

Recombinant expression and characterization of a L-amino acid oxidase from the fungus *Rhizoctonia solani*

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Abstract L-Amino acid oxidases (L-AAOs) catalyze the oxidative deamination of L-amino acids to the corresponding α -keto acids, ammonia, and hydrogen peroxide. L-AAOs are homodimeric enzymes with FAD as a non-covalently bound cofactor. They are of potential interest for biotechnological applications. However, heterologous expression has not succeeded in producing large quantities of active recombinant L-AAOs with a broad substrate spectrum so far. Here, we report the heterologous expression of an active L-AAO from the fungus *Rhizoctonia solani* in *Escherichia coli* as a fusion protein with maltose-binding protein (MBP) as a solubility tag. After purification, it was possible to remove the MBP-tag proteolytically without influencing the enzyme activity. MBP-*rs*LAAO1 and 9His-*rs*LAAO1 converted basic and large hydrophobic L-amino acids as well as methyl esters of these L-amino acids. The progress of the conversion of L-phenylalanine and L-leucine into the corresponding α -keto acids was determined by HPLC and ¹H-NMR analysis of reaction mixtures, respectively. Enzymatic activity was stimulated 50–100-fold by SDS treatment. K_m values ranging from 0.9–10 mM and v_{max} values from 3 to 10 U mg⁻¹ were determined

after SDS activation of 9His-*rs*LAAO1 for the best substrates. The enzyme displayed a broad pH optimum between pH 7.0 and 9.5. In summary, a successful overexpression of recombinant L-AAO in *E. coli* was established that results in a promising enzymatic activity and a broad substrate spectrum for biotechnological application.

Keywords L-Amino acid oxidase · Heterologous expression · *E. coli* · Maltose-binding protein · Solubility tag

Introduction

L-Amino acid oxidases (L-AAOs, EC 1.4.3.2) have been identified in diverse organisms such as snakes, bacteria, fungi, and algae (Bender and Krebs 1950; Geueke and Hummel 2002; Nuutinen and Timonen 2008; Pollegioni et al. 2013). They catalyze the oxidative deamination of L-amino acids under formation of the corresponding α -keto acids, ammonia, and hydrogen peroxide. Flavin adenine dinucleotide (FAD) acts as a tightly but not covalently bound cofactor, which accepts a hydride under formation of α -imino acid intermediates. The latter hydrolyse spontaneously to α -keto acids and ammonia (Faust et al. 2007; Moustafa et al. 2006). The reduced FAD reacts with oxygen to regenerate the oxidized FAD under formation of hydrogen peroxide. Most L-AAOs are active as homodimers, but there are also monomeric enzymes (Pollegioni et al. 2013). The protein structures of the homodimers have been determined for L-AAOs from snake venoms and from the bacterium *Rhodococcus opacus* (Faust et al. 2007; Moustafa et al. 2006; Ullah et al. 2012; Zhang et al. 2004). Some L-AAOs such as the enzymes from *R. opacus* and from the fungus *Hebeloma cylindrosporum* convert most proteinogenic α -amino acids (Geueke and Hummel 2002; Nuutinen et al. 2012). Other oxidases are more

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specific. For example, cobra venom L-AAO prefers hydrophobic amino acids (Bender and Krebs 1950).

The related D-amino acid oxidases are already used successfully for various biotechnological applications (Pollegioni and Molla 2011). Accordingly, several potential biotechnological applications for L-AAOs can be envisaged. L-AAOs could be used for the production of α -keto acids of L-arginine, L-lysine, L-cysteine, and L-methionine. These α -keto acids are not substrates in biosynthetic pathways of the corresponding L-amino acids. Therefore, they cannot be produced by engineering biosynthetic pathways directly as described for, e.g., α -keto acids in branched chain amino acid biosynthesis (Tashiro et al. 2015). A further application is the use of L-AAOs for the preparation of enantiomerically pure D-amino acids from racemic mixtures of α -amino acids by enzymatic resolution via L-AAOs. Finally, L-AAOs could be used as components of biosensors. However, a major limitation so far is the lack of suitable simple heterologous expression systems enabling a production of large quantities of active recombinant L-AAOs with a broad substrate spectrum (Hossain et al. 2014; Pollegioni et al. 2013). The L-AAO from *R. opacus* expressed in *Escherichia coli* was insoluble and could not be refolded into an active form (Geueke and Hummel 2003). An active *R. opacus* L-AAO was obtained after recombinant expression in *Streptomyces lividans*. However, the specific L-AAO activity was only 0.029 U mg⁻¹ in crude extracts from *S. lividans* while crude extracts derived from the wild-type organism *R. opacus* contained 0.071 U mg⁻¹ (Geueke and Hummel 2003). An L-AAO of the fungus *H. cylindrosporum* belonging to the division of *Basidiomycota* has been expressed recombinantly in *E. coli* as an active but almost completely insoluble fusion protein with an N-terminal 6-His tag (Nuutinen et al. 2012). These results underline the challenge of heterologously overexpressing L-AAOs with a broad substrate spectrum.

Heterologous expression of L-AAOs with a narrow substrate spectrum has been more successful. An L-aspartate oxidase from the archaea *Sulfolobus tokodaii* has been expressed in *E. coli* with a His-tag (Bifulco et al. 2013). The L-AAO from the bacterium *Streptococcus oligofermens*, which can utilize seven amino acids as substrate, has also been expressed as a soluble 6His-tagged fusion protein in *E. coli* (Tong et al. 2008). Moreover, L-phenylalanine and L-histidine have been converted by the L-AAO from the fungus *Trichoderma harzianum* ETS323 after expression as 6His-tagged fusion proteins in *E. coli* and refolding from inclusion bodies (Cheng et al. 2011). A truncated L-amino acid deaminase from the bacterium *Proteus mirabilis* with a 6His-tag has been refolded from *E. coli* inclusion bodies and deaminates L-histidine, L-arginine, L-phenylalanine, and L-glutamate under formation of the corresponding α -keto acids (Liu et al. 2013). The yeast *Pichia pastoris* has been used as expression system for the L-AAO from the fish *Platichthys stellate* with a 6-His tag for application as an antibacterial agent (Kasai et al. 2015).

Since genomes of dozens of fungi have been sequenced recently (e.g., Cubeta et al. 2014; Floudas et al. 2012; Tang et al. 2012), putative L-AAOs can be identified by sequence similarities especially in the FAD and substrate-binding sites. In the following, we report the successful overexpression of a fungal putative L-AAO recombinantly in *E. coli* with a high yield and a detailed characterization of the biochemical properties of the enzyme as well as initial applications in biotransformations for α -keto acid syntheses.

