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Identification of nitrogen mineralization enzymes, L-amino acid oxidases, from the ectomycorrhizal fungi *Hebeloma* spp. and *Laccaria bicolor*

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ABSTRACT

Amino acids are major nitrogen sources in soils and they harbour a central position in the nitrogen metabolism of cells. We determined whether *Hebeloma* spp. and *Laccaria bicolor* expressed the enzyme L-amino acid oxidase (LAO), which catalyses the oxidative deamination of the α -amino group of L-amino acids. We measured LAO activities from the mycelial extracts of seven laboratory-grown fungal strains with three methods, and we measured how LAO activities were expressed in one *Hebeloma* sp. strain grown on four nitrogen sources. *Hebeloma* spp. and *L. bicolor* converted L-phenylalanine, but not D-phenylalanine, to hydrogen peroxide, 2-oxoacid, and ammonia, suggesting that they expressed LAO enzymes. The enzymes utilized five out of seven tested L-amino acids as substrates. LAO activities were maximal at pH 8, where Michaelis constant (K_m) values were 2–5 mM. The LAO of *Hebeloma* sp. was expressed on every nitrogen source analysed, and the activities were the highest in mycelia grown in nitrogen-rich conditions. We suggest that LAO is a mechanism for cellular amino acid catabolism in *Hebeloma* spp. and *L. bicolor*. Many soil bacteria and fungi also express LAO enzymes that have broad substrate specificities. Therefore, LAO is a potential candidate for a mechanism that catalyses nitrogen mineralization from amino acids at the ecosystem level.

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Introduction

Basidiomycete and ascomycete fungi, including those that form ectomycorrhizal (ECM) symbioses with tree roots, are major inhabitants in soils of boreal and temperate forests (Landeweert *et al.* 2003; O'Brien *et al.* 2005; Rosling *et al.* 2003). The ECM fungi promote the fitness and growth of their host plants, for example, via enhanced nutrient acquisition from the soils. In particular, the importance of ECM fungi in nitrogen nutrition of their host trees is well established

(Read & Perez-Moreno 2003). Soils of boreal and temperate forests have a high organic material content. Proteins and amino acids are major constituents of the soil nitrogen pool in these ecosystems, and are often present in higher concentrations than mineral forms of nitrogen (Johnsson *et al.* 1999). Soil bacteria and saprotrophic fungi can break down these compounds and release easily accessible mineral nitrogen in soil. Most ECM fungi can grow using amino acids as their sole nitrogen source (Ahmad *et al.* 1990; Guidot *et al.* 2005; Keller 1996). Therefore, amino acids may play a major role in

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the nitrogen nutrition of ECM fungi and their host plants. A number of molecular mechanisms from ECM fungi, which are involved in nitrogen transport and amino acid synthesis, have been characterized (Brun *et al.* 1992; Jargeat *et al.* 2000; Javelle *et al.* 2003; Wipf *et al.* 2002). However, relatively little is known about the enzymes breaking down amino acids (Ahmad *et al.* 1990; Garnier *et al.* 1997; Marmeisse *et al.* 2004; Quoreshi *et al.* 1995).

The aim of this study was to characterize novel mechanisms of amino acid catabolism from ECM fungi. We hypothesized that ECM fungi expressed the enzyme L-amino acid oxidase [LAO; L-amino acid: oxygen oxidoreductase (deaminating); EC 1.4.3.2], which catalyses nitrogen mineralization by oxidative deamination of L-amino acids (Fig 1). To test the hypothesis, we grew strains of *Hebeloma* spp. and *L. bicolor* as laboratory cultures and analysed whether the extracts of the fungal mycelia converted L-amino acids to the expected reaction products: H_2O_2 , 2-oxoacids, and NH_4^+ . We then measured the pH optima, Michaelis constant (K_m), and substrate specificities of LAO. Finally, we characterized the expression pattern of LAO from one *Hebeloma* sp. strain grown on different nitrogen sources.

Materials and methods

Fungal strains and growth conditions

Hebeloma sp. strain SE5 was obtained from the fungal culture collection of the Mycorrhizal Interactions group, Department of Applied Biology, University of Helsinki. *Hebeloma* sp. strains F-NB01 (sporocarp, *Picea abies* nursery), F-RS01 (sporocarp, roadside), and R-RS01 (root tip, *Picea abies* on roadside) were isolated by Taina Pennanen (Finnish Forest Research Institute) from material collected from southern Finland. *Hebeloma* sp. strain SIV [sporocarp, under *Picea abies*; Nancy, France (Kiffer 1974)] and *Laccaria bicolor* strain S238N (Di Battista *et al.* 1996), were from Jean Garbaye (INRA-Nancy, France). *H. cylindrosporum* strain CBS 558.96 was from Centraalbureau voor Schimmelcultures (Utrecht).

