L-Amino acid oxidase of the fungus *Hebeloma cylindrosporum* displays substrate preference towards glutamate

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Catabolism of amino acids is a central process in cellular nitrogen turnover, but only a few of the mechanisms involved have been described from basidiomycete fungi. This study identified one such mechanism, the L-amino acid oxidase (Lao1) enzyme of *Hebeloma cylindrosporum*, by 2D gel separation and MS. We determined genomic DNA sequences of *lao1* and part of its upstream gene, a putative pyruvate decarboxylase (*pdc2*), and cloned the cDNA of *lao1*. The two genes were also identified and annotated from the genome of *Laccaria bicolor*. The *lao1* and *pdc2* gene structures were conserved between the two fungi. The intergenic region of *L. bicolor* possessed putative duplications not detected in *H. cylindrosporum*. Lao1 sequences possessed dinucleotide-binding motifs typical for flavoproteins. Lao1 was less than 23% identical to Lao sequences described previously. Recombinant Lao1 of *H. cylindrosporum* was expressed in *Escherichia coli*, purified and refolded with SDS to gain catalytic activity. The enzyme possessed broad substrate specificity: 37 L-amino acids or derivatives served as effective substrates. The highest activities were recorded with L-glutamate, but positively charged and aromatic amino acids were also accepted. Michaelis constants for six amino acids varied from 0.5 to 6.7 mM. We have thus characterized a novel type of Lao-enzyme and its gene from the basidiomycete fungus *H. cylindrosporum*.

**INTRODUCTION**

Filamentous fungi are abundant inhabitants of forest soils, where they participate in a number of ecologically essential nutrient transformations (Dighton, 2003; O’Brien et al., 2005). Some of these fungi, the ectomycorrhizal basidiomycete fungi (ECM), live in symbiosis with tree roots from which they acquire sugars, and in return provide to their plant partners soil nutrients such as nitrogen, phosphorus and trace elements (Finlay, 2008; Nehls, 2008). The genus *Hebeloma* occurs worldwide in boreal and temperate forests, and forms ECM with angio- and gymnosperm trees. The many species of the genus reside in diverse habitats, e.g. sandy dunes, calcareous and nutrient-rich soils, and peatlands (Vesterholt, 2005). *H. cylindrosporum* abounds on sandy soils throughout Europe, and is one of the pioneer ECM species in such habitats. It is also one of the major model organisms for ECM research (Marmeisse et al., 2004).

Amino acids are important nitrogen compounds in soils due to their ubiquity, availability and fast turnover times (Jones & Kielland, 2002). Microbial utilization of amino acids consists of the processes of extracellular proteolysis, uptake and intracellular catabolism (recycling or assimilation) (Schimel & Bennett, 2004). Cellular catabolism of amino acids allows these nitrogen resources to be further distributed into other compounds. In this way, the microbes may better adapt to amino acid resources that display compositional heterogeneity, and are variably available spatially and temporally (Rothstein, 2009). It

**Abbreviations:** ECM, ectomycorrhizal fungi; JGI, Joint Genome Institute; Lao, L-amino acid oxidase; Pdc, pyruvate decarboxylase; UTR, untranslated region.

The GenBank/EMBL/DDJB accession number for *H. cylindrosporum* *lao1* coding sequence is HQ172162, and for the genomic DNA contig is HQ172163. The third-party annotation for *H. cylindrosporum* *pdc2* coding sequence is BK007877, and for *L. bicolor* *lao1* gene is BK007876.

Supplementary methods, four figures and six tables are available with the online version of this paper.
has been hypothesized that ammonia originating from catabolism of amino acids may be one source of nitrogen transferred from ECM fungi to their symbiotic plant hosts (Chalot et al., 2006).

Cellular catabolism of the amino acid nitrogen may occur by several enzymic mechanisms: transamination, hydrolysis or redox-mediated deamination reactions. l-Amino acid oxidases (Lao; EC1.4.3.2) catalyse stereospecific oxidative deamination of l-amino acids. Lao-enzymes contain a flavin adenine dinucleotide (FAD) cofactor that participates in the two-electron redox reaction, a recurrent feature of many flavoenzymes (Fraaije & Mattevi, 2000). The electron transfer from the substrate to the FAD has been proposed to occur via hydride transfer mechanism. Subsequently, the formed imino acid intermediate is oxidized to 2-oxoacid and ammonia, and the reduced FAD is oxidized by molecular oxygen to yield the third reaction product, hydrogen peroxide (Faust et al., 2007; Hafner & Wellner, 1971).

We have previously described Lao-enzyme activities from two genera of ECM-forming fungi, Hebeloma spp. and Laccaria bicolor (Nuutinen & Timonen, 2008). The activities were localized intracellularly and they were especially high in nitrogen-rich growth conditions. These observations suggest a central role of Lao in intracellular deamination of amino acids (N mineralization). However, the genes coding for Lao are not known from these fungi. Lao genes and/or purified enzymes have been described from several other organisms, including asco- and basidiomycete fungi, cyanobacteria, several metazoan lineages (mammals, snakes, fish and molluscs), and algae (Bockholt et al., 1995; Chavan et al., 2002; Geuke & Hummel, 2002; Kitani et al., 2007; Niedermann & Lerch, 1990; Raibekas & Massey, 1998; Stasyk et al., 2010; Yang et al., 2005). Some of these Lao-enzymes mineralize nitrogen from many chemically distinct amino acids. The enzymes from snake venoms and actinobacteria, for example, display the highest activities towards amino acids and actinobacteria, for example, display the highest activities towards amino acids with hydrophobic or positively charged side chains (Geuke & Hummel, 2002; Ponnudurai et al., 1994). Because of such a broad substrate range, the microbial Lao-enzymes may represent potent mechanisms for the cellular catabolism of amino acid nitrogen.

Since the amino acids hold a central position in the nitrogen economy of soil fungi and bacteria, a detailed understanding is needed of the organisms and molecular mechanisms catalysing the catabolic reactions. To gain insights into these mechanisms in ECM fungi, we describe here the cloning and functional characterization of the major Lao from Hebeloma cylindrosporum.