Materials and methods

Amplification and cloning of the *rsLAAO1*

A synthetic gene (Life Technologies) of a putative *Rhizoctonia solani* AG-3 Rhs1AP L-AAO1 (EUC54418.1, Cubeta et al. 2014) codon optimized for expression in *P. pastoris* (sequence in GenBank under accession number KX925447) was cloned into the expression vector pMALc5X (New England Biolabs) encoding an N-terminal maltose-binding protein (MBP) followed by a recognition site for the Factor Xa protease under the control of a tac promoter via the restriction sites *Nde* I and *Eco* RI generating pKHRi1 (MBP-*rsLAAO1*). To insert a sequence encoding a 9His-tag after the sequence encoding the recognition site for the Factor Xa protease, a forward primer (GCGG CCGCATGCATCATC ACCATCACCACCATCACCAT TCCTCCAAAGAGTTCAAGGAC) with a *Not* I site (underlined sequence) and a 9His-tag (bold) and a reverse primer (GCGGCCGCGCATATGTGAAATCCTTCCCTC) with a *Not* I site were used to PCR-amplify pKHRi1. The PCR-product was digested with *Not* I and ligated yielding pKHRi2. To enable the low-temperature cleavage of the fusion protein, a sequence encoding the PreScission protease recognition site was introduced upstream of the sequence encoding the N-terminal 9His-tag by PCR amplification of pKHRi2 with the following primers (restriction sites are underlined, 9His-tag are in bold, and *PreScission protease* sequences are in italics): GCGG CCGCCTCGAAGTAC TGTTCCAGGGTCCCTATGC ATCATCACCATCACCAC and GCGGCCGCCATA TGTGAAATCCTTCCCTC, generating pKHRi4 (MBP-P-9His-*rsLAAO1*) after *Not* I digestion and ligation. The vector pKHRi7 was generated by cloning the synthetic gene into pET28b (Novagen) via the restriction sites *Nde* I and *Eco* RI for expression of 6His-*rsLAAO1* with an N-terminal 6His-tag.

Expression

pKHRi1 and pKHRi7 were transformed into *E. coli* BL21 CodonPlus (DE)-RP and Arctic Express (DE) (Ferrer et al. 2004) competent cells and cultured in LB broth supplemented with antibiotics (50 μ g mL⁻¹ kanamycin, 100 μ g mL⁻¹ ampicillin, 20 μ g mL⁻¹ gentamycin, 25 μ g mL⁻¹

chloramphenicol). MBP-*rsLAAO1* expression was induced at an OD₆₀₀ of 0.8 by 0.05 or 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 16 and 24 °C for 5 h. The test expression in Arctic Express cells (Ferrer et al. 2004) was performed following the manufacturer's instructions using 0.05 mM. Cells taken at the indicated time points were resuspended in PBS (20 mM sodium phosphate buffer, pH 7.4, 150 mM NaCl), briefly sonicated, and centrifuged. The soluble fraction and the remaining pellet (resuspended in the same volume) were separated by SDS-PAGE and stained with Coomassie Brilliant Blue R-250. For expression on a 500-mL scale, Arctic Express (DE3) cells with pKHRi1 or pKHRi4 were grown overnight at 37 °C in LB broth with 20 μg mL⁻¹ gentamycin for selection of the *cpn10/cpn60* chaperonin expression plasmid. The culture was diluted to an OD₆₀₀ of 0.15 with LB broth and incubated at 30 °C until the OD₆₀₀ reached 1.3–1.5. Expression was induced after a 15-min preincubation at 11 °C by 0.05 mM IPTG for 18 h at 11 °C.

Purification of the *rsLAAO1* protein

Purification of the tagged proteins was carried out at 4 °C. Cells of a 500-mL culture were pelleted, resuspended in 20 mL MBP buffer (20 mM Tris-HCl, pH 8.2, 200 mM NaCl, 1 mM EDTA), and disrupted by French press. After centrifugation at 27,000×*g* for 30 min, the resulting soluble fraction was applied to an amylose column, which was equilibrated with MBP buffer. After washing, MBP-*rsLAAO1* or MBP-P-9His-*rsLAAO1* was eluted by supplementing the MBP buffer with 10 mM maltose.

For the two-step purification of MBP-P-9His-*rsLAAO1*, the elution fractions were pooled and diluted with His buffer (50 mM sodium phosphate buffer, pH 8.2, 300 mM NaCl) in a ratio of 1:1. One microgram of PreScission protease was added to 100 μg fusion protein for cleaving the MBP-tag and incubated for 18 h at 4 °C, and the solution was applied to a Ni²⁺-NTA column. The free MBP-tag and the PreScission protease were in the flow through. After washing with 10 column volumes of His buffer, 9His-*rsLAAO1* was eluted with His buffer containing 500 mM imidazole (pH 8.2). The fractions were pooled, rebuffed to His buffer via ultrafiltration (Vivaspin 6 30,000 MWCO, Sartorius), and stored at 4 °C.

Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with 11% acrylamide in the separating gel (Laemmli 1970). Proteins were stained with Coomassie Brilliant Blue R-250. Native PAGE was performed in 8% polyacrylamide gels using a sample buffer without SDS and 2-mercaptoethanol. Some samples were

supplemented with 0.025% SDS. Hydrogen peroxide (H₂O₂) production in the native PAGE gels was detected by incubating the gels at room temperature in 50 mM triethanolamine (TEA) buffer (pH 8.0), 10 mM L-arginine, 2 mg mL⁻¹ *o*-dianisidine, and 5 U mL⁻¹ horseradish peroxidase until the bands were visible (Geueke and Hummel 2002).

Protein determination

The concentration of *rsLAAO1* proteins was determined according to the method developed by Bradford (1976) using bovine serum albumin as standard. The purity and expression rates of *rsLAAO1* samples were monitored by SDS-PAGE.

Spectroscopy

Absorbance spectra were recorded using a UV-2450 UV-VIS spectrophotometer (Shimadzu). The FAD content in 9His-*rsLAAO1* was determined by absorbance in 50 mM potassium phosphate buffer, pH 8.5, containing 300 mM NaCl. The concentration of FAD bound to *rsLAAO1* was calculated from the absorbance at 450 nm ($\epsilon = 11,300 \text{ M}^{-1} \text{ cm}^{-1}$; Whitby 1953). The cofactor occupancy of 9His-*rsLAAO1* was calculated from the ratio of the absorbance at 280 and 450 nm ($\epsilon_{280\text{nm}} = 20,552 \text{ M}^{-1} \text{ cm}^{-1}$ for free FAD, $\epsilon_{280\text{nm}} = 128,230 \text{ M}^{-1} \text{ cm}^{-1}$ for 9His-*rsLAAO1* calculated with EXTCOEF; <http://workbench.sdsc.edu>).

Enzyme assay

To determine the oxidase activity of *rsLAAO1*, the initial production rate of hydrogen peroxide was measured.

