INTRODUCTION

l-amino acid oxidases (LAAOs) are mostly homodimers and contain a non-covalently bound FAD cofactor. These enzymes catalyze the oxidative deamination of l-amino acids to corresponding α-keto acids, ammonia (NH₄⁺), and hydrogen peroxide (H₂O₂) (Pollegioni et al., 2013; Yu & Qiao, 2012). LAAOs are found in a wide range of species such as bacteria (Geueke & Hummel, 2002), fungi (Nuutinen & Timonen, 2008), mollusks (Yang et al., 2005), algae (Piedras et al., 1992), fish (Kasai et al., 2010), mammals (Sun et al., 2002) and in venoms of snakes (Bender & Krebs, 1950) or insects (Ahn et al., 2000). LAAOs from snake venoms (svLAAOs) are the best known and best-characterized enzymes, due to the simple purification from venoms (Izidoro et al., 2014). LAAOs contribute to the toxicity of venoms and also show antibacterial, antiviral, antimicrobial, or antitumoral activities in various organisms (Izidoro et al., 2014; Yu & Qiao, 2012). Due to the formation of hydrogen peroxide, they also are used as a defense mechanism (Derby et al., 2018).

LAAOs are of interest for biotechnological applications such as the production of α-keto acids from l-amino acids or d-amino acids from...
Abbreviations: A, Ascomycota; B, Basidiomycota; n.d., not determined; SN, culture supernatant.

To explore more divergent enzymes, a predicted LAAO from the Ascomycota Colletotrichum gloeosporioides (35% amino acid identity) was selected. The purification of a highly active LAAO from the Ascomycota Neurospora crassa and its cloning was reported (Niedermann & Lerch, 1990) but it has not been expressed heterologously. ncLAAO1 is very different from hcLAAO4 (23% amino acid identity). In addition, frLAAO1 was expressed as an enzyme without an ER-signal sequence from the Basidiomycota Fibroporia radiculosa. The synthetic genes lack the information for ER-signal sequences and were codon-optimized for expression in P. pastoris. Initially, these putative LAAOs were expressed in E. coli as 6His- or maltose-binding protein fusion proteins. However, the fusion proteins were either completely insoluble or the low amounts of soluble MBP-fusion proteins were inactive as in the case of MBP-cgLAAO1 (Figure A1). The genes coding for these LAAOs were cloned into the P. pastoris expression vector pPIC9K, which encodes the prepro-sequence of the α-mating factor of S. cerevisiae as an N-terminal ER import signal sequence. Linearized plasmids were integrated into the HIS4 locus of P. pastoris SMD1163. Clones were selected according to (Scorer et al., 1994). Expression was induced in a medium at pH 6.0 with 1% methanol at 15°C and samples were taken every 24 h for 96 h (Figure 1).

Two LAAOs, 9His-nclAAO1 (nc: Neurospora crassa, Figure 1a) and 9His-icLAAO2 (ia: Laccaria amethystina, Figure 1d), could be detected in western blots of supernatants after an expression for 48 h. 9His-frLAAO1 (fr: Fibroporia radiculosa) and 9His-cgLAAO1 (cg: Colletotrichum gloeosporioides) were visible after 72 h (Figure 1b,c). 9His-frLAAO1 was not further characterized due to low expression levels. Only 0.8 U of purified 9His-icLAAO2 were obtained per 1 liter of culture due to poor binding to the column material. The substrate spectrum of 9His-icLAAO2 was similar to that of 9His-hcLAAO4. 9His-cgLAAO1 was expressed at 360 U L⁻¹, but only 34 U L⁻¹ of the purified enzyme were obtained due to poor binding to the column

### RESULTS

#### Expression of fungal LAAOs in P. pastoris

Recently, we were able to express 9His-rsLAAO1 and 9His-hcLAAO4 as active enzymes (Hahn, Neumeister, et al., 2017, Heß et al., 2020). They do not convert all proteinogenic l-amino acids even though both enzymes have a fairly broad substrate spectrum. Databases were searched for fungal sequences encoding predicted LAAOs. We focused on the small number of LAAOs with predicted ER-signal sequences with the rationale that a naturally secreted protein may offer a higher chance of active expression as a secreted protein in P. pastoris (Table 1). A LAAO from Laccaria amethystina (LAAO2) was selected as a more closely related enzyme (52% amino acid identity with hLAAO4).

