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Primary structure and expression of peroxisomal acetylspermidine oxidase in the methylotrophic yeast *Candida boidinii*

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Abstract Acetylspermidine oxidase (ASOD) belongs to a family of FAD-containing amine oxidases and catalyzes the oxidation of *N*-acetylated spermidine in polyamine metabolism. ASOD was purified to apparent homogeneity from cells of the methylotrophic yeast *Candida boidinii* grown on spermidine as the sole nitrogen source. *C. boidinii* ASOD catalyzed the oxidation of only N^1-acetylspermidine. Based on partial amino acid sequences, oligonucleotide primers were designed for polymerase chain reaction, and the ASOD-encoding gene, *ASO1*, was cloned. The open reading frame encoding *ASO1* was 1530 bp long and corresponded to a protein of 509 amino acid residues (calculated molecular mass = 57 167 Da). *ASO1* contained a FAD-binding motif of G-A-G-I-A-G in the N-terminal region and carried an amino acid sequence of -S-K-L at the C-terminal, representing a typical peroxisome targeting signal 1. ASOD was localized in the peroxisomes in overexpressed *C. boidinii*. To our knowledge, this is the first report on the gene coding for ASOD that can catalyze the oxidation of *N*-acetylated polyamine as a substrate, from any type of organism.

Key words: Polyamine oxidase; Acetylspermidine; Peroxisomal enzyme; Flavin-containing enzyme; *Candida boidinii*

1. Introduction

Polyamine oxidase (PAO; EC 1.5.3.-), which belongs to the family of FAD-containing amine oxidases, is assumed to be involved in the degradation or inter-conversion of polyamines. PAOs can be divided into two groups on the basis of their substrate specificity: one catalyzes the oxidation of *N*-acetylated polyamines and the other catalyzes that of free polyamines [1,2]. We herein classify acetylspermidine oxidase (ASOD) under the former group, and PAO under the latter one. PAO is found in the cell wall of monocotyledonous plants, such as oat, barley and maize [2,3], and is postulated to be involved in cell wall development [4]. Recently, the primary structure of maize PAO was determined [5]. PAO is also detected in nematodes [6].

Since polyamines are important substances for cell proliferation and differentiation [1], their intracellular levels must be regulated properly. In mammalian cells, spermidine is *N*-acetylated with spermidine/spermine *N*-acetyltransferase (SSAT) [7] and then oxidized to diamine and acetamidaldehyde in the presence of ASOD [8]. This SSAT/ASOD pathway was reported to participate in the regulation of intracellular polyamine levels in concert with the polyamine biosynthetic pathway that involves ornithine decarboxylase [9]. It was also suggested that mammalian ASOD participates in general detoxification reactions other than catalyzing the oxidation of acetylspermidine [10]. ASOD activity was found not only in mammalian cells [10] but also in methylotrophic yeasts and amoebae [11,12]; however, it was not observed in *Escherichia coli* and *Saccharomyces cerevisiae* [13–16]. ASODs from mammalian and methylotrophic yeast cells could catalyze the oxidation at the secondary amino group of N^1-acetylspermidine to produce putrescine, 3-acetamidopropionaldehyde and H_2O_2 [8,11]. In contrast to PAO, ASOD was reported to be localized in peroxisomes in both mammalian and yeast cells. However, its physiological role is not clear and its primary structure is not known.

The methylotrophic yeast *Candida boidinii* is a good model organism to study polyamine metabolism because of the following reasons: (1) it can grow well on several monoamine and polyamine compounds, e.g. methylamine, spermidine or D-alanine, as the sole nitrogen source. Therefore, we can analyze polyamine metabolism in relation to other amine-related compounds in one organism. (2) ASOD activity is induced together with diamine acetyltransferase activity (corresponding to mammalian SSAT) [17,18]; (3) the subcellular fractionation procedure is well established since *C. boidinii* has been used as a model organism to study peroxisomal protein transport and (4) a powerful methanol-inducible expression system is available for genes from native or foreign sources [19]. In this study, we purified ASOD from *C. boidinii* cell-free extract, cloned the ASOD-encoding gene (*ASO1*) and determined its primary structure. Then we overexpressed the *ASO1*, and examined the subcellular localization of gene products. The primary structure of ASOD revealed that it is distinct from another polyamine oxidase family protein, PAO, not only in terms of substrate specificity but also in terms of its primary structure and subcellular localization.