1. The Ampliflu Red (10-acetyl-3,7-dihydroxyphenoxazine) reagent was used to measure H₂O₂ release in enzyme reactions (Zhou et al. 1997). Fluorescence signals (excitation at 550 nm, emission at 585 nm) were recorded using a Tecan Infinite 200 microplate reader at 30 °C. Unless otherwise stated, the final reaction mixture contained 10 mM L-arginine, 10 U mL⁻¹ peroxidase, 10 μM Ampliflu Red, and *rsLAAO1* in limiting amounts in 200 mM TEA/HCl buffer (pH 8.5) in a final volume of 200 μL. The H₂O₂ concentrations in the calibration standards were 0.05 to 7.5 μM.
2. The coupled peroxidase/dye assay determined H₂O₂ by detection of oxidized *o*-dianisidine (Vanboven et al. 1988). This was monitored spectrophotometrically at 436 nm ($\epsilon = 8100 \text{ M}^{-1} \text{ cm}^{-1}$). The standard assay mixture contained 10 mM L-arginine, 50 mM TEA/HCl buffer (pH 8.5), 0.2 mg mL⁻¹ *o*-dianisidine, 5 U mL⁻¹ peroxidase, and *rsLAAO1* in limiting amounts. Reactions were carried out in 96-well plates at 30 °C in a Tecan Infinite 200 microplate reader.

One unit was defined as the amount of enzyme that catalyzes the conversion of 1 μmol L-amino acid per minute. K_m and v_{max} were determined with L-amino acid concentrations of 0.1–30 mM.

Reversed-phase high performance liquid chromatography

Five millimolars of L-phenylalanine, 0.05 U SDS-activated 9His-*rsLAAO1* (24 μg), and 32.5 U catalase were incubated in 50 mM TEA buffer (pH 8.5) in a total volume of 650 μL for different periods of time at 30 °C. Samples were boiled to stop the reaction, and 20 μL of the reaction products were separated by HPLC (Jasco) with a Macherey-Nagel RP 18 column (NUCLEODUR® C18 HTec, 4.6×150 mm, 5 μm with a guard column) using eluent A (water 0.1% (v/v) trifluoroacetic acid (TFA)) and eluent B (methanol 0.1% (v/v) TFA). Separation was obtained at a flow rate of 1 mL min^{-1} and a linear gradient from 60 to 30% A over 8 min followed by a linear gradient from 30 to 60% A over 4 min; effluent fractions were monitored at $\lambda = 220$ nm. The products were identified by comparison with L-phenylalanine and phenylpyruvate.

Proton NMR measurements

9His-*rsLAAO1* was dialyzed against 80 mM sodium phosphate buffer pH 8.0 in D_2O . Twenty millimolars of L-leucine, 0.8 U SDS-activated 9His-*rsLAAO1* (260 μg), and 200 U catalase were incubated in 80 mM sodium phosphate buffer pH 8.0 (in D_2O , final volume 4 mL) at 30 °C. After several time points, samples were boiled (5 min 95 °C) to stop the reaction and centrifuged, and the supernatants were analyzed. NMR measurements were conducted on a Bruker Avance 600 spectrometer (^1H : 600.13 MHz) at 25 °C. Chemical shifts of resonances in spectra were referenced to residual solvent peaks (H_2O , $\delta = 4.79$ ppm). The identities of components responsible for the resonances present in the spectra were assigned by comparison with ^1H spectra of authentic standards.

Results

The complete genome sequences of many fungi have been determined in recent years revealing dozens of candidates for L-amino acid oxidases (L-AAOs). A Blast search with the protein sequence of L-AAO1 from *H. cylindrosporium* revealed a putative protein from *R. solani* AG-3 Rhs1AP (GenBank accession number EUC54418.1; Cubeta et al. 2014). The alignment showed that 33% of the amino acid residues were identical including residues predicted to be important for the interaction with FAD and substrates. This *rsLAAO1* does not contain a signal sequence according to the prediction programs PSORT II (Nakai and Horton 1999) and SignalP 4.1 (Petersen et al.

2011). *Rhizoctonia* belongs to the same class *Agaricomycetes* in the division of *Basidiomycota* as *Hebeloma*. A synthetic gene encoding this sequence optimized for expression in *Pichia pastoris* (sequence in GenBank under accession number KX925447) was cloned into an *E. coli* expression plasmid to produce fusion proteins with an N-terminal 6His-tag. Expression was tested at 16 and 24 °C. Soluble proteins in the cell lysate (Fig. 1a, S) and pellets (P) were separated by SDS-PAGE. 6His-*rsLAAO1* was barely detected in the soluble fraction even though large amounts were found in the pellet after expression at 24 °C. Maltose-binding protein (MBP) has been used to increase the solubility of fusion proteins (Kapust and Waugh 1999), but may interfere with activity due to its size of 40 kDa. Almost half of the MBP-*rsLAAO1* was detected in the soluble fraction (Fig. 1b) under the same expression conditions. These data indicate that the MBP-tag increased the solubility compared to a 6His-tag. Expression conditions were further tested with different concentrations of IPTG for different amounts of time. Soluble fractions were analyzed by western blotting with antiserum against the MBP-tag. A fusion protein band was detected, which had the expected mobility slightly above 100 kDa (Fig. 1c). Maximal amounts of soluble MBP-*rsLAAO1* were obtained at 24 °C. However, the L-AAO activity per milligram of MBP-*rsLAAO1* protein was about 3-fold higher in cleared cell lysates after expression at 16 °C compared to lysates after expression at 24 °C (Fig. 1d) indicating that part of the soluble protein was inactive at 24 °C.

As lower expression temperatures improved yields of active protein, we tested an expression system, which is optimized for expression at even lower temperatures. The *E. coli* strain Arctic Express produces two cold-adapted chaperonins allowing for protein expression at 4–12 °C (Ferrer et al. 2004). Then, conducting such an expression, less fusion protein was lost in the pellet after induction for 18 h at 11 °C (Fig. 1b). The soluble fraction (Fig. 2, S) was loaded onto amylose resin. After washing (W), the fusion protein was eluted by 10 mM maltose (E1–E7) and high amounts of soluble, active MBP-*rsLAAO1* were obtained.

The activity of MBP-*rsLAAO1* was assayed with different amino acids and derivatives (10 mM) to determine the substrate spectrum. The activities relative to L-arginine were calculated (Table 1). The best substrates were on the one hand the basic amino acids L-arginine, L-lysine, and L-ornithine and on the other hand the large hydrophobic amino acids L-phenylalanine, L-leucine, norleucine, and L-methionine. Polar and acidic amino acids were not converted. Interestingly, methyl esters derived from α -amino acids were also substrates as shown for L-phenylalanine methyl ester, L-leucine methyl ester, and L-methionine methyl ester even though with lower relative activity compared to the corresponding free α -amino acids. Next, different derivatives of L-phenylalanine were tested in a systematic approach (Fig. 3a). D-Phenylalanine was not converted, demonstrating the influence of the absolute