#### TABLE 1 Overview of expressible fungal L-amino acid oxidases

| Original organism | Division | ER-signal sequence | Put. N-sites | Glycosylated | Predicted MW (kDa) | % sequence identity | Yield (U L⁻¹) |
|-------------------|----------|--------------------|--------------|--------------|--------------------|-------------------|--------------|
| H. cylindrosporum  | LAAO4    | B                  | +            | 4            | +                  | 68.6              | 100          |
| H. cylindrosporum  | LAAO2    | B                  | +            | 6            | +                  | 70.9              | 52           |
| C. gloeosporioides | LAAO1    | A                  | +            | 10           | +                  | 67.9              | 35           |
| N. crassa         | LAAO1    | A                  | +            | 10           | +                  | 64.2              | 23           |

Abbreviations: A, Ascomycota; B, Basidiomycota; n.d., not determined; SN, culture supernatant.

*Heß et al., 2020.*
material. 9His-cgLAAO1 had a broad substrate spectrum (Table A2) including l-tryptophan, which is a poor substrate for 9His-hcLAAO4. $K_m$ values were around 1 mM and $v_{max}$ values around 10 U mg$^{-1}$ for the best substrates l-glutamine, l-leucine, and l-methionine (Table A3). 9 U L$^{-1}$ of 9His-nclLAAO1 could be obtained from culture supernatants with 30 U L$^{-1}$. The very broad substrate spectrum of 9His-nclLAAO1 includes amino acids not or poorly converted by other recombinant fungal LAAOs such as l-valine, l-serine, l-threonine, l-aspartic acid, and l-phenylglycine at a substrate concentration of 10 mM (Table A4). N-$\alpha$-acetyl-l-lysine was a very good substrate indicating that the enzyme could also oxidase the $\varepsilon$-amino group. The ability to oxidize N-$\alpha$-acetyl-l-lysine in addition to N-$\varepsilon$-acetyl-l-lysine and many l-amino acids has also been observed for a LAAO isolated from Rhodococcus sp. AIU Z-35-1 (Isobe & Nagasawa, 2007). $K_m$ values were below or at 10 $\mu$M and $v_{max}$ values around 1 U mg$^{-1}$ for the best substrates l-arginine, l-phenylalanine, and l-leucine (Table A5). However, activities were lower at a substrate concentration of 10 mM than at 0.1 mM for these and good substrates such as l-tryptophan and l-histidine (Table A4). This inhibition was much stronger for l-arginine (specific activity 0.13 U mg$^{-1}$) than for l-phenylalanine (0.53 U mg$^{-1}$, Figure A2a). Substrates with a high $K_m$ value such as l-isoleucine were not affected (Figure A2a). As 9His-hcLAAO4 by exposure to acidic pH (Heß et al., 2020) we determined the influence of these two treatments and pH 9.0 on 9His-cgLAAO1 and 9His-nclLAAO1. The activity of 9His-nclLAAO1 increased slightly after these treatments (Figure A3a) while 9His-cgLAAO1 was not influenced (Figure A3b).

2.2 | Proof of N-glycosylation for fungal LAAOs and analysis of activity after deglycosylation

All four expressed LAAOs were detected at a higher position in western blots (Figure 1) than expected from the predicted molecular weight (Table 1). These enzymes contain between 2 and 10 possible
N-glycosylation sites predicted by the NetNGlyc 1.0 webserver (Table 1). LAAOs purified from culture medium were deglycosylated with PNGaseF or incubated with water as controls (Figure 2).