2. Materials and methods

2.1. Yeast strain and large-scale cultivation

C. boidinii IFO10574 was grown on synthetic dextrose (SD) medium [20] that contained 1.5% (w/v) glucose, 0.17% (w/v) yeast nitrogen base w/o amino acids and ammonium sulfate (Difco). As the sole nitrogen source, spermidine trihydrochloride was added at a concentration of 12 mM. The yeasts were cultured in SD medium using jar

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fermentors (B.M. Marubishi, type MDL-751S, volume = 7 l, working volume = 4.5 l) under 250 rpm of agitation and 0.67 v.v.m. of aeration at 28°C for 64 h.

2.2. Purification of ASOD

Collected cells (ca. 900 g wet cells obtained from 36 l of culture) were spheroplasted in lysis buffer (50 mM Tris–HCl (pH 7.4), 120 mM sorbitol, 1 mM dithiothreitol) containing 2 mg/ml Zymolyase 20T (Seikagaku Kogyo) and were lysed on ice by a supersonic disrupter (Tomy, type UD201). During the following purification procedures, enzyme samples were kept below 10°C.

After centrifugation at 16 000 × *g* for 20 min, proteins in the supernatant were precipitated by adding 50–90% saturated ammonium sulfate. The pellet was dispersed and dialyzed against H-buffer (50 mM HEPES, pH 8.2). The dialyzed sample was loaded onto a column of POROS HS (PerSeptive) equilibrated with H-buffer. ASOD was eluted with 300 mM NaCl in H-buffer. The eluate was adjusted to an appropriate salt concentration and loaded onto a column of ω-aminohexyl agarose (Sigma) equilibrated with 100 mM NaCl in H-buffer. ASOD was eluted with 10 mM spermidine trihydrochloride in the same buffer. The obtained ASOD was precipitated by adding 95% saturated ammonium sulfate and the pellet was dispersed in 50 mM P-buffer (potassium-phosphate, pH 6.8). The solution was desalted and loaded onto a column of ceramic hydroxyapatite (Bio-Rad) equilibrated with 50 mM P-buffer. After the column was washed with 200 mM P-buffer, ASOD was eluted with 300 mM P-buffer. ASOD was concentrated using an ultrafiltration tube, Centricon 10 (Amicon), and then the buffer concentration was adjusted to 50 mM. This sample was applied to a small-scale chromatography system SMART (Pharmacia) equipped with a column of Mini S (Pharmacia). ASOD was eluted at about 160 mM with a linear gradient of increasing potassium-phosphate concentration.

2.3. Measurement of protein concentration and relative molecular mass

Protein was determined using a Bradford Protein Assay Kit (Bio-Rad) with bovine IgG as the standard. The purity and the molecular weight were determined by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The molecular weight was also estimated by gel-filtration chromatography on a Superdex 200 PC 3.2/30 (Pharmacia) in H-buffer containing 300 mM NaCl.

2.4. Assay for ASOD activity

ASOD activity was measured in terms of H_2O_2 production in a spectrophotometric assay [11] using N^1-acetylspermidine dichloride as the substrate. For the blank, the substrate was omitted. One unit of ASOD activity was expressed as the production of 1 nmol H_2O_2 min^{-1}.

2.5. Gene cloning of ASO1

Internal amino acid sequences of purified ASOD were determined using the Edman degradation method following lysyl-endopeptidase fragmentation. Then, we designed degenerated polymerase chain reaction (PCR) primer sets: priF2, 5′ AAY GAA GTH GGW WSW ATY GAR TGG 3′, and priR3, 5′ TTC ACC DGC RAA WCK WAC WCK WCC 3′. PCR templates were extracted from *C. boidinii* IFO10574. According to the sequence data from the first PCR run, additional 3′ RACE and inverse-PCR were carried out.