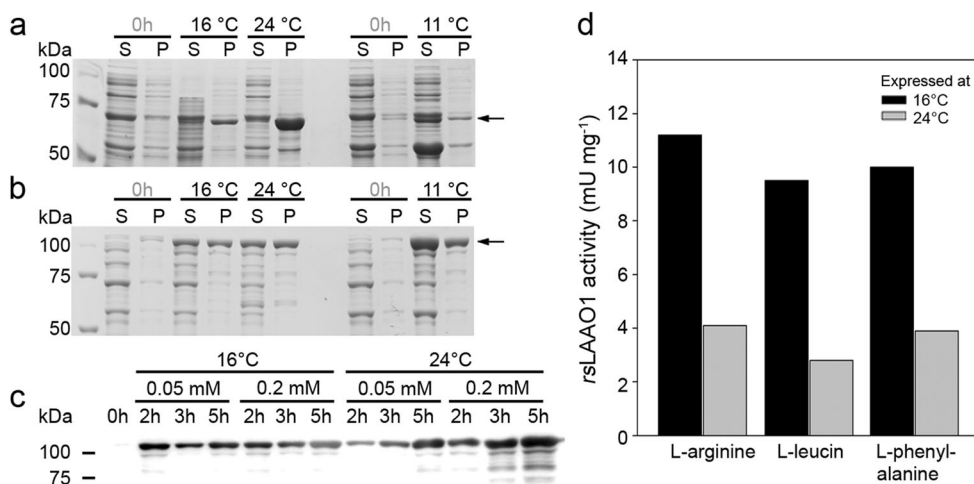


Fig. 1 Expression of an active recombinant *Rhizoctonia solani* L-amino acid oxidase fusion protein in *E. coli*. **a** 6His-*rsLAAO1* was expressed in the *E. coli* strain BL21-CodonPlus (DE3)-RP at 16 °C (left) or 24 °C (middle) for 3 h with 0.2 mM IPTG or in the *E. coli* strain Arctic Express at 11 °C (right) for 11 h with 0.05 mM IPTG. Control cells were harvested before induction (0 h). Cells were lysed by sonification and separated into soluble fractions (S) and pellets (P) by centrifugation. Fractions were analyzed by SDS PAGE. 6His-*rsLAAO1* (arrow) was detected in the pellet after expression at 16 and 24 °C while low amounts were expressed at 11 °C. **b** MBP-*rsLAAO1* was expressed and analyzed as above. MBP-*rsLAAO1* (arrow) was found in the pellets and soluble fractions at 16 and 24 °C. MBP-*rsLAAO1* was predominantly soluble after expression at 11 °C. **c** MBP-*rsLAAO1* was expressed in the *E. coli*

strain BL21-CodonPlus (DE3)-RP at 16 °C (left) or 24 °C (right) for 2, 3, or 5 h after induction with 0.05 or 0.2 mM IPTG. Control cells were harvested before induction (0 h). Soluble fractions were analyzed by SDS-PAGE. The band above 100 kDa was identified as MBP-*rsLAAO1* by immunoblotting with antiserum directed against the MBP-tag. **d** The specific activity of MBP-*rsLAAO1* is higher after expression at 16 °C (black) than at 24 °C (gray). Soluble fractions of cell lysates after induction with 0.2 mM IPTG for 3 h were analyzed for LAAO activity using a coupled peroxidase Ampliflu Red assay with 10 mM L-arginine, L-leucine, or L-phenylalanine as substrates. The amounts of MBP-*rsLAAO1* (0.95 mg mL^{-1} at 16 °C and 3.5 mg mL^{-1} at 24 °C) were estimated in Coomassie-stained gels by comparison to serial dilutions of bovine serum albumin

configuration. L-Tyrosine as a polar derivative was converted very slowly even taking into account the low substrate concentration (about 2 mM due to limited solubility). L-Phenylglycine did not serve as substrate, indicating that the lack of the β -CH₂ group is unfavorable. The presence of the α -methyl group in L- α -methyl phenylalanine prevented conversion as well. N-methyl-L-phenylalanine was not converted, demonstrating the detrimental effect of a methylation at the

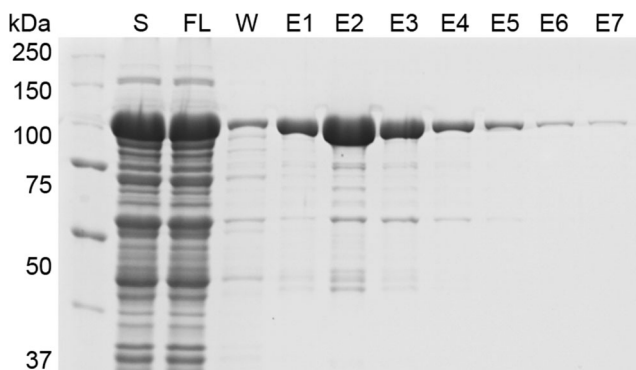


Fig. 2 Purification of recombinant MBP-*rsLAAO1*. MBP-*rsLAAO1* was expressed in the *E. coli* strain Arctic Express at 11 °C. Cells were lysed, debris and insoluble proteins removed by centrifugation, and the soluble fractions (S) loaded onto an amylose resin. After collection of the flow through (FL) and wash fractions (W), proteins were eluted using 10 mM maltose (E1–E7). Fractions were separated by SDS PAGE and stained with Coomassie

amino group. A β -amino acid did not serve as substrate as shown for L- β -phenylalanine ((S)-3-amino-3-phenylpropionic acid). The amine 2-phenylethylamine was not converted demonstrating the importance of the carboxyl group. Isomers of L-leucine and L-valine were compared to evaluate the influence of branched alkyl chains (Fig. 3b). The linear isomer D/L-norleucine was assayed at 5 mM of L-norleucine because of its limited solubility. At this concentration, norleucine was converted with a comparable rate to 5 mM of L-leucine (data not shown) but slower than 10 mM of L-leucine (Fig. 3b). L-Isoleucine bearing a branched β -position was a very poor substrate. In addition, L-valine and L-*tert*-leucine bearing three CH₃ groups at the β -position were not converted.

The pH dependence of MBP-*rsLAAO1* activity was analyzed between pH 5 and pH 11 for L-arginine (Fig. 4a), L-leucine (Fig. 4b), and L-phenylalanine (data not shown). Citrate buffers, TEA buffers, and glycine buffers were prepared to cover this pH range. The enzyme was more active in TEA buffer (circles) than in citrate buffer (squares) at pH 7.0 and glycine buffer (triangles) at pH 8.5 for all substrates. In addition, MBP-*rsLAAO1* showed a broad pH optimum between pH 7.0 and 9.5.