The fungi were grown for 18–45 d at room temperature (22–25 °C) on cellophane-covered, agar-solidified (12 g l⁻¹) Hagem's media (Modess 1941), with 0.63 g l⁻¹ glucose. The pH was adjusted to 4.8–5 with HCl or NaOH before autoclaving. Thiamine (100 µg l⁻¹) was added after autoclaving. For nitrogen experiments, the *Hebeloma* sp. SE5 was grown as above, but on basal Melin-Norkrans media (Marx 1969), with modifications. The medium composition was (g l⁻¹): 0.76 g l⁻¹ KH_2PO_4 , 0.05 g l⁻¹ CaCl_2 , 0.025 g l⁻¹ NaCl, 0.15 g l⁻¹ $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.5 g l⁻¹ glucose, 0.012 g l⁻¹ $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$. The medium was

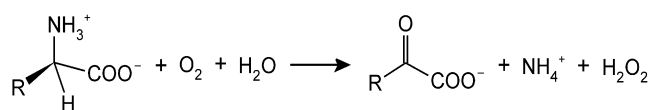


Fig 1 – The reaction catalysed by LAO. The enzyme mineralizes nitrogen from L-amino acids by oxidative deamination, which yields 2-oxoacid, ammonia, and H_2O_2 as products. R = side chain of the amino acids.

amended with NaNO_3 , $(\text{NH}_4)_2\text{SO}_4$, L-glutamate or, after autoclaving, with sterile-filtered bovine serum albumin (BSA) as nitrogen sources. Each compound was used at 0.4, 4, and 40 mM concentrations of nitrogen.

Molecular identification and sequence analyses of *Hebeloma* strains

DNA from fungal mycelia of all six *Hebeloma* spp. strains used in this study were extracted by CTAB and proteinase K protocol (Timonen *et al.* 1997). ITS regions of the DNA were amplified with primers ITS1 and LR21 (Tedersoo *et al.* 2003) using Phusion polymerase (Finnzymes, Espoo). PCR reactions were checked for products on 1 % agarose–ethidium bromide gel. The products were precipitated with ethanol and/or cleaned with phenol and treated by exonuclease and alkaline phosphatase (Exo/SAP) prior to sequencing at the Haartman Institute Sequencing unit (University of Helsinki). Acquired sequences were manually checked using Vector NTI® Suite version 7 (InforMax®).

Reference sequences of *Hebeloma* spp., *Alnicola* spp., and *Laccaria bicolor* were retrieved from GenBank and UNITE (Köljalg *et al.* 2005) databases and aligned with ClustalW [European Bioinformatics Institute; Chenna *et al.* (2003)]. The parsimony analyses were performed by WinClada Ver. 1.00.08 (Nixon 2002) as in Timonen & Hurek (2006), except that *L. bicolor* was fixed as the cladogram outgroup. The ITS sequences of the *Hebeloma* spp. strains were deposited to GenBank with accession numbers EF564167–EF564172.

Preparation of crude mycelial extracts

Mycelia from one to seven agar plates grown in parallel were harvested and combined. The mycelia were freshly ground in mortars in extraction buffer (EB), modified from Brun *et al.* (1992) and from Juuti *et al.* (2004). The composition of the EB was: 100 mM Tris–HCl, pH 8, 6 mM dithiothreitol, 5 mM MgCl_2 , 1 mM EDTA, 8.8 % glycerol, 2.5 % soluble polyvinylpyrrolidone (molecular weight 10 000), 5 % insoluble polyvinylpyrrolidone (Polyclar V), 0.5 % Triton-X 100, 10 µg ml⁻¹ phenylmethylsulphonyl fluoride, 5 µl ml⁻¹ protease inhibitor mixture (Sigma P-9599, containing AEBSF, bestatin, pepstatin A, E-64, leupeptin, and 1,10-phenanthroline). The volume of added EB was four to six times the fresh weight of the mycelium.

In the case of *Hebeloma* sp. strains F-NB01, F-RS01, and R-RS01, the mortar-ground mycelia was ultrasonicated (Labsonic U, B. Braun, Melsungen) twice for 60 s on ice, incubated at room temperature for 3–6 h, and ultrasonicated again as above. All extracts were clarified by centrifugation (8000 × g, 5 min, 4 °C). The extracts were retrieved, the resulting pellet washed once with EB, centrifuged, and the wash supernatant was combined with the extract. They were stored at –80 °C, where they retained most of their enzyme activities for at least three years. One extract was defined as one biological replicate.

Protein assay

Proteins in the fungal extracts were precipitated by adding 100 % (w/v) trichloroacetic acid to yield final concentrations of 10–20 % acid. The precipitates were collected by

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