2.6. Expression of ASO1 under the control of AOD1 promoter

In order to realize a high-level expression of *ASO1*, its coding sequence was connected to the promoter of alcohol oxidase, *AOD1* on a *C. boidinii* expression vector pNoteI [21]. The coding sequence was obtained by PCR amplification using primers pri20F, 5′ ATT GCG GCC GCA ATG ACA ACA GTA AGA ACA GAT GCT ATA GTT ATT GG 3′, and pri21R, 5′ TTT AAG CTT CTC GAG GTG GTA GAG GTG GTG GCT TTA GAG GTA G 3′. The recombinant plasmid pNoteI-*ASO1* was introduced into the *ura3* region of a host *C. boidinii aod1*Δ [22]. The transformant TF-ASO1 was cultured in SD medium. Induction of the *AOD1* promoter was performed by changing carbon and nitrogen sources as described previously [23]. Strain GC [24] was used as the control strain. Cells recovered from mid-log-phase culture were disrupted by sonication and subjected to ASOD assay.

2.7. Subcellular fractionation

TF-ASO1 cells grown on methanol plus glycerol medium [25] were spheroplasted and lysed gently by osmotic shock. The lysate was subjected to organelle fractionation experiments, which were performed using continuous Nycodentz (Sigma) gradient as previously described [25]. Each fraction was assayed for ASOD and cytochrome *c* oxidase activities [26] or subjected to Western analysis using anti-Pmp47 antibody.

3. Results and discussion

3.1. Purification of ASOD

ASOD was purified ca. 21 000-fold to apparent homogeneity based on SDS–PAGE. The specific activity of the purified enzyme was 993 000 U (mg protein)$^{-1}$. The apparent molecular mass of the purified enzyme was estimated to be 57 kDa by SDS–PAGE or 70 kDa by gel-filtration chromatography. Therefore, ASOD is assumed to be a monomeric enzyme. ASOD was most active toward N^1-acetylspermidine among the tested amines. ASOD could also catalyze the oxidation spermine and N^8-acetylspermidine at the ratios of 8 and 6% of the activity toward N^1-acetylspermidine, respectively, but did not have any detectable activity toward free spermidine, putrescine, several aliphatic monoamines and several D-amino acids. These data indicated that ASOD was distinct from PAO in terms of substrate specificity.

3.2. Cloning of the ASO1 gene and its 5′- and 3′-flanking regions

Nucleotide sequences were compiled to a candidate for a 1530 bp open reading frame (ORF) (Fig. 1). This ORF was identified as *ASO1* from the following results: (1) the deduced amino acid sequence of this ORF contained four internal peptide sequences: -AYQYLLK-, -ITFSDWRK-, -NEVG-SIEW- and -GRVRFAGE-, which had been determined by

Table 1
Expression of *C. boidinii ASO1* under the control of *AOD1* promoter

Strain (relevant character)	Culture medium	ASOD activity units (mg protein)$^{-1}$
Transformant TF-ASO1	Glc, AS	0.19
(*ASO1 AOD1*pro-*ASO1* Δ*aod1*)	Met/Gly, MA	1140
	Glc, SPD	91.4
Control strain GC	Glc, AS	0.15
(*ASO1 AOD1*)	Met/Gly, MA	0.41
	Glc, SPD	97.4
IFO10574	Glc, AS	0.35
(*ASO1 AOD1*)	Glc, SPD	96.6

Culture medium was prepared using 0.17% of yeast nitrogen base w/o ammonium sulfate and amino acids (Difco) as basal medium and adding the following supplements. Glc, 1% (w/v) glucose; Met/Gly, 0.5% (v/v) methanol plus 3% (w/v) glycerol; AS, 0.5% (w/v) ammonium sulfate; MA, 0.5% (w/v) methylamine·HCl; SPD, 12 mM spermidine·3HCl.