**METHODS**

**Fungal growth and protein extraction.** *H. cylindrosporum* strains h7 (Gay et al., 1993) and CBS 558.96 (Centraalbureau voor Schimmelcultures, Utrecht, Netherlands) were grown for 18–35 days on cellophane-covered modified Hagem’s medium. Soluble cellular enzymes were extracted by first grinding the freshly collected hyphae in mortar and then disintegrating with ultrasound (Nuutinen & Timonen, 2008).

**1D and 2D protein electrophoresis.** For SDS-PAGE the proteins were diluted into half-strength Laemmli sample buffer (Bio-Rad), with 6 mM DTT. The sample mixes were used as such for electrophoresis (Lao activity staining) or they were boiled for 5 min (protein staining). Proteins were separated on 7–8 % acrylamide gels at 4 °C. Precision Plus molecular mass markers were from Bio-Rad.

For 2D electrophoresis, the fungal extracts were treated (at room temperature, 3.5 h) with digestion buffer (200 mM Tris/HCl, pH 7, protease inhibitor cocktail, 50 mg PMSF ml$^{-1}$, 5 mM MgCl$_2$, 2 mM CaCl$_2$, 50 μg RNase ml$^{-1}$, 500 nU DNase ml$^{-1}$, 10 μM chitinase ml$^{-1}$), and exchanged to low-salt buffer [10 mM Tris/HCI, 5 % (v/v) glycerol, 0.5 % (v/v) Triton X-100] by centrifugal ultrafiltration (30 kDa cut-off, Amicon). The protein (20–40 μg) was mixed with IEF buffer containing 0.5 % (w/v) Bio-Lyte 3–10 ampholytes (Bio-Rad), 0.5 % (w/v) Triton X-100, 6 mM DTT. Native IEF was performed on 7 cm strips with pH 3–10 (GE Healthcare) or 3–6 (Bio-Rad), at 12 700 V h$^{-1}$, 16 °C (Ettan IPGphor, GE Healthcare). For the second dimension, the strips were equilibrated 30 min in sample buffer and run on SDS-PAGE gels as described above.

SDS-PAGE gels were stained for Lao activity (*H$_2$O$_2$* production) with the peroxidase–o-dianisidine method, with l-phenylalanine or l-leucine as the substrates (Nuutinen & Timonen, 2008). The amino acids were omitted from negative control staining. Total proteins were stained with Coomassie G-250 (Bio-Safe; Bio-Rad) or Sypro Ruby (Molecular Probes) as described in the manufacturer’s instructions.

**Protein identification by MS.** Lao spots from 2D gels were in-gel digested (sequencing grade modified trypsin, Promega) as described by Shevchenko et al. (1996), with modifications. After digestion and elution out of the gels, the tryptic peptides were desalted and concentrated using Zip Tip MC-18 reverse phase (Millipore). Peptides were eluted onto the MALDI target plate with 0.1 % TFA, 50 % acetonitrile, after which the matrix solution was added [saturated z-cyano-4-hydroxycinnamic acid (Sigma) in 33 % acetonitrile, 0.1 % TFA].

MALDI-TOF/TOF analyses were carried out with Autoflex III (Bruker Daltonics) operated in positive and reflective modes. Data were acquired by accumulating mass spectra from 2000 laser shots in the m/z range between 700 and 4200 Da, and up to 10 000 for MS/MS spectra. The peptide mass fingerprinting analyses used 19 kV acceleration voltages. In tandem MS mode, the ion source voltage was 6 kV and the fragmented ions were further accelerated in the lift device with 19 kV. The mass spectrometer was externally calibrated using a peptide calibration standard (Bruker Daltonics). Tryptsin and putative keratin peptide mass peaks were removed from the data before database search submissions.

Proteins were identified by searching the peptide monoisotopic masses (peptide mass fingerprints) or the fragment ion spectra (MS/MS ion search) with the Mascot search engine (Perkins et al., 1999) against NCBI nr and EST-others databases, or a locally created database. The search parameters were as follows: precursor tolerance 0.1–0.8 Da; fragment tolerance MS/MS 0.6–1 Da; one trypsin missed cleavage was allowed; fixed and variable modifications were carbamidomethylated Cys and oxidized Met, respectively. Significance threshold for ion score matches was Pe<0.05.
Cloning lao1 cDNA and genomic DNA sequencing. Total cellular RNA was isolated from fungal mycelia with Tri-reagent (Sigma) following the manufacturer’s instructions. cDNA was prepared from total RNA templates with M-MuLV RNase H- (Finnzymes) according to Smart RACE kit (Clontech) instructions. The 5’ and 3’ ends of lao1 were amplified (Phusion polymerase, Finnzymes) by using the nested PCR protocol (Supplementary Table S1 and Supplementary Methods, available with the online version of this paper). The full-length lao1 cDNA was cloned with ligation-independent cloning (pET46 EK/LIC kit; Novagen) according to the manufacturer’s instructions. Briefly, the H. cylindrosporum lao1 was amplified from the cDNA template, with primers cDNAflr (Supplementary Table S1), and transformed into Escherichia coli NovabluGigaSingles competent cells (Novagen). Clones were screened for the correct inserts by colony-PCR according to the Novagen kit instructions. Plasmids were isolated (GeneJET kit, Fermentas) from three positive clones, and their inserts were sequenced.

Genomic DNA was isolated from H. cylindrosporum mycelia by using the CTAB method, and purified by precipitation with chloroform:isoamylalcohol (Heinonsalo et al., 2001). Three fragments of genomic DNA were amplified with primers given in Supplementary Table S1, designed based on 3’-RACE results, EST data and the L. bicolor genome sequence. The sequences of the three amplicons were then determined by direct sequencing of the PCR products, with a 4 x sequencing depth on average.