A band at the position expected for the theoretical molecular weight of these four enzymes (determined by ExPASy ProtParam tool, see also Table 1) was obtained after the addition of PNGaseF (Figure 2, lanes +PNGaseF). Two bands were detected after deglycosylation of 9His-cgLAAO1 (Figure 2c) suggesting that it was only partially deglycosylated.

In the first set of experiments, the impact of deglycosylation on activity was determined for 9His-nclLAAO1 and 9His-cgLAAO1 (Figure 3) according to (Heß et al., 2020). The activity was measured with a coupled peroxidase/o-dianisidine assay. Deglycosylation of 9His-nclLAAO1 with PNGaseF overnight at 37°C did not alter activity compared to controls with additions of water (Figure 3a, +PNGaseF, 37°C: 0.43 ± 0.06 U mg⁻¹; H₂O, 37°C: 0.49 ± 0.07 U mg⁻¹; H₂O, 4°C: 0.37 ± 0.11 U mg⁻¹). This suggests that N-glycosylation is not essential for activity but may support the proper folding of the enzyme during biosynthesis. The activity of 9His-cgLAAO1 was reduced by incubation overnight at 37°C (Figure 3b).

### 2.3 Site-directed mutagenesis of 9His-hcLAAO4

We have already shown that deglycosylation does not reduce the activity of 9His-hcLAAO4 expressed in P. pastoris (Heß et al., 2020). 6His-hcLAAO4 expressed in E. coli has a much lower specific activity (2 U mg⁻¹) compared to 9 U mg⁻¹ by 9His-hcLAAO4 expressed in P. pastoris (Bloess et al., 2019, Heß et al., 2020). However, similar specific activity is reached after acid activation. This means that the maximal activity is not affected by glycosylation. Therefore, glycosylation may have a role during biosynthesis resulting in a stable, more active conformation.

hcLAAO4 has four predicted N-glycosylation sites according to NetNGlyc 1.0 webserver at amino acid residues N54, N164, N193, N331.

These asparagine residues were changed to alanine residues. Single mutants (N54A, N164A, N193A, N331A), a triple mutant (N164A N193A N331A), and the mutant without putative N-glycosylation sites (ΔN-Glyc.) were expressed and purified as described in experimental procedures.

The enzyme variants were analyzed via western blotting (Figure 4). The ΔN-Glyc. mutant enzyme was detected at the same position as 6His-hcLAAO4 expressed in E. coli and the deglycosylated 9His-hcLAAO4 indicating that there was no additional posttranslational modification present in the enzyme expressed in P. pastoris (Heß et al., 2020). All single mutant enzymes (N54A, N164A, N193A, and N331A) were detected at a lower position than the wild-type enzyme (Wt). These results suggest that 9His-hcLAAO4 carries N-glycans at all four putative N-glycosylation sites. The N54A mutant had a lower position than the other single mutants. The N54A and the ΔN-Glyc. variants could be detected as clear bands, whereas all other single mutants, the N164A N193A N331A triple mutant as well as the wild-type enzyme showed a diffuse band (Figure 4). This could be due to a heterogenous hyperglycosylation on N54 and a more frequently observed N-glycosylation with Man₈-9GlcNAc₂ (Bretthauer & Castellino, 1999) at the three other N-glycosylation sites. Furthermore, the band of the N54A mutant was on the same height as the upper band of the partially deglycosylated 9His-hcLAAO4 (Figure 6, lane +PNGaseF, 37°C, and N54A) indicating that the N-glycan at N54 was more efficiently removed than the other N-glycans.

The activity was determined using a coupled peroxidase/o-dianisidine assay with 10 mM l-glutamine. For comparisons, we used 6His-hcLAAO4 expressed in E. coli (E. coli Wt) (Bloess et al., 2019) and fully glycosylated 9His-hcLAAO4 expressed in P. pastoris (Wt).