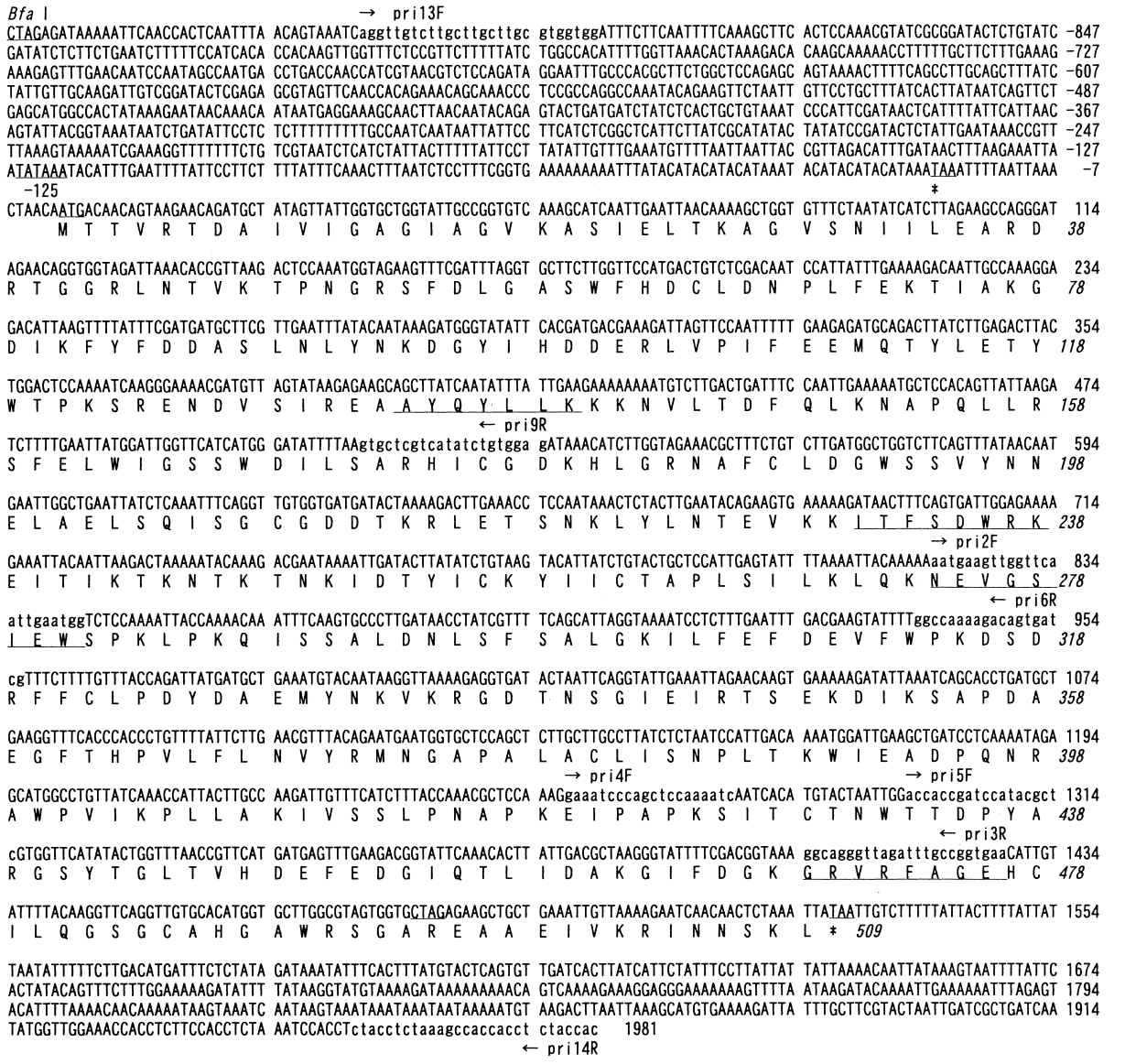

Fig. 1. Nucleotide sequence and predicted amino acid sequence of the *ASO1* gene of *C. boidinii*. Numbering of nucleotides and amino acids begins at A in the translation initiating ATG and methionine, respectively. An in-frame stop codon near the 5′ end and chemically identified amino acid residues are underlined. Small lettered sequences were used for PCR amplification. The nucleotide sequence reported here has been submitted to the DDBJ with accession number AB018223.

amino acid sequencing. (2) The molecular mass for the ORF was calculated to be 57 167 Da, which was consistent with the value of 57 kDa estimated by SDS–PAGE analysis. (3) The cell-free extract from the overexpressed strain of *C. boidinii* exhibited a much higher ASOD activity than that from the control strain (see below).