Since the MBP-tag may influence the enzymatic properties of *rsLAAO1*, an expression plasmid was generated that codes for an N-terminal MBP followed by a recognition sequence for PreScission protease, a 9His-tag, and *rsLAAO1* (MBP-P-

Table 1 Substrate spectrum of MBP-*rsLAAO1*

	L-Amino acids	Relative activity (%)
Hydrophobic amino acids	L-Alanine	0
	L-Leucine	70
	L-Valine	0
	L-Isoleucine	3.1
	D/L-Norleucine ^a	53
	L-Phenylalanine	88
	L-Tryptophane	6.4
	D-Phenylalanine	0
	L-Phenylglycine	0
	(<i>S</i>)- β -Phenylalanine	0
	L-Proline	0
	<i>L-tert</i> -Leucine	0
	L-Methionine	23
Polar amino acids	L-Serine	0
	L-Cysteine	0
	L-Threonine	0
	L-Glutamine	1.6
	L-Asparagine	1
	L-Tyrosine ^b	4
Basic amino acids	L-Arginine	100
	L-Lysine	55
	L-Ornithine	60
	L-Canavanine	0
	L-Histidine	5.2
Acidic amino acids	L-Aspartic acid	0
	L-Glutamic acid	0
Amino acid derivatives	L-Leucine methyl ester	61
	L-Phenylalanine methyl ester	67
	<i>N</i> -Methyl-L-Phenylalanine	0
	L-(α)-Methylphenylalanine	0
	<i>N</i> -Acetyl-L-Phenylalanine methyl ester	0
	2-Phenylethylamine	0
	<i>N</i> - α -Acetyl-L-Arginine	0
	L-Methionine methyl ester	12

The standard substrate concentration was 10 mM amino acid in the assay

^a In the case of racemic D/L-amino acids, the concentration of L-amino acids was 5 mM in the assay mixture

^b A saturated solution was used for amino acids with low solubility

9His-*rsLAAO1*). Treatment of the purified recombinant protein with PreScission protease removes the MBP-tag. The 9His-tag can be used for an additional affinity purification step and for detection by antibodies. Large amounts of soluble MBP-P-9His-*rsLAAO1* with a mobility of slightly above 100 kDa (Fig. 5a, S) were obtained while a considerable fraction was detected in the pellet (P) after expression in the *E. coli* strain Arctic Express at 11 °C. The soluble fraction (S) was loaded onto amylose resin. After washing (W1), the fusion protein was eluted by 10 mM maltose (E1). Incubation of the eluate with PreScission protease resulted in the appearance

of a 65-kDa band consistent with 9His-*rsLAAO1* and a 40-kDa band corresponding to the size of MBP (C) indicating that the MBP-tag was removed. This mixture was loaded onto Ni-NTA resin, and a 65-kDa band was eluted by 500 mM imidazole (E2). Western blotting with antibodies directed against the MBP- and 9His-tag confirmed the identity of these bands (data not shown). Accordingly, purified 9His-*rsLAAO1* was obtained successfully.

L-Amino acid oxidases require a FAD cofactor, which is bound tightly but not covalently. UV-Vis spectroscopy was used to analyze the recombinant purified 9His-*rsLAAO1*

Fig. 3 Activity of MBP-*rsLAAO1* towards substrates related to L-phenylalanine (a) and L-leucine (b). MBP-*rsLAAO1* converted methyl esters of L-phenylalanine and L-leucine with a reduced rate. L-Tyrosine was a poor substrate. D-phenylalanine, L-phenylglycine, N-methyl-L-phenylalanine, and β -phenylalanine were not converted. The linear L-norleucine served as good and L-isoleucine as very poor substrates while L-valine and L-*tert*-leucine did not react. A coupled peroxidase *o*-dianisidine assay was used with 10 mM substrate concentration except for L-norleucine (5 mM) and L-tyrosine (below 2 mM)

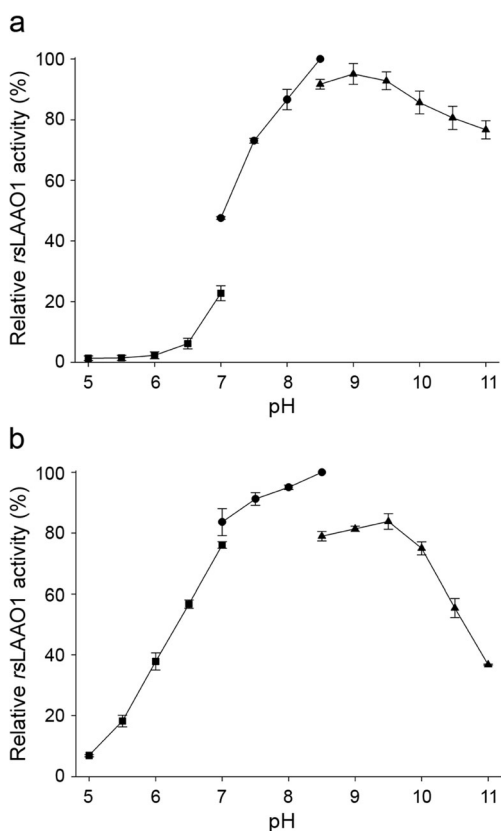
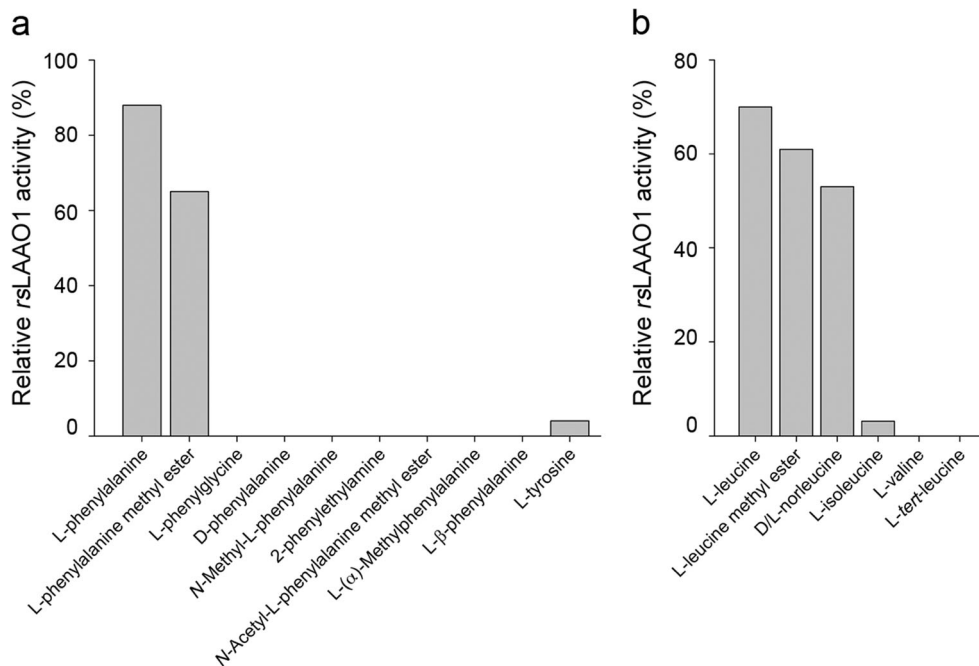


Fig. 4 pH optimum of MBP-*rsLAAO1* was between pH 8.5 and 9.5 for L-arginine (a) and L-leucine (b). The enzymatic activity was assayed with peroxidase and *o*-dianisidine in citrate buffers (squares), TEA buffers (circles), and glycine buffers (triangles) of the indicated pH. Data are mean of three independent experiments; error bars are standard deviations