Sequence analyses. DNA contigs were built with Vector NTI 10 (Invitrogen). Sequence coverage for the genomic and cDNA ranged from two to nine sequence traces per site. Nucleotide and protein sequence searches were performed with BLAST queries against GenBank or against the L. bicolor genome database at the Joint Genome Institute (JGI) (Martin et al., 2008). Nucleotide and protein sequences were aligned with CLUSTAL W (Thompson et al., 1994), Dialign (Morgenstern, 2004) or Mcoffe (Wallace et al., 2006). The alignments were edited and visualized with BioEdit 7.0 or JalView 2.3 (Waterhouse et al., 2009). All software was run with default parameters.

Putative signal peptides in Lao1 protein sequences were predicted with SignalP 3.0 (Bendtsen et al., 2004). Cellular localization signals were predicted with several methods (with parameters for the taxa given in parentheses): TargetP 1.1 (non-plant model) (Emanuelsson et al., 2000), Cello 2.5 (eukaryotes) (Yu et al., 2006), Euk-mPloc 1.0 (Chou & Shen, 2007), BaCelLo (fungi) (Pierleoni et al., 2006) and WoLF Psort (fungi) (Horton et al., 2007).

Global pairwise sequence identities of DNA and proteins, and theoretical protein sequence properties (pi, molecular mass), were calculated with EMBOSS (Rice et al., 2000). Local nucleotide alignments were generated with Lalign ver. 35.04 (http://fasta.bioch.virginia.edu). DNA scoring matrix was +5/-4, and gap opening and extension costs were -12 and -4, respectively.

Motifs with E-values <2 in the intergenic regions of H. cylindrosporum and L. bicolor were identified with MEME ver. 4.3.0 (Bailey & Elkan, 1994). For phylogenetic analysis the tandem occurrences of the two motifs with E-values <0.01 (A and B) were combined, which yielded one sequence from H. cylindrosporum and four sequences from L. bicolor. The phylogeny was estimated with PhyML and GTR substitution model as implemented in phylogeny.fr (Dereeper et al., 2008). Gamma distribution (four categories), proportion of invariable sites and transition/transversion ratios were all estimated from the data. One hundred bootstrap replicates were calculated. The resulting tree was rooted with midpoint rooting.

Transcription factor binding sites in the intergenic regions were searched from fungal matrices in the Transfac 6.0 database with algorithms P-Match and Match (Chekmenev et al., 2005; Kel et al., 2003). The matrix similarity cut-offs were set to minimize the sum of false-negative and false-positive predictions. Results were limited to those transcription factors involved in nitrogen-, carbon- and stress-related processes.

Protein expression and enrichment. The PET46-HcLao1 plasmid was transformed into the expression host E. coli BL21(DE3) (Novagen) as described in the manufacturer’s instructions. Bacteria were grown in LB broth to OD600 0.5–1.0, then amended with 50 μM FAD and 1 mM IPTG, and grown overnight (16 °C, 350 r.p.m.). The cells were harvested by centrifugation, and soluble proteins were extracted to CL buffer [50 mM potassium phosphate, pH 8.0, 250 mM NaCl, 8.8 % glycerol, 2 mM MgCl2, 0.1 % (w/v) Triton X-100, 0.5 μl Benzonase ml−1, 0.5 mM lystopine ml−1, 2 μl protease inhibitor cocktail ml−1, 50 mg PMSF ml−1, 0.2 mM β-mercaptoethanol] by incubation with shaking (4 °C, 20 min, 150 r.p.m.), sonication (20 s, tip-sonicator Labsonic U, B. Braun), and two freeze-thaw cycles. The extracts were clarified by centrifugation (4 °C, 16,000 g, 10 min).

Recombinant Lao1 (rLao1) protein was batch-purified from extracts with Ni-NTA affinity matrix (Sigma) at 4 °C, as described in the manufacturer’s instructions. Wash buffer was 50 mM phosphate, pH 8.0, 8.8 % glycerol, 0.1 % Triton X-100, 250 mM NaCl. The rLao1 was eluted with wash buffer containing 500 mM imidazole. The eluates were further purified with gel permeation chromatography (Akta Prime system, Superdex 200 prep grade column, 600 × 16 mm, GE Healthcare), 14 cm h−1 flow, in wash buffer. The eluates were analysed for proteins by SDS-PAGE or Bradford assay, and for Lao activities (H2O2 production).

Lao1 refolding and functional characterization. For the enzymic assays in solution, the rLao1 was activated by diluting the purified enzyme 1:6–1:10 into 0.1 M potassium phosphate, pH 8.0, 0.5 % SDS. Lao activity measurements and data analyses were performed as described by Nuutinen & Timonen (2008) with the spectrophotometric peroxidase–o-dianisidine method, revealing H2O2 production from the amino acid substrate, with few modifications. Freshly activated rLao1 (0.4–2 μg ml−1) was added to the reaction, and the absorbance was measured at 15 or 30 s intervals for 30–80 min at room temperature. The linear least-squares method, as implemented in Microplate Manager ver. 5.1 (Bio-Rad), was utilized to calculate enzyme activities from the raw data of absorbance as functions of time. Standard errors of the activities from two or three replicate experiments were calculated with Microsoft Excel. Positive control for the assays was crude snake venom from Crotalus atrox (Sigma).

For the determination of kinetic parameters (Vmax, Km), the rLao1 activities were measured as above. Activities were recorded at 15 concentration points within the range of 0.04–100 mM (L-Phe and L-Leu), or 0.03–300 mM (L-Glu, L-Orn, L-Gln and L-Ala). Assay buffer was 0.1 M potassium phosphate, pH 8.0, 0.02 % Triton X-100. The parameter values were obtained by fitting the data to Michaelis-Menten or to substrate inhibition equations by non-linear least-squares method (Origin 7.5, OriginLab). pH optima were determined by measuring H2O2 production as above in two buffer systems: 200 mM phosphate/acetate (pH interval 5–8), and 200 mM Tris/ phosphate/carbonate (pH interval 8–12), at intervals of one pH unit. The substrates were L-Glu, L-Orn or L-Phe, each at 10 mM in the buffers above.