The upstream region of *ASO1* contained some sequence elements. The TATA box was identified at nucleotide (nt) −125 relative to A in the putative start codon. There were tandem repeats consisting of 5′ CATA CATA CATA AATA 3′ at nt −51. In the 3′ regions of *ASO1*, an obvious transcription termination signal was not identified. Although the potential polyadenylation signal (AATAAA) was situated at nt 1833, 1837 and 1844, the recovered 3′ RACE products contained a polyA sequence at nt 1631, 1640 or 1682. Therefore, the functional polyadenylation signal was not authenticated.

3.3. Comparison of the deduced amino acid sequence of ASO1 with those of related proteins

A sequence database search for proteins with a comparable amino acid sequence to *ASO1* using the FASTA program gave PAO from maize (24% identity in 527 amino acid (aa) overlap)[5], monoamine oxidase from human (24% identity, 508 aa) [27] and tyramine oxidase from *Micrococcus luteus* (26%, 312 aa) [AB010716]. These proteins were members of the FAD-containing amine oxidase family. BLAST search identified three significantly conserved regions between ASOD and PAO (Fig. 2). The first conserved region in the N-terminal region included a FAD-binding motif, G-X-G-X-X-G. The second and third conserved regions were also found in plant and mammalian genes related to a FAD-containing amine oxidase family. The phylogenic tree suggests that ASOD and PAO evolved separately from a common ancestor gene of amine oxidase (Fig. 3). Nevertheless, there were dis-

Fig. 2. Sequence alignment of *C. boidinii* ASOD with maize PAO. Sequences were aligned using the Clustalx program. Shaded parts are homologous sequences proposed by the BLAST method. GxGxxG shows a FAD-binding motif consisting of Gly-Xaa-Gly-Xaa-Xaa-Gly. *ASO1*, *C. boidinii* asetylspermidine oxidase; PAO, maize polyamine oxidase.

tinct sequence differences between PAO and ASOD particularly in the interspace between P_{324} and A_{393} of ASOD. Results from the BLAST search using P_{324} to A_{393} from ASOD or Y_{349} to S_{389} from PAO as queries did not reveal related proteins.

3.4. Role of ASOD in utilization of nitrogen source is distinct from that of methylamine oxidase

It is noteworthy that the use of spermidine as the nitrogen source induced ASOD activity 270 to 650-fold in *C. boidinii* strains GC and IFO10574 (Table 1). In contrast, the use of methylamine as the nitrogen source induced methylamine oxidase activity, but not ASOD activity in the control strains (data not shown). By analogy with the methylotrophic yeast *Hansenula polymorpha* [28], this methylamine oxidase activity is probably attributed to a peroxisomal copper-containing enzyme. Therefore, ASOD and methylamine oxidase, both of which are localized in the peroxisome as described below, play distinct roles in the utilization of nitrogen sources in *C. boidinii*.

3.5. Expression of ASO1 under the control of AOD1 promoter and peroxisomal localization

The *ASO1* coding sequence was placed under the control of the *AOD1* promoter and overexpressed in *C. boidinii* strain *aod1*Δ (Table 1). As reported previously, the use of strain *aod1*Δ as the host strain effectively produced several heterologous oxidases in the *C. boidinii* expression system and methylamine, which was added as the nitrogen source, accelerated the expression of the heterologous oxidases in the peroxisomes of *C. boidinii* [22]. The same strategy was employed for the expression of *ASO1*.

When TF-ASO1 cells were induced on methanol plus glycerol medium, ASOD activity was 1140 U (mg protein)$^{-1}$, which was 2780-fold higher than that of control strain GC. In contrast, when grown on glucose and ammonium sulfate (under repressed conditions), both strains showed a similar repressed level of ASOD activity. These results showed that the cloned ORF could express the enzyme activity.