Among the untreated enzymes with l-alanine in one N-glycosylation site, the N54A exchange showed the biggest reduction of activity (Figure 5 and Table A6). The specific activity of the N54A mutant enzyme was 2.8-fold lower in comparison to the wild-type enzyme (4.9 U mg⁻¹ to 14.1 U mg⁻¹). In contrast, small reductions in activity could be detected in proteins without one of the other N-glycosylation sites or with N54 as the only remaining site (13.4 U mg⁻¹ to 11.6 U mg⁻¹, Figure 5 and Table A6). These data suggest that N54 carries the most important N-glycan for basal activity of 9His-hcLAAO4. The enzyme without N-glycosylation sites (ΔN-Glyc.) showed nearly the same activity as 6His-hcLAAO4 expressed in E. coli (2.4 U mg⁻¹ to 1.1 U mg⁻¹ for E. coli Wt). However, the specific activities reached similar values for all enzyme variants expressed in P. pastoris after pH3 activation (39.6 U mg⁻¹ to 44.5 U mg⁻¹ compared to 42.8 U mg⁻¹ for the wild-type enzyme). Acid-activated 6His-hcLAAO4 expressed in E. coli reached 33.3 U mg⁻¹ (Figure 5 and Table A6).
ΔN-Glyc. (13.46 ml) behaved very similar to the glycosylation sites 11.45 ml, which corresponds to 216 kDa. The enzyme without N-untreated wild-type enzyme (Wt, black line) eluted at a volume of already observed at 30°C. Enzyme variants with asparagine to alanine 9His-10–120 min before the activity assay at 30°C. Fully glycosylated 9His-hcLAAO4 lost only 10% of its activity as described before (Heß et al., 2020). The enzyme lacking all four N-glycosylation sites (ΔN-Glyc) was inactivated by 60 min (Figure 7). Inactivation was already observed at 30°C. Enzyme variants with asparagine to alanine exchanges in one N-glycosylation site were slightly less stable than the wild-type enzyme. All variants were quickly inactivated at 80°C.

To investigate the hydrodynamic radius of the enzymes we performed analytical size exclusion chromatography (Figure 6). The untreated wild-type enzyme (Wt, black line) eluted at a volume of 11.45 ml, which corresponds to 216 kDa. The enzyme without N-glycosylation sites ΔN-Glyc. (13.46 ml) behaved very similar to the E. coli expressed 6His-hcLAAO4 (13.19 ml, Figure 6a). The PNGaseF treated wild type 9His-hcLAAO4 (+PNGaseF, 37°C) eluted at 12.07 ml (159 kDa) indicating that the partial deglycosylation reduced the hydrodynamic radius. Acid activation shifted the E. coli expressed 6His-hcLAAO4 to 12.23 ml in comparison to the untreated enzyme (13.19 ml) as already described (Bloess et al., 2019).

Expressed in P. pastoris neither wild type nor ΔN-Glyc. enzyme similarly changed mobility upon acid activation (11.25 ml and 13.10 ml, Figure 6b).

Finally, we analyzed whether glycosylation affected temperature stability. Acid-activated enzymes were incubated at 70°C for 10–120 min before the activity assay at 30°C. Fully glycosylated 9His-hcLAAO4 lost only 10% of its activity as described before (Heß et al., 2020). The enzyme lacking all four N-glycosylation sites (ΔN-Glyc) was inactivated by 60 min (Figure 7). Inactivation was already observed at 30°C. Enzyme variants with asparagine to alanine exchanges in one N-glycosylation site were slightly less stable than the wild-type enzyme. All variants were quickly inactivated at 80°C.