Enzymatic conversion of l-leucine to 2-oxoacid was detected with the dinitrophenylhydrazine assay (Nuutinen & Timonen, 2008). UV/VIS spectra of rLao1 (2 mg ml−1 in wash buffer) were recorded by using a 1 cm path length quartz cuvette on an 8453 spectrophotometer (Agilent).
RESULTS

Lao1 is a major protein of H. cylindrosporum

When total cellular proteins of H. cylindrosporum h7 were separated by SDS-PAGE, the Lao activity migrated as two major species of 67 and 140 kDa. The former species co-localized with a major Coomassie-stained band (Fig. 1a). The Lao profiles and total protein patterns of H. cylindrosporum CBS 558.96 were very similar to those of strain h7 (data not shown). In 2D-PAGE, the 67 kDa Lao species of strain h7 separated into two groups of spots. The pl of the major group was approximately 5.4, and it consisted of seven to nine spots (Fig. 1b). Again, the spots that displayed the highest Lao activities and the most intensive Coomassie stains were co-localized. Taken together, the electrophoresis results suggest that Lao may be a major cellular protein of H. cylindrosporum in the assay conditions used.

MALDI-TOF/TOF fragmentation patterns of five peptides derived from the most abundant protein of the pl 5.4 group yielded significant matches to two ESTs of H. cylindrosporum (Supplementary Table S2). The results suggest that these EST sequences represented the expressed Lao1 gene of this fungus.

Structures of the lao1 and pdc2 genes of H. cylindrosporum, and identification of their homologues from L. bicolor genome

The 5’ and 3’ coding sequence ends of H. cylindrosporum lao1 were verified with data from RACE-PCR and the ESTs. Amplification of the full-length lao1 from total cDNA population yielded a single band on agarose electrophoresis (data not shown). All three sequenced clones harboured identical lao1 transcripts (HQ172162). The sequenced and assembled genomic DNA contig from H. cylindrosporum was 4440 nt (HQ172163). This contig covered the whole lao1 gene, which consisted of five exons, and its 5’-upstream and partial 3’-untranslated regions (Fig. 2).

BLAST searches of the genome of the basidiomycete fungus L. bicolor with H. cylindrosporum Lao1 revealed the probable lao1 of L. bicolor, which was, however, annotated as a hypothetical protein. Subsequent BLAST searches of the EST database confirmed that lao1 was expressed in both fungi (Supplementary Table S3). Based on the EST data, the gene structure of L. bicolor lao1 was revised (BK007876; Supplementary Tables S4 and S5). The lao1 genes of H. cylindrosporum and L. bicolor were 79% identical at nucleotide level, and the intron numbers and phases were conserved between the two fungi (Fig. 2 and Supplementary Table S4). Overall, the sequence and structural similarities between the two genes support the proposed annotation transfer of the BK007876 gene of L. bicolor as lao1.

The 5’-upstream region of the lao1 gene in the genome of L. bicolor possessed a gene annotated as putative pyruvate decarboxylase pdc2 (JGI ID 396786), which consisted of 14 exons (Fig. 2, Supplementary Table S5). The full-length coding sequence of the H. cylindrosporum pdc2 was inferred from EST data (BK007877; Supplementary Table S3). The H. cylindrosporum genomic DNA contig contained a pdc2 gene fragment, which consisted of eight exons, and covered 44% of the whole gene (Fig. 2, Supplementary Tables S4 and S5). Taken together, these experiments resulted in sequencing and annotation of the transcript and genomic DNA sequences of the lao1 and partial pdc2 genes of H. cylindrosporum, and identification of the homologous genes from the genome of L. bicolor.

Comparative analysis of the pdc2–lao1 intergenic regions

Alignment and pattern-matching methods were used to find the putative regulatory sequences from the intergenic regions between the pdc2 and lao1 of H. cylindrosporum and L. bicolor. Putative TATA boxes, 5’-untranslated and core promoter regions were located within 150 nt upstream of the translation start sites of pdc2 and lao1, as these regions displayed the greatest sequence similarities within the intergenic regions (Fig. 3, Supplementary Fig. S1). However, global alignments showed that the overall pairwise nucleotide identities of the intergenic regions were only 47%.

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**Fig. 1.** Lao-enzyme species and total protein profiles of H. cylindrosporum hyphal extracts (not heat-denatured). (a) Proteins separated by SDS-PAGE. Staining: L, Lao activity with peroxidase–o-dianisidine method; C, total protein staining with Coomassie; L+C, sequential Lao and Coomassie. The dashed arrow marks the band that displayed the highest Lao activity and co-localized with a major cellular protein. (b) Proteins separated by 2D SDS-PAGE. The solid arrow marks the protein spot identified by MALDI-TOF/TOF MS of tryptic peptides. Molecular masses (kDa) and pl of proteins are also shown.
Local alignments of the intergenic regions revealed an imperfect palindrome sequence from *H. cylindrosporum*, and a repeat-enriched region from *L. bicolor* (Fig. 3, Supplementary Fig. S2). MEME analyses revealed that three ungapped motifs (A–C) occurred in tandem four times in *L. bicolor* (Fig. 3 and Supplementary Fig. S3). Unexpectedly, these motifs occurred only once in tandem in *H. cylindrosporum*. The motifs co-localized with the regions that displayed similarities in local alignments (Fig. 3 and Supplementary Fig. S4). Phylogenetic analysis of the combined motifs A and B clustered the four *L. bicolor* sites together, indicating that the sequences of the four motifs of this fungus were more similar to each other than to those of *H. cylindrosporum* (data not shown).

Transfac database search revealed 20 and 13 binding sites for transcription factors from *H. cylindrosporum* and *L. bicolor*, respectively.
respectively (Fig. 3). Only seven of these sites occurred in similar positions in the two fungi (large and bold type in Fig. 3), which is consistent with the large sequence divergence of the intergenic regions. The most abundant binding sites were NIT2 and ADRI1. Recognition sequences for these two factors co-localized to all but one of the motifs A and B of L. bicolor, and also in the sole tandem occurrence of these motifs in H. cylindrosporum (Fig. 3). Altogether, these results have pinpointed a few conserved sequence stretches that may be involved in gene regulation from the otherwise divergent intergenic regions of the two fungi.