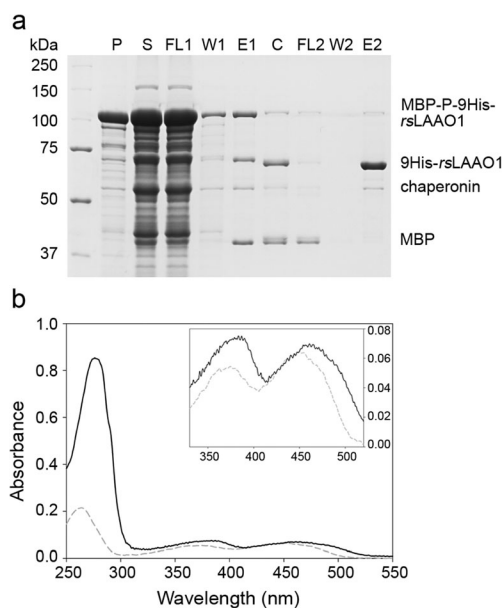


Fig. 5 Purification of MBP-P-9His-*rsLAAO1*, digestion to 9His-*rsLAAO1*, and determination of FAD content. **a** A fusion protein consisting of maltose-binding protein (MBP), a consensus sequence for the PreScission protease (–P–), a 9His-tag, and *rsLAAO1* was expressed in the *E. coli* strain Arctic Express. Cells were lysed, a pellet (P) removed by centrifugation, and the soluble fractions (S) loaded onto an amylose resin. After collection of the flow through (F1) and wash fractions (W1), proteins were eluted using 10 mM maltose (E1). The fusion protein was incubated with PreScission protease to remove MBP (C), loaded onto Ni-NTA resin (flow through: F2), and washed (W2), and 9His-*rsLAAO1* eluted with 500 mM imidazole (E2). Fractions were separated by SDS-PAGE and stained with Coomassie. **b** The UV-Vis spectrum of purified 9His-*rsLAAO1* (solid line) showed an absorption spectrum between 350 and 500 nm that is typical for flavoproteins and differs from free FAD (dashed line). Quantification revealed an occupancy of 9His-*rsLAAO1* with FAD of 70–100% in different preparations

because FAD in proteins has a characteristic absorption spectrum between 300 and 500 nm (Fig. 5b, solid line). The cofactor occupancy was determined from the spectrum using the extinction coefficients for FAD at 280 and 450 nm and the calculated extinction coefficient at 280 nm for the apoprotein. As a result, between 70 and 100% of the subunits contained a molecule of the cofactor FAD in different preparations of 9His-*rsLAAO1* as well as MBP-*rsLAAO1*.

It has been reported that SDS treatment is required to activate recombinant *H. cylindrosporium* LAAO1 expressed in *E. coli* (Nuutinen et al. 2012). Therefore, we tested whether low amounts of SDS influenced the activity of 9His-*rsLAAO1* and MBP-P-9His-*rsLAAO1*. Soluble proteins from cell extracts (Fig. 6a, S), MBP-P-9His-*rsLAAO1* eluted from an amylose column (E1), and 9His-*rsLAAO1* eluted from Ni-NTA agarose (E2) were either left untreated or were incubated with 0.025% SDS before separation by native PAGE. Gels were assayed for L-arginine-dependent production of H₂O₂ with peroxidase and *o*-dianisidine. LAAO1 activity was low in the absence of SDS (Fig. 6a, left). By contrast, strong activity was observed after an incubation with 0.025% SDS (Fig. 6a, right). To quantify this effect, MBP-

rsLAAO1 and 9His-*rsLAAO1* were assayed spectrophotometrically after preincubation with different amounts of SDS (Fig. 6b). Enzymatic activity was 0.05–0.15 U mg⁻¹ in the absence of SDS. It was dramatically increased to 5–10 U mg⁻¹ in the presence of 1–2 mM SDS at an optimal weight ratio of three parts of SDS to one part of protein. MBP-*rsLAAO1* and 9His-*rsLAAO1* had similar specific activities and were stimulated to a similar extent by comparable SDS concentrations. These data indicate that the MBP-tag did not influence enzymatic activities.

The effect of SDS treatment was quantified further. A 1-L culture of *E. coli* Arctic Express yielded 236 mg of protein in the soluble fraction of cell lysates with a total activity of 54 U (specific activity 0.2 U mg⁻¹ with L-arginine) upon expression of MBP-P-9His-*rsLAAO1*. After two affinity chromatography steps, 4.4 mg 9His-*rsLAAO1* with a specific activity of 9 U mg⁻¹ was obtained (total activity 40 U). To determine the kinetic properties of 9His-*rsLAAO1*, initial velocities were determined for different substrate concentrations after SDS activation. *K_m* and *v_{max}* (Table 2) were calculated from Hanes-Woolf plots. L-Arginine had a *K_m* of 0.9 mM and *v_{max}* of 8.3 U mg⁻¹. The presence of a methyl ester moiety increased *K_m* strongly (2.3 mM for L-phenylalanine, 13.9 mM for L-phenylalanine methyl ester, 2.4 mM for L-leucine, and 6.3 mM L-leucine methyl ester) while *v_{max}* values were in a comparable range. These values were compared to enzymatic activities of MBP-*rsLAAO1* without SDS activation (Table 2). For L-arginine, *v_{max}* was only 0.1 U mg⁻¹ without SDS but 8.3 U mg⁻¹ with SDS. Similar differences in *v_{max}* of 50–100-fold were obtained for all substrates tested. In contrast, the effect of SDS activation on the *K_m* values was much smaller. Substrate spectra were very similar for untreated MBP-*rsLAAO1* (Table 1) and SDS-activated 9His-*rsLAAO1* (data not shown).

In addition, we applied the expressed 9His-*rsLAAO1* in initial biotransformations, which also enables us to obtain a direct proof for the formation of α -keto acids by two different methods. A procedure was developed to separate L-phenylalanine (retention time 3 min) from phenylpyruvate (retention time 5.7 min) by reversed-phase HPLC (data not shown). SDS-activated 9His-*rsLAAO1* was incubated with L-phenylalanine for 0, 15, 30, or 60 min in the presence of catalase to avoid oxidation of phenylpyruvate by H₂O₂. Reaction mixtures were boiled to stop the reaction and separated by reversed-phase HPLC. A peak with a retention time of 5.7 min was absent at the time point 0 min, but appeared after a 15-min conversion time and increased further after 30 and 60 min (Fig. 7a). Side products were not detected. These data indicate that L-phenylalanine was converted to phenylpyruvate with high selectivity.