ASOD has a tripeptide of -S-K-L at the C-terminal (see Fig. 1), which belongs to a typical 'peroxisome targeting signal 1' for peroxisomal matrix enzymes. The localization of ASOD in the overexpressing transformant was studied. The organelle pellet fraction, which contained mainly peroxisomes and mitochondria, was prepared from transformant cells grown on glycerol plus methanol medium,and was fractionated by Nycodentz-gradient ultracentrifugation. Since yeast peroxisomes are fragile, only a portion of catalase and ASOD corresponding to approximately 65 to 75% ASOD activity was recovered in the pellet fraction. Fractionation experi-

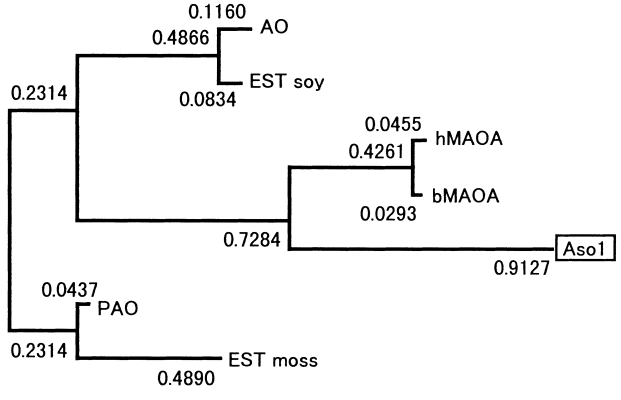

Fig. 3. Phylogenic tree of ASOD and related amine oxidases. The tree was estimated using the NJ method on conserved portions near the C-terminal. *ASO1*, ASOD from *C. boidinii*, A_{348}–C_{485}; hMAOA, human monoamine oxidase type A (P21397), A_{348}–Y_{444}; bMAOA, bovine monoamine oxidase type A (P21398), A_{348}–Y_{444}; PAO, maize polyamine oxidase (AJ002204), V_{372}–Y_{467}; AO, putative amine oxidase from *Arabidopsis thaliana* (AAD22129), V_{349}–S_{442}; EST moss, EST fragment from *Physcomitrella patens* (AW509769); EST soy, EST fragment from *Glycine max* (AW423551).

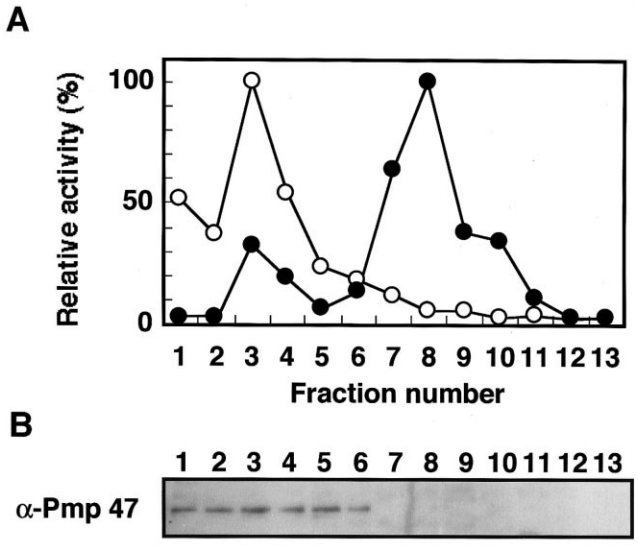

Fig. 4. Subcellular localization of ASOD in overproduced strain TF-ASO1 of *C. boidinii*. Cells were grown on glycerol plus methanol medium as the carbon source and methylamine as the nitrogen source. A: Open circles, ASOD; closed circles, cytochrome *c* oxidase. B: Western analysis against peroxisomal membrane protein, Pmp47.

ments indicated that the overexpressed ASOD co-migrated with the peroxisome membrane protein Pmp47 but showed a peak distinct from that of the mitochondrial marker enzyme, cytochrome *c* oxidase (Fig. 4). We concluded that the overexpressed ASOD is transported efficiently to the peroxisomes of strain TF-ASO1 under methanol-induced conditions. ASOD is the third example of proteins produced in large amounts within the peroxisomes of *C. boidinii* strain *aod1*Δ [22].

To our knowledge, this is the first report on the ASOD-encoding gene. Although the deduced amino acid sequence of *ASO1* showed some similarity to that of other types of amine oxidases, ASOD could constitute a new family of FAD-containing amine oxidases based on (1) sequence analysis, (2) substrate specificity, (3) peroxisomal localization and (4) regulatory features. Cloning and analyses of the *C. boidinii ASO1* gene reported here should facilitate further studies on the metabolism and regulation of polyamines in both lower and higher eukaryotes.

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