Properties of the predicted Lao1 protein sequences

The Lao1 transcripts of H. cylindrosporum and L. bicolor were inferred to code for proteins of 641 and 651 amino acids, respectively. Both proteins had a theoretical pI of 6.2. The sequences displayed 77% pairwise identity and 84% similarity. Lao1 sequences possessed the dinucleotide-binding motif GxGxxG, which is well conserved in Laos (Wierenga et al., 1986). However, the two sequences displayed only 23% local identity (41% similarity) to the partial Lao sequence (Fig. 4) and several other flavoenzymes (Wierenga et al., 1986).

Putative N-glycosylation sites were predicted for the Lao sequences of the two basidiomycetes and the snake Calloselasma rhodostoma with the methods NetNGlyc and EnsembleGlyc. Two predictions in homologous sequence positions were common to H. cylindrosporum and L. bicolor (Asn 40/54 and Asn 445/450, respectively). Two predictions were unique to H. cylindrosporum (Asn 176 and 417). In comparison, both methods correctly predicted the two experimentally verified N-glycosylation sites of C. rhodostoma Lao (Asn 190 and 379, from sequence P81382). Of these predictions, the sequence region surrounding the Asn 445/450 of basidiomycetes displayed moderate similarity to Asn 379 of C. rhodostoma. Thus, this position may be a conserved glycosylation site. In contrast, the positions of Cys residues that form disulphide bridges in C. rhodostoma Lao (Pawełek et al., 2000) are not conserved in the two fungal sequences (data not shown).

SignalP predicted no signal sequence to be present in H. cylindrosporum or L. bicolor Lao1. Computational predictions of cellular localization suggested that the fungal Lao1 proteins may reside in cytoplasm or peroxisomes (Table 1).

Recombinant Lao1 expressed in E. coli possessed enzymic activity after refolding

Cellular extracts of E. coli induced to express the recombinant H. cylindrosporum Lao1 (rLao1) contained a 70 kDa protein that was not present in cells grown under non-inducing conditions (Fig. 5a). This suggests that the soluble rLao1 could be expressed in E. coli. Most importantly, the protein showed high Lao activity in in-gel activity assays, while the parent E. coli genotype did not (Fig. 5b). However, most of the expressed protein resided in insoluble inclusion bodies and was devoid of activity (Fig. 5a, b, lane 3).

The rLao1 was then enriched from the soluble protein extracts with Ni-affinity and gel permeation chromatography. Lao activity eluted from the latter column as a single peak (data not shown), which consisted of a 70 kDa protein that co-localized with the Lao-active band in SDS-PAGE activity assays (Fig. 5c, d, lane 5). However, the rLao1 preparation did not possess the 140 kDa Lao-active band that was present in fungal extracts (Fig. 5d). Peptide mass fingerprinting of the purified 70 kDa protein yielded 18 peptides that originated from deduced Lao1 protein sequence, with 39% sequence coverage (data not shown). These results confirm that the lao1 gene of H. cylindrosporum codes for a protein with Lao activity.

Interestingly, the apparent electrophoretic mobility of rLao1 in SDS-PAGE was slightly slower than that of the fungal Lao1 (Fig. 5c), accounting for a protein size difference of about 2–4 kDa. The sequences of rLao1 originating from the expression vector (6×His-tag and enterokinase cleavage site) accounted for only 1.2 kDa of the weight discrepancy. Thus, the fungal Lao1 protein may undergo yet unidentified modifications of the protein sequence.

Catalytic properties of recombinant Lao1

The maximal rLao1 activities were very similar when measured with either the dinitrophenylhydrazine assay, showing 2-oxoacid production, or the coupled peroxidase assay, revealing the production of hydrogen peroxide in the rLao1 reaction with the L-amino acid substrate (data not shown). The peroxidase assay was used in all subsequent activity measurements.

The substrate specificities of the rLao1 were very broad. rLao1 displayed more than 2% relative activity with 37 amino acids or derivatives out of 62 analysed (Table 2). L-Glutamate produced the highest activity, followed by amino acids possessing positively charged or polar side chains (e.g. L-glutamine, L-ornithine and L-asparagine). Many amino acids with hydrophobic side chains were also
accepted as substrates (Table 2). The substrates had backbones consisting of between three and eight atoms, and they did not contain bulky or hydrophilic residues at the \( \beta \) carbon. A number of aromatic and cyclic compounds related to phenylalanine, with and without ring derivatization and substituents, were also rLao1 substrates (Table 2). None of the five analysed D-amino acids, proline or the N-methylated phenylalanine served as substrates (Table 2). These results indicate that L-enantiomeric amino acids with free amino groups were required for the catalysis to occur. In contrast, two esters of phenylalanine yielded 42–27 % of the maximal Lao1 activities, indicating that some steric hindrance on the carboxy group was allowed.

The apparent Michaelis constants (\( K_m \)) of six amino acids displayed only 10-fold variation, and ranged from 0.5 mM for L-leucine to 6.7 mM for L-alanine (Table 3). Slight substrate inhibition occurred at very high substrate concentrations (\( K_i \>100 \) mM), most notably for L-ornithine and L-glutamine (Table 3). Also the limiting rates (\( V_{max} \)) for the six amino acids were quite similar and agreed well with the relative activities presented in Table 2. The specificity constants (\( k_{cat}/K_m \)) were in the range 24.6–1.2 \( \times 10^3 \) s\(^{-1}\) M\(^{-1}\) (Table 3), thus displaying maximum 20-fold differences between the substrates. According to this measure, L-leucine, L-glutamine and L-glutamate were the most specific substrates for the rLao1. The pH optimum of rLao1 was 8 for all amino acids analysed. L-Ornithine had the broadest and L-glutamate the narrowest pH range (Table 3).

The purified rLao1 protein preparation was coloured yellow. In the wavelength range 325–530 nm the rLao1 displayed a two-peak UV/vis spectra, with the peaks at 392 nm and 472 nm. Treatment of rLao1 with 5 % TCA yielded yellow supernatant and white precipitate. These results suggest that rLao1 possessed non-covalently attached flavin cofactor.