¹H NMR was used as an alternative analytical method, which can distinguish L-leucine from α -keto leucine and allows for a quantification of these components. The protons at

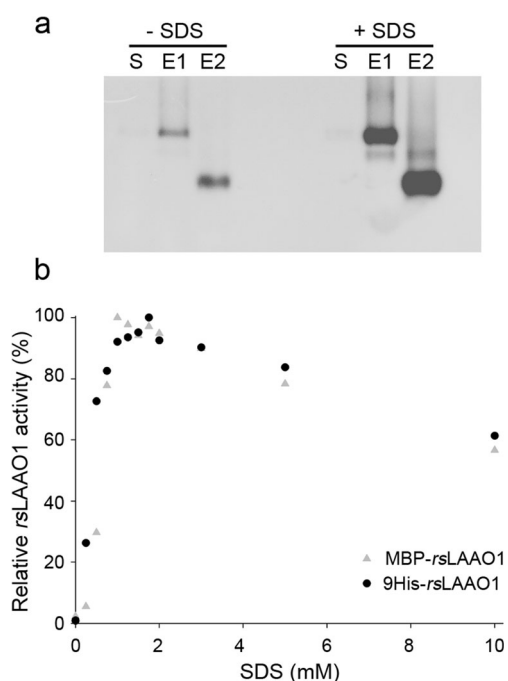


Fig. 6 Low amounts of SDS increased the activity of MBP-*rsLAAO1* and 9His-*rsLAAO1* by a factor of 20. **a** Soluble proteins from cell extracts (S), MBP-P-9His-*rsLAAO1* eluted from an amylose column (E1), and 9His-*rsLAAO1* eluted from Ni-NTA agarose (E2) were incubated without (left) or with (right) 0.025% SDS, separated by native PAGE, and assayed for L-AAO activity with arginine as substrate. **b** MBP-*rsLAAO1* (gray triangles) and 9His-*rsLAAO1* (black circles) were incubated at a protein concentration of 0.1 mg mL⁻¹ with SDS at the indicated concentration for 5 min. L-AAO activity was measured using a coupled peroxidase *o*-dianisidine assay

Table 2 Kinetic properties of native MBP-*rsLAAO1* (top) and SDS-activated 9His-*rsLAAO1* (bottom)

Substrate	ν_{\max} (U·mg ⁻¹)	K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m
L-Arginine	0.10 ± 0.02	0.4 ± 0.01	0.13	0.30
L-Phenylalanine	0.13 ± 0.02	6.8 ± 0.3	0.16	0.02
L-Leucine	0.10 ± 0.02	6.1 ± 0.1	0.13	0.02
L-Phenylalanine methyl ester	0.13 ± 0.01	11.7 ± 0.2	0.16	0.01
L-Lysine	0.07 ± 0.01	1.3 ± 0.1	0.09	0.07
L-Ornithine	0.08 ± 0.01	5.6 ± 0.2	0.10	0.02
L-Methionine	0.06 ± 0.01	20.0 ± 1.1	0.08	0.004
Substrate	ν_{\max} (U·mg ⁻¹)	K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m
L-Arginine	8.3 ± 0.2	0.9 ± 0.04	10.4	11.4
L-Phenylalanine	9.7 ± 0.4	2.3 ± 0.1	12.2	5.3
L-Leucine	8.6 ± 0.1	2.4 ± 0.3	10.8	4.6
L-Phenylalanine methyl ester	8.1 ± 0.2	13.9 ± 0.6	10.2	0.7
L-Lysine	3.6 ± 0.1	2.3 ± 0.2	4.6	2.0
L-Ornithine	6.0 ± 0.5	12.4 ± 0.2	7.5	0.6
L-Methionine	5.7 ± 0.2	5.4 ± 0.3	7.2	1.3
L-Leucine methyl ester	9.5 ± 0.4	6.3 ± 0.2	11.8	1.9

K_m and ν_{\max} were calculated from Hanes-Woolf plots. Data are means of three independent experiments with standard deviations

the β -carbon and γ -carbon are found at a chemical shift of 2.6 and 2.1 ppm, respectively, in α -keto leucine (Fig. 7b). By contrast, a multiplet between 1.6 and 1.7 ppm is observed for these protons in L-leucine. The proton attached to the α -carbon of L-leucine at 3.7 ppm is removed during the enzymatic reaction. The ¹H NMR signal of the protons of both -CH₃ groups remains unchanged at 0.9 ppm and can be used as reference for quantification. As water produces a strong ¹H NMR signal, all buffers were prepared in D₂O and the purified 9His-*rsLAAO1* was dialyzed against a buffer in D₂O and activated by SDS. The reaction was stopped after 0, 30, 60, or 120 min and the spectra analyzed (Fig. 7b). Peaks at a chemical shift of 2.6 and 2.1 ppm characteristic for α -keto leucine appeared and increased with time while peaks for L-leucine vanished. Accordingly, 95% of the L-leucine was converted into α -keto leucine within 120 min.

Discussion

Here, we described the successful heterologous expression of a fusion protein with *R. solani* L-AAO1 in *E. coli*. A key factor was the usage of an MBP-fusion protein as solubility tag (Kapust and Waugh 1999). Sufficient amounts of soluble protein were detected upon expression of MBP-*rsLAAO1* and MBP-P-9His-*rsLAAO1*. MBP as solubility tag was required only during biosynthesis in *E. coli*. Once being correctly folded, the purified 9His-*rsLAAO1* was stable in solution even after proteolytic removal of the MBP-tag. The second key to success was lowering the expression temperature to

11 °C. A higher proportion of the soluble MBP-*rsLAAO1* was inactive after expression at 24 °C compared to 16 and 11 °C. As lower temperatures reduce the rate of protein synthesis, more time is available for correct folding, incorporation of the cofactor FAD, and homodimerisation. As a result, between 70 and 100% of the subunits contained FAD after purification. However, a larger fraction of MBP-*rsLAAO1* and MBP-P-9His-*rsLAAO1* did not bind to the amylose column and was found in the flow through (Figs. 2 and 5, FL) even though the column had high binding capacity. In addition, the MBP-P-9His-*rsLAAO1* activity in the soluble fraction was much lower than expected from the protein amount in the native gel (Fig. 6a). These data indicate that even after expression at 11 °C, the soluble MBP-P-9His-*rsLAAO1* was partially misfolded and inactive. The situation was worse for an MBP-fusion protein with a putative L-AAO from the fungus *Fibroporia radiculosa* (GenBank accession number CL98847; Tang et al. 2012), which shares 35% of the amino acid residues with LAAO1 from *Hebeloma cylindrosporum* and 30% of the amino acid residues with *rsLAAO1*. This fusion protein was partially soluble after expression at 11 °C, but did not bind to the amylose column and no L-AAO activity could be detected (data not shown). Problems in folding could be due to the lack of posttranslational modifications. However, both proteins do not contain endoplasmic reticulum-import signals and are most likely cytosolic according to prediction programs. Therefore, they should not be glycosylated. We obtained a specific L-AAO activity of 0.2 U mg⁻¹ after SDS activation in *E. coli* crude lysates, which is much higher than 0.029 U mg⁻¹ measured after