Here we describe that four fungal l-amino acid oxidases could not be expressed as active proteins in E. coli but were successfully expressed as secretory proteins in the yeast P. pastoris. All of these enzymes were N-glycosylated in P. pastoris even though only three of them contain predicted ER-signal sequences indicating that they are secretory proteins originally. Likely, the N-glycosylation improved the folding and solubility of these enzymes as carbohydrates are very hydrophilic. A similar observation was made for the snake venom LAAO from Calloselasma rhodostoma (crLAAO, Kommoju et al., 2007). While expression in E. coli resulted in inclusion bodies, crLAAO was expressed as an active, N-glycosylated enzyme in P. pastoris. N-glycosylation is required for solubility of this LAAO as deglycosylation of crLAAO expressed in P. pastoris and isolated from snake venom resulted in precipitation of the protein and inactivation. Deglycosylation did not decrease the activity of 9His-ncLAAO1 as well as 9His-hcLAAO4 (Heß et al., 2020). 9His-cgLAAO1 was partially inactivated due to the incubation at 37°C overnight but did not lose activity completely. These data indicate that N-glycosylation is not a prerequisite for solubility for these fungal LAAOs. Deglycosylation did not influence the activity of LAAOs isolated from snake venom of different species of Bothrops: Bothrops pirajai (Izidoro et al., 2006).
Bothrops pauloensis (Rodrigues et al., 2009), Bothrops alternatus (Stabeli et al., 2004); and Cerastes cerastes (Abdelkafi-Koubaa et al., 2014). Glycosylation in fungal LAAOs has been rarely studied. It has been shown that the LAAO isolated from the Agaricomycetes fungus Amanita phalloides (ApLAAO) is N-glycosylated (Sabotic et al., 2020). However, the effect of deglycosylation on activity was not analyzed.

9His-cgLAAO1 is of potential interest for biotechnological applications because it can convert L-tryptophan with higher specific activity compared to 9His-rsLAAO1 or 9His-hcLAAO4 (Hahn, Neumeister, et al., 2017, Heß et al., 2020). However, \( v_{\text{max}} \) for the best substrates is lower for 9His-cgLAAO1 than for 9His-hcLAAO4 (13 U mg\(^{-1}\) compared to 30 U mg\(^{-1}\)). 9His-ncLAAO1 has low \( v_{\text{max}} \) (1 U mg\(^{-1}\)) and very low \( K_{m} \) values in the range of 5–10 \( \mu \text{M} \) for the best substrates, which is about 100-fold lower than \( K_{m} \) values for the three other fungal LAAOs studied. In addition, the activity of 9His-ncLAAO1 is inhibited at 10 mM substrate concentration for many L-aminos acids. On the other hand, 9His-ncLAAO1 converts substrates, which are not accepted by the other three fungal LAAOs such as L-valine, L-serine, L-threonine, L-aspartic acid, and L-phenylglycine. 9His-ncLAAO1 and 9His-cgLAAO1 are not activated by acidic pH or by SDS in contrast to 9His-rsLAAO1 and 9His-hcLAAO4.

Site-directed mutagenesis was used to analyze the roles of the different N-glycans in detail. In contrast to S. cerevisiae, P. pastoris contains mostly Man\(_{g}\)GlcNAc\(_{g}\) glycans but is also capable to synthesize N-glycans with more mannose residues (Bretthauer & Castellino, 1999). The diffuse band of 9His-hcLAAO4 and its N164A, N193A, N331A and N164A N193A N331A variants (Figure 4) suggest the occurrence of a hypermannosylated N-glycan. A sharp band appeared due to the removal of the N-glycosylation site at N54. These data indicate N54 is hypermannosylated while N164, N193, and N331 carry Man\(_{g}\)GlcNAc\(_{g}\) glycans.

Similar specific activation was reached after acid activation regardless of the removal of one or more N-glycosylation sites (Figure 5). These data indicate that activation and maintenance of the active conformation did not depend on N-glycosylation.

The N-glycan at N54 had the highest impact on untreated activity (Figure 4 and Table A6). The N54A exchange reduced the specific activity to 4.9 U mg\(^{-1}\) while removal of one or all of the three other N-glycosylation sites did not change the specific activity or had little impact. The specific activity was even lower in the absence of all N-glycans (2.4 U mg\(^{-1}\)). Deglycosylation of 9His-hcLAAO4 did not reduce the specific activity of the untreated or acid-activated enzyme (Figure 5 and (Heß et al., 2020)). These data suggest that hcLAAO4 adopts a conformation of low activity if synthesized without N-glycans. The N-glycan at N54 induces folding into a conformation of intermediate activity during biosynthesis. Once this conformation is formed it is not sensitive to the removal of N-glycans by deglycosylation. The most active conformation can be reached by acid activation independent of N-glycans.