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**Table 1.** Computational predictions of cellular localizations of *H. cylindrosporum* and *L. bicolor* Lao1

<table>
<thead>
<tr>
<th>Method</th>
<th><em>H. cylindrosporum</em></th>
<th><em>L. bicolor</em></th>
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<tbody>
<tr>
<td>SignalP 3.0</td>
<td>No signal sequence other than mitochondria</td>
<td>No signal sequence other than mitochondria</td>
</tr>
<tr>
<td>TargetP 1.1</td>
<td>or secretory pathway</td>
<td>or secretory pathway</td>
</tr>
<tr>
<td>WoLF Psort*</td>
<td>Cytoplasm 7.0 Extracellular 7.0</td>
<td>Plasma membrane 9.0</td>
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<tr>
<td>Cello 2.5*</td>
<td>Cytoplasm 1.7 Peroxisome 1.1</td>
<td>Cytoplasm 2.0</td>
</tr>
<tr>
<td>Euk-mPLoc</td>
<td>Peroxisome</td>
<td>Peroxisome</td>
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<tr>
<td>BaCelLo</td>
<td>Cytoplasm</td>
<td>Cytoplasm</td>
</tr>
</tbody>
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*The numbers in WoLF Psort and Cello display the quality values associated with the predictions.
Lao1 enzyme species of *H. cylindrosporum* originate from one gene

To verify the fungal Lao1 protein sequence and to identify the Lao protein species, the peptide mass fingerprint identification of 2D gel-separated protein spots that displayed Lao activity was performed as in Fig. 1(b). Nine protein spots from three 2D gels were analysed, and all of them matched to the translated protein sequence of Lao1. Individual spots yielded 10–25 matching peptide molecular masses in MALDI-TOF, and the obtained Lao1 sequence coverage ranged from 24 to 47 %. Supplementary Table S6 presents the peaks identified as Lao1 peptides from one experiment. These results are consistent with the hypothesis that the electrophoretic species of Lao were derived from modifications of a protein coded by one *lao1* gene.

**DISCUSSION**

**Characteristics of the nucleotide and protein sequences**

We have previously shown that *Hebeloma* spp. and *L. bicolor* possessed Lao-enzyme activity, which may deaminate amino acids in these fungi (Nuutinen & Timonen, 2008). The present study describes protein-, cDNA- and genomic DNA-level characterization of the Lao1 of *H. cylindrosporum*, and identification of their homologues from the genome sequence of *L. bicolor*. The comparisons of gene structures showed that the organization and the structures of *lao1* and partial *pdc2* genes were nearly identical in *H. cylindrosporum* and *L. bicolor*, suggesting that the corresponding proteins are also functionally similar. In contrast, the sequence similarities and GC contents of the intergenic and intronic DNA were markedly lower than those of the coding sequences. In particular, the intergenic regions harboured four repeated sequence motifs in *L. bicolor*, but only one in *H. cylindrosporum*. The motifs of *L. bicolor* may have arisen via duplications, as suggested by the tandem arrangement of the motifs, and phylogenetic clustering of their sequences. A very interesting observation was the co-localization of transcription factor binding sites for ADR1 and NIT2 in the two motifs. Thus, the predicted duplications may have amplified these regulatory sites for the *pdc2* (and/or *lao1*) gene(s). This, in turn, may predispose the *L. bicolor* genes under differential regulatory constraints compared with *H. cylindrosporum*. As an affirmation, a previous study observed that all seven *Hebeloma* spp. strains analysed expressed higher levels of Lao activity than *L. bicolor* (Nuutinen & Timonen, 2008).

Lao1 protein sequences of *H. cylindrosporum* and *L. bicolor* displayed low global identities compared with several other characterized Lao-sequences, including those from the basidiomycete *A. phalloides* and two ascomycete fungi (Davis et al., 2005; Niedermann & Lerch, 1990; Stasyk et al., 2010). Similarly, phylogenetic analyses have previously shown that large evolutionary distances separated the Lao sequences from ascomycetes, snakes, bacteria and algae (Macheroux et al., 2001). Since the Lao-sequences from these taxa had undergone extensive diversification at the sequence level, it is likely that the enzymes may also display functional divergence.

Lao1 of *H. cylindrosporum* may undergo several modifications of the protein sequence in vivo, since the molecular mass and pI heterogeneity of the fungal enzyme was absent.
Table 2. Substrate specificities of the recombinant Lao1 of *H. cylindrosporum*, expressed in *E. coli*

The amino acids that displayed more than 2 % relative activity are shown. The activities were <2 % with the following compounds as substrates: L-aspartate, L-aspartate-β-methyl-ester, L-threonine, glycine, proline, 2-aminoheptanedioic acid, L-2-amino-3-guadininopro-pionic acid, L-methionine sulfoximine, glufosinate, L-tert-leucine, D-ornithine, D-phenylalanine, D-glutamate, D-histidine, D-alanine, DL-β-phenylalanine, N-methyl-L-phenylalanine, 3-methyl-DL-phenylalanine, β-methyl-DL-phenylalanine, L-phenylalaninol, phenethylamine, L-phenylalaninamide and the dipeptides Phe-Phe, Phe-Gly and Gly-Gly.

