-1}\cdot M^{-1}$) as opposed to those for D-glutamate (25.0 $s^{-1}\cdot M^{-1}$) and *N*-methyl-D-aspartate (447 $s^{-1}\cdot M^{-1}$). This clearly demonstrates that the recombinant enzyme is highly specific for D-aspartate equally to the native enzyme. Taken collectively, the data indicate that the recombinant enzyme is essentially identical to the native enzyme in all of these properties (*8*, *16*).

In the present study, we describe the cloning and characterization of the *DDO* gene from the yeast *C. humicola* UJ1. Additionally, the gene was expressed in *E. coli* and the recombinant enzyme was purified and characterized. This is the first report of a *DDO* gene from a microorganism. The gene will be useful for fully elucidating the physiological function of DDO and for studies on the regulatory mechanism of *DDO* gene expression. Moreover, the heterologous expression of the *ChDDO* gene could facilitate studies of its structure-function relationship, in addition to practical applications, such as the determination and identification of D-aspartate in several organisms.

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