Activation by acid (6His-hcLAAO4, Bloess et al., 2019) or SDS (9His-rsLAAO1, Hahn, Hertle, et al., 2017) after expression in E. coli results in a conformational change, which can be detected as an increase in size by size exclusion chromatography. When expressed in P. pastoris, 9His-hcLAAO4 had a higher hydrodynamic radius than 6His-hcLAAO4 expressed in E. coli, which was reduced by deglycosylation (Figure 6). 9His-hcLAAO4 did not elute at a lower retention volume after acid activation. The untreated \( \Delta N\text{-Glyc.} \)-protein eluted at a retention volume similar to the untreated 6His-hcLAAO4 expressed in E. coli (E. coli Wt) consistent with the identical mobility in SDS-PAGE (Figure 4). We did not observe a change in the elution volume of the \( \Delta N\text{-Glyc.} \)-protein after acid treatment. This behavior may point toward differences in activation of the \( \Delta N\text{-Glyc.} \)-protein expressed in P. pastoris and 6His-hcLAAO4 expressed in E. coli.

Temperature stability was greatly reduced in the \( \Delta N\text{-Glyc.} \)-enzyme. These data indicate that N-glycosylation contributes to the temperature stability of 9His-hcLAAO4 expressed in P. pastoris. However, 6His-hcLAAO4 expressed in E. coli is not inactivated at
70°C even though it is not glycosylated (Heß et al., 2020). Enzymatic deglycosylation of 9His-hcLAAO4 overnight at 37°C does not inactivate the enzyme (Heß et al., 2020). Four hydrophilic asparagine residues were replaced with less hydrophilic alanine residues on the surface of the ΔN-Glyc.-enzyme. These changes in the surface may also destabilize the enzyme.

Our results contribute to understanding the effect of glycosylation at fungal LAAOs and will help for further biochemical characterizations of fungal LAAOs.

4 | EXPERIMENTAL PROCEDURES

4.1 | Amplification and cloning of LAAOs

The synthetic genes (GenBank accession numbers MW752153 (cgLAAO1), MW752154 (ncLAAO1), MW752155 (JoLAAO2), and MW752156 (frLAAO1)) of the LAAOs (Table 1) were amplified with the forward primers in Table A1 introducing an EcoRI recognition site (underlined sequence) followed by a 9His-tag (bold) upstream of the LAAO coding sequence. The reverse primers in Table A1 contained the stop-Codon (bold) and the NotI site (underlined sequence). The PCR products were cloned in an intermediate step into pGEM-TEasy (Promega), digested with EcoRI and NotI, and ligated into the EcoRI and NotI digested pPIC9K (Invitrogen), which encodes the pre-pro-sequence of α-mating factor. The cloning of 9His-hcLAAO4 (GenBank accession number MH751433, (Bloess et al., 2019)) was briefly described (Heß et al., 2020). The four N-glycosylation sites of hcLAAO4 predicted by NetNGlyc 1.0 webserver were changed into alanine residues one after the other via overlap PCR as described (Heß et al., 2020). For the expression in E. coli, LAAO-genes were inserted in pET28b-vector (Novagen) encoding an N-terminal 6His-tag via restriction sites Ndel and NotI according to (Bloess et al., 2019).

4.2 | Genome integration into P. pastoris and selection of clones

Genomic integration was carried out according to (Heß et al., 2020). Cells of P. pastoris SMD1163 yeast strain (Invitrogen) were speroplasted and linearized plasmids were transformed via electroporation (linearization with Sall for integration into the HIS4 locus). After electroporation, 1 M ice-cold sorbitol was added immediately and the cells were spread on MD-His agar plates (0.17% yeast nitrogen base, 0.4 µg L⁻¹ biotin, 2% glucose, amino acid mix (50 mg L⁻¹ each of L-glutamic acid, L-methionine, L-lysine, L-leucine, L-isoleucine), 1.5% Agar). Plates were incubated at 30°C for 2 days as described (Wu & Letchworth, 2004). The selection was performed according to (Scorer et al., 1994) on YEPD-agar-plates with increasing concentration of G-418 (Geneticin, Calbiochem) (1 mg ml⁻¹, 2.5 mg ml⁻¹, 5 mg ml⁻¹, 7.5 mg ml⁻¹, 10 mg ml⁻¹). Plates were cultured at 30°C for several days.