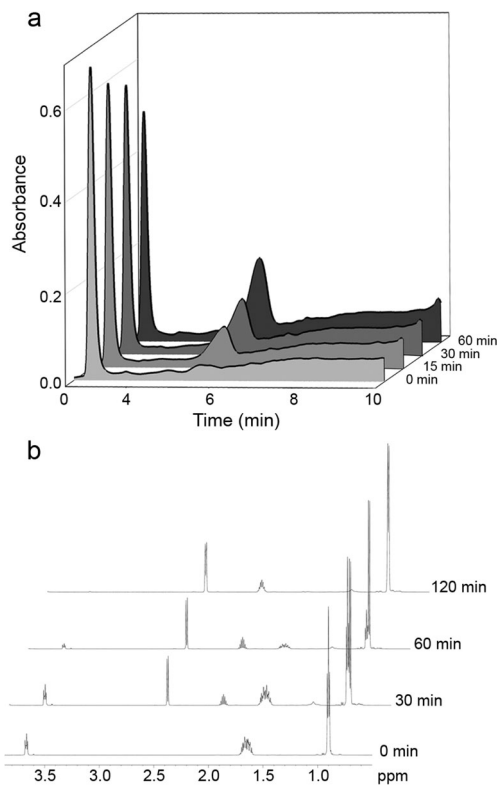


Fig. 7 Detection of α -keto acids produced by 9His-*rsLAAO1* by HPLC analysis (**a**) and ^1H NMR spectroscopy (**b**). **a** The reaction mixture with 5 mM of L-phenylalanine, SDS-activated 9His-*rsLAAO1*, and catalase was incubated for 0, 15, 30, and 60 min at 30 °C. After boiling, the reaction mixtures were analyzed by HPLC on a reversed-phase C18 column. L-Phenylalanine (retention time 3 min) was converted with high selectivity to phenylpyruvate (retention time 5.7 min) without the formation of any side products. **b** The reaction mixtures containing 20 mM L-leucine, SDS-activated 9His-*rsLAAO1*, and catalase in D_2O were incubated for the indicated time periods at 30 °C and boiled. The supernatants were investigated by proton NMR at room temperature. α -Keto leucine (4-methyl-2-oxovaleric acid) was obtained in high purity without any side products. Characteristic peaks for leucine are at 3.7 ppm (hydrogen at α -carbon) and at 1.6 and 1.7 ppm (hydrogens at β - and γ -carbon). In α -keto leucine, the peak at 2.5 ppm is due to hydrogens at β -carbon and at 2.1 ppm caused by the hydrogen at the γ -carbon

heterologous expression of *R. opacus* L-AAO in crude extracts from *S. lividans* (Geueke and Hummel 2003). However, even higher activities are desirable for biocatalytic applications.

Purified MBP-*rsLAAO1* and 9His-*rsLAAO1* had very low specific activities of 0.06–0.13 U mg^{-1} , which could be increased 50–100-fold by brief incubation with 1–2 mM SDS to specific activities of 3–10 U mg^{-1} depending on the substrate. A similar effect of SDS was detected after the heterologous expression of *H. cylindrosporium* LAAO1 in *E. coli* (Nuutinen et al. 2012): no activity was observed without SDS, while specific L-AAO activities of 6–14 U mg^{-1} were measured after SDS treatment. However, L-AAO activity was detected in crude mycelial extracts (Nuutinen and Timonen 2008) indicating that the endogenous protein is produced in an active

form. On the other hand, L-AAO folding during biosynthesis in *E. coli* may differ slightly from folding in the fungal cells. SDS binds to hydrophobic regions of proteins and can change the structure of proteins. In most cases, this results in inactivation and finally denaturation of proteins. Interestingly, SDS treatment affected the K_m values of MBP-*rsLAAO1* and 9His-*rsLAAO1* much less than v_{max} . These data suggest that SDS does not influence the binding of the substrate in the active site. There have also been reports that SDS can activate some endogenous enzymes. Tyrosinases and polyphenol oxidases can be synthesized as latent, inactive enzymes in plants but also in the frog *Xenopus laevis*. Different treatments including incubation with SDS induce conformational changes, which activate these enzymes and induce enzymatic browning of fruits and vegetables (Espin and Wichers 1999; Moore and Flurkey 1990; Wittenberg and Triplett 1985).

Basic and large hydrophobic α -amino acids are the best substrates for MBP-*rsLAAO1* and 9His-*rsLAAO1*. Polar or acidic amino acids as well as amino acids with substituents in the β -position are converted poorly or not at all. This substrate spectrum is narrower and differs greatly from that of *H. cylindrosporium* LAAO1 (Nuutinen et al. 2012), which shares 33% of the amino acid residues with *rsLAAO1*. The best substrate for *H. cylindrosporium* LAAO1 is L-glutamate followed by L-glutamine. Basic, polar, and hydrophobic amino acids are accepted as well with good activities by *hcLAAO1*. Interestingly, MBP-*rsLAAO1* and 9His-*rsLAAO1* also convert methyl esters of L-phenylalanine and L-leucine with v_{max} values similar to that of the corresponding free α -amino acids. However, K_m values were increased by a factor of 6 and 3, respectively, by the presence of methyl esters. These lower binding affinities indicate that the carboxyl group contributes to binding of the substrate. *Hebeloma cylindrosporium* LAAO1 also converts L-phenylalanine and L-phenylalanine methyl ester with similar relative activities (Nuutinen et al. 2012). However, neither a L-AAO from the fungus *Neurospora crassa* (division *Ascomycota*, Thayer and Horowitz 1951) nor L-AAO from the bacterium *R. opacus* (Geueke and Hummel 2002) is able to react with methyl esters of amino acids. The L-AAO isolated from king cobra venom could also convert L-leucine methyl ester, but the K_m was 3.2 mM compared to 0.2 mM for L-leucine (Tan and Saifuddin 1991). An extremely low activity towards methyl ester was observed for the L-AAO from the venom of the rattle snake *Crotalus adamantus* (0.006 U mg^{-1} for $N\epsilon$ -CBZ-L-lysine methyl ester compared to 0.405 U mg^{-1} for $N\epsilon$ -CBZ-L-lysine) (Hanson et al. 1992). The structures of *R. opacus* L-AAO and snake venom L-AAO1 from *Calloselasma rhodostoma* revealed that the amino acid substrate is bound in a similar way in the active site. In both cases, the carboxyl group of the substrate interacts with a guanidinium group of an arginine residue, the hydroxyl group of a tyrosine residue, and a water molecule coordinated by a lysine residue of the L-

AAO (Faust et al. 2007; Moustafa et al. 2006). These three amino acid residues are conserved in *R. solani* L-AAO1. Differences in the conversion of the amino acid methyl esters must be due to minor differences in the active site. The ability to accept methyl esters of amino acids is a very interesting property for biocatalytic applications because α -keto acids are less stable than their corresponding esters.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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