<table>
<thead>
<tr>
<th>Side chain</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acidic side chains</strong></td>
<td></td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>100</td>
</tr>
<tr>
<td>L-Homoglutamic acid</td>
<td>18</td>
</tr>
<tr>
<td><strong>Basic/amide side chains</strong></td>
<td></td>
</tr>
<tr>
<td>L-2,3-Diaminopropanoic acid</td>
<td>5</td>
</tr>
<tr>
<td>L-Asparagine</td>
<td>74</td>
</tr>
<tr>
<td>L-Ornithine</td>
<td>76</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>79</td>
</tr>
<tr>
<td>L-Citrulline</td>
<td>25</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>12</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>25</td>
</tr>
<tr>
<td>L-Homoarginine</td>
<td>31</td>
</tr>
<tr>
<td>DL-2,6-Diaminopimelic acid</td>
<td>4</td>
</tr>
<tr>
<td>L-Canavanine</td>
<td>6</td>
</tr>
<tr>
<td><strong>Aromatic/cyclic side chains</strong></td>
<td></td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>44</td>
</tr>
<tr>
<td>L-Phenylalanine methyl ester</td>
<td>42</td>
</tr>
<tr>
<td>L-Phenylalanine tert-butyl ester</td>
<td>27</td>
</tr>
<tr>
<td>DL-Homophenylalanine</td>
<td>19</td>
</tr>
<tr>
<td>2-Chloro-L-phenylalanine</td>
<td>8</td>
</tr>
<tr>
<td>4-Bromo-L-phenylalanine</td>
<td>8</td>
</tr>
<tr>
<td>3-Cyclohexyl-L-alanine</td>
<td>31</td>
</tr>
<tr>
<td>3-Cyclopentyl-DL-alanine</td>
<td>31</td>
</tr>
<tr>
<td>3-(2-Pyridyl)-L-alanine</td>
<td>42</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>25</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>46</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>19</td>
</tr>
<tr>
<td>3-(1-Naphthyl)-L-alanine</td>
<td>4</td>
</tr>
<tr>
<td><strong>Aliphatic side chains</strong></td>
<td></td>
</tr>
<tr>
<td>L-Alanine</td>
<td>27</td>
</tr>
<tr>
<td>L-2-Aminobutanoic acid</td>
<td>58</td>
</tr>
<tr>
<td>L-Valine</td>
<td>2</td>
</tr>
<tr>
<td>L-Norvaline</td>
<td>66</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>61</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>11</td>
</tr>
<tr>
<td>L-Norleucine</td>
<td>58</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>39</td>
</tr>
<tr>
<td>DL-2-Aminooctanoic acid</td>
<td>5</td>
</tr>
<tr>
<td><strong>Hydroxyl side chains</strong></td>
<td></td>
</tr>
<tr>
<td>L-Serine</td>
<td>3</td>
</tr>
<tr>
<td>L-Homoserine</td>
<td>26</td>
</tr>
</tbody>
</table>

from the recombinant Lao1. The snake venom Lao has also been shown to possess at least 18 electrophoretic enzyme species (Hayes & Wellner, 1969). Laos from snake venoms are N-glycosylated, which may present one source of heterogeneity (Pawelek et al., 2000). Between two and four putative glycosylation sites were computationally predicted from the basidiomycete Lao1 sequences, although it is not yet known if basidiomycete Lao1s are glycosylated. Most eukaryotic Laos are known or predicted to be processed by the removal of a signal- and/or propeptide (Niedermann & Lerch, 1990; Sun et al., 2002), and vertebrate Laos localize into lysosomes or venom glands (Chavan et al., 2002; Mason et al., 2004). However, sequence comparisons revealed no conserved signal sequences or cleavage sites from the two basidiomycete Lao1 sequences. Both the slightly smaller size of the native Lao1 of *H. cylindrosporum* compared to its recombinant counterpart, and the presence of several protein species of different sizes strongly argue for proteolytic processing of the fungal enzyme. However, our attempts to determine the N-terminal amino acid sequence of Lao1 by Edman degradation were unsuccessful.

Stability and catalytic properties of recombinant Lao1

Expression of the recombinant Lao1 in *E. coli* confirmed the functional assignment of the gene as Lao. rLao1 possessed a non-covalently bound flavin cofactor, and the protein sequence carried the dinucleotide-binding motif (Wierenga et al., 1986). The flavin cofactor is tightly bound within a stable protein scaffold, since both the native and rLao1 enzymes tolerated treatment with SDS, while the snake Lao was inactivated with such treatment. A similar SDS protocol has been used for the solubilization and activation of another flavoenzyme, rat dimethylglycine dehydrogenase expressed in *E. coli*, but this enzyme possessed a covalently bound flavin cofactor (Brizio et al., 2004). The relative amount of Lao in fungal extracts was higher after the nuclease and chitinase digestion steps than in non-treated extracts. Similarly, no Lao activity was lost from the crude cellular extract of *Hebeloma* sp. ‘Denudata’ after several hours of incubation at room temperature (Nuutinen & Timonen, 2008). These results suggest that Lao of *Hebeloma* spp. may be very resistant to inactivation by chemicals and proteolysis.

Of the amino acid substrates tested, L-glutamate yielded the highest relative activities and limiting velocities, and it was among the best substrates with regard to catalytic efficiency. Such high competence for a negatively charged amino acid is unusual. The Lao-enzymes from other eukaryotes oxidize glutamate only slowly or not at all (relative activities 0–17 %) (Bender & Krebs, 1950; Zhong et al., 2009), while the highly agile Lao of the actinobacterium *Rhodococcus opacus* achieved a relative activity of 44 % (Geuake & Hummel, 2002). Interestingly, this apparent shift in relative substrate utilization in Lao1 of *H. cylindrosporum* did not cause gross discrimination against other L-amino acids, since significant activities were also
recorded with a number of positively charged, hydrophilic, hydrophobic and aromatic L-amino acids as substrates. In contrast, the bacterium Streptomyces possesses L-glutamate oxidase, a flavoenzyme related to Lao that is very specific for this substrate (Böhmer et al., 1989). The high activity of Lao1 towards L-glutamate likely arises from highly localized charge interactions within the Lao1 active site, since the relative activities were markedly lower if the backbone of the negatively charged substrate was one methylene group shorter or longer than L-glutamate. The Lao-enzymes of basidiomycete fungi may be mechanisms for utilizing as nitrogen sources a number of amino acids, even the rare ones not occurring in proteins. Unusual amino acids may represent significant nitrogen compounds in certain environments, e.g. δ-acetylornithine in the underground parts of an alpine herb, Bistorta bistortoides (Lipson et al., 1996).