4.3 | Analysis of expression of LAAOs

Clones were screened for the highest LAAO expression after induction with methanol as described (Heß et al., 2020). For this, clones were cultivated overnight at 30°C in 20 ml BMGY medium (2% peptone, 1% yeast extract, 100 mM potassium phosphate pH 6.0, 1.34% yeast nitrogen base, 0.4 µg ml⁻¹ biotin, 1% glycerol). 50 ml
BMGY medium (2% peptone, 1% yeast extract, 100 mM potassium phosphate pH 6.0, 1.34% yeast nitrogen base, 0.4 µg ml⁻¹ biotin, 1% methanol) was inoculated with 1 OD600 of cells. Expression was performed at 15°C and 190 rpm for 96 h. Methanol was added every 24 h to a final concentration (v/v) of 1%. Every 24 h a sample was taken and centrifuged (4°C, 4600 g, 5 min). 30 µl of the supernatants were analyzed via western blotting.

4.4 | Expression of LAAOs

Cells were cultivated in 20 ml BMGY medium at 30°C for at least 16 h, as described in Heß et al., 2020. Cells were centrifuged and diluted into 500 ml BMGY medium for 72 h at 15°C. Methanol was added every 24 h to a final concentration (v/v) of 1%. After centrifugation of cells (4°C, 4,600 g, 10 min), the supernatants (medium) with secreted LAAOs were stored at 4°C and used for purification of the enzymes. Expression in E. coli Arctic express (DE3) was done according to Bloess et al., 2019 in 250 ml LB-broth (10 g L⁻¹ bacto tryptone, 5 g L⁻¹ yeast extract, 5 g L⁻¹ NaCl, 1 g L⁻¹ glucose) or 500 ml HSG-medium (13.5 g L⁻¹ soy peptone, 7 g L⁻¹ yeast extract, 14.9 g L⁻¹ glycerol (99%), 2.5 g L⁻¹ NaCl, 2.3 g L⁻¹ K₂HPO₄, 1.5 g L⁻¹ KH₂PO₄, 0.14 g L⁻¹, MgSO₄·H₂O, pH 7.4) with 50 µg ml⁻¹ kanamycin and 25 µg ml⁻¹ gentamycin at 11°C and was induced by 0.05 mM IPTG.

4.5 | Purification of the secreted LAAO

Purification was performed at 4°C as described (Heß et al., 2020). The supernatant with the secreted LAAO was loaded onto a Ni²⁺-NTA resin. The flow-through was collected, mixed, and reloaded onto the Ni²⁺-NTA resin via a peristaltic pump for 24 h with a flow velocity of 1 ml min⁻¹. The column was washed with 10 column volumes (cv) of His-washing buffer (50 mM Na₂HPO₄ pH 7.0, 300 mM NaCl and 20 mM imidazole). LAAO was eluted with 10 ml His buffer (50 mM Na₂HPO₄ pH 7.0, 300 mM NaCl and 500 mM imidazole). The enzyme was concentrated via ultrafiltration (Vivaspin 6 30.000 MWCO, Sartorius), rebuffered into HEPES buffer pH 7.0 (100 mM HEPES, 150 mM NaCl), and stored at 4°C. Size exclusion chromatography was performed according to Bloess et al., 2019 with an Etan LC (GE Healthcare Life Sciences, Chicago, IL, USA) on a Superdex⁸⁰ 200 Increase 10/300 GL column (GE Healthcare Life Sciences, Chicago, IL, USA).