The rLao1 had very similar limiting velocities to the enzyme from R. opacus, and slightly lower than the enzymes from snake venoms (Geueke & Hummel, 2002; Zhong et al., 2009). Lao1 of H. cylindrosporum, belonging to subgroup ‘Mycocybe’ of the genus, possessed very similar pH optima and Michaelis constants to the Lao-enzymes of Hebeloma subgroups ‘Velutipes’ and ‘Denudata’, measured from the crude mycelial extracts (Nuutinen & Timonen, 2008). This supports the results obtained from the functional assays using these non-pure enzyme sources (Nuutinen & Timonen, 2008).

Altogether, the results support the hypothesis that the Lao of Hebeloma spp. possesses very broad substrate specificities, comparable to those of actinobacteria, ascomycete fungi and snake venoms (Nuutinen & Timonen, 2008). In addition, the Lao1 of H. cylindrosporum displayed unique functional and stability properties among the known Lao-enzymes. This emphasizes the need to explore relatively less scrutinized taxonomic groups – like basidiomycete fungi – when searching for enzymes with novel properties.

### Lao in amino acid catabolism

Lao was one of the major soluble proteins of H. cylindrosporum in the growth conditions used, arguing for its major role in cellular amino acid catabolism. Accordingly, the highest Lao activities of a Hebeloma sp. strain were recorded when it was grown in nitrogen-rich conditions (Nuutinen & Timonen, 2008). Glutamate (and glutamine), alanine and aspartate (and asparagine) are among the most abundant intracellular amino acids in several basidiomycete fungi, e.g. Schizophyllum commune and Coprinopsis cinerea (Hanks et al., 2003; Ulrich et al., 2007 and references therein). Interestingly, in these two fungi, the intracellular ammonium content has been shown to increase approximately twofold in response to nitrogen starvation, concomitantly to a fourfold decrease in the amino acid pool (Hanks et al., 2003; Ulrich et al., 2007). Similarly, under excessive amino acid supply, several basidiomycete fungi, including the ones that form ectomycorrhiza, have been shown to produce ammonia into the growth medium, as discussed by Nuutinen & Timonen (2008). Thus, deamination (mineralization) of amino acids may be a significant process of amino acid turnover in basidiomycetes. The rapid decline of the intracellular amino acid pools indicates that the catabolic machinery is constitutively present in the cells or is very rapidly upregulated. The relatively high Michaelis constants of Lao (this study and Nuutinen & Timonen, 2008) may ensure that the intracellular concentrations of amino acids also remain sufficient for anabolic processes.

Werdin-Pfister et al. (2009) summarized the results of eight studies on soluble amino acid compositions of forest soils. L-Glutamate was among the five most abundant amino acids in all of the soils. Asparagine and glutamine were also abundant in most sites studied. Thus, the composition of amino acid pools of soils resembles the intracellular composition of basidiomycete fungi. Of the most abundant amino acids in these soils, only aspartate and serine would be poor substrates for the Lao1 of H. cylindrosporum. With respect to the substrate utilization profile, the Lao1 is well adapted to the prevailing amino acid composition of its environment. Thus, the Lao-expressing fungi may be adapted to mineralize the amino acids from nutrient-rich patches of soils, like pollen, seeds and animal remnants (Read & Perez-Moreno, 2003; Schimel & Bennett, 2004).

### Table 3. Kinetic properties of the H. cylindrosporum Lao1, expressed as recombinant protein in E. coli

<table>
<thead>
<tr>
<th>Substrate amino acid</th>
<th>V&lt;sub&gt;max&lt;/sub&gt; [nkat (mg protein)&lt;sup&gt;-1&lt;/sup&gt;] ±</th>
<th>K&lt;sub&gt;m&lt;/sub&gt; (mM) ±</th>
<th>K&lt;sub&gt;i&lt;/sub&gt; (mM) ±</th>
<th>k&lt;sub&gt;cat&lt;/sub&gt; (s&lt;sup&gt;-1&lt;/sup&gt;) ±</th>
<th>k&lt;sub&gt;cat&lt;/sub&gt;/K&lt;sub&gt;m&lt;/sub&gt; (s&lt;sup&gt;-1&lt;/sup&gt; M&lt;sup&gt;-1&lt;/sup&gt;×10&lt;sup&gt;3&lt;/sup&gt;) ±</th>
<th>pH optimum ±</th>
<th>pH range* ±</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Alanine</td>
<td>105 ± 5</td>
<td>6.7 ± 1.4</td>
<td></td>
<td>7.7</td>
<td>1.2</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>L-Ornithine</td>
<td>165 ± 15</td>
<td>5.8 ± 2.3</td>
<td>114 ± 19</td>
<td>12.1</td>
<td>2.1</td>
<td>8</td>
<td>6–9</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>151 ± 6</td>
<td>0.5 ± 0.1</td>
<td></td>
<td>11.1</td>
<td>24.6</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>131 ± 4</td>
<td>1.1 ± 0.2</td>
<td></td>
<td>9.7</td>
<td>8.6</td>
<td>8</td>
<td>6–10</td>
</tr>
<tr>
<td>L-Glutamate</td>
<td>239 ± 6</td>
<td>1.1 ± 0.1</td>
<td></td>
<td>17.6</td>
<td>16.5</td>
<td>8</td>
<td>6–9</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>214 ± 13</td>
<td>0.9 ± 0.2</td>
<td>257 ± 74</td>
<td>15.8</td>
<td>17.9</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

* pH range yielding >10% of the maximal activity for that amino acid.
ACKNOWLEDGEMENTS

We are grateful to Raphaël Lambilliotte and Hervé Sentenac for providing the H. cylindrosporum strain h7. We thank Marc Baumann and Eeva Kauppi for guidance in the proteome analyses, Paula Kristo (Haartman Institute Sequencing Core facility) for help in the DNA sequencing and Sanna Koutaniemi and Teemu Teeri for help with the Akta system. Jari Valkonen is thanked for his continuous support. We acknowledge CSC for use of computational resources. Academy of Finland (grant no. 1115962) and Department of Applied Chemistry and Microbiology supported this study financially. K.H. was supported by grants from the European Union (PIEF-GA-2009-236714) and the Finnish Cultural Foundation.

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L-Amino acid oxidase of H. cylindrosporum


Edited by: J. M. Becker