4.6 | Enzymatic assay

Enzymatic activity of LAAOs was measured using a coupled peroxidase/o-dianisidine assay with 10 mM l-amino acid as described (Hahn, Neumeister, et al., 2017). The initial rate of H₂O₂ production was measured. A standard assay mixture contained 10 mM l-glutamine (for 9His-hcLAAO4 or 9His-cgLAAO1) or l-leucine ethyl ester (for 9His-nLAAO1), 50 mM TEA/HCl buffer (pH 7.0), 0.2 mg ml⁻¹ of o-dianisidine, 5 U ml⁻¹ peroxidase and LAAO in limiting amounts (0.75–2 µg in a 200 µl assay). Absorption was measured in 96-well plates at 30°C in a Tecan Spark microplate reader at 436 nm. One unit was defined as the amount of enzyme that catalyzes the conversion of 1 µmol l-amino acid per minute. To determine the impact of various conditions on the activity, enzymes were preincubated with 1.5 mM SDS or different pH values (pH 3.0, 4.5, 7.0, 9.0) for 10 min and then measured under standard conditions. Initial velocities of H₂O₂ production were determined with different l-amino acid concentrations between 0.02 and 20 mM for 9His-cgLAAO1 or 9His-nLAAO1. Kₘ and vₘₐₓ values were calculated from Hanes-Woolf plots.

4.7 | Deglycosylation of LAAOs

Deglycosylation was performed by PNGaseF (NEB). To analyze enzymatic activity the PNGaseF mixture contained 100 mM EDTA pH 8.0, 100 mM Tris-HCl pH 8.0, 1 mM PMSF in DMSO, 1% (w/v) CHAPS and 500 U PNGaseF. The digestion was done overnight at 37°C. As controls, PNGaseF was replaced with water, and samples were incubated at 37°C or 4°C overnight. For SDS-PAGE the mixture containing 5 µg of glycoprotein and 1 µl 10× glycoprotein denaturing buffer (NEB) in a total of 10 µl was heated at 95°C for 10 min. After adding 2 µl 10× GlycoBuffer 2 (NEB), 2 µl 10% NP40 (NEB), 5 µl H₂O und 500 U PNGaseF, the mixture was incubated at 37°C and 300 rpm for 1 h.

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CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

Marc Christian Heß: Conceptualization (supporting); Data curation (lead); Investigation (lead); Visualization (lead); Writing-original draft (equal); Writing-review & editing (supporting). Marvin Grollius: Investigation (supporting). Valentin Duhay: Investigation (supporting). Simon Koopmeiners: Investigation (supporting); Writing-review & editing (supporting). Svenja Bloess: Investigation (supporting); Writing-review & editing (supporting). Gabriele Fischer von Mollard: Conceptualization (lead); Data curation (supporting); Supervision (lead); Visualization (supporting); Writing-original draft (equal); Writing-review & editing (lead).

ETHICS STATEMENT

Work with recombinant DNA has been performed according to national requirements (Dt.55.3.5-5/94-Bi, Anlage Nr. 412).
DATA AVAILABILITY STATEMENT
Data associated with this article can be found at PUB (Publikationen an der Universität Bielefeld) under https://doi.org/10.4119/unibi/2956041.

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**APPENDIX**

**TABLE A1** Overview of used primer for cloning of the LAAOs

| Primer | Sequence (5′-3′) | Plasmid | Yeast strain |
|--------|------------------|---------|--------------|
| 9His-ncLAAO1 | | | |
| EcoRI_Neurospora_for5 | AAGAATTCCATCATCATCACCATCACCCACCATATGGCTACTAAGCCATCTGACTCCG | pMCH15 | MCH13 |
| NotI_Neurospora_rev6 | GCGGCCGCTTAACGGATCAGTC | | |
| 9His-frLAAO1 | | | |
| EcoRI_Fibroporia_for3 | AAGAATTCCATCATCATCACCATCACCCACCATATGGCTACTAGGAGT | pMCH21 | MCH17 |
| NotI_Fibroporia_rev4 | AGCGGCCGCTTAACGGATCAGTC | | |
| 9His-cgLAAO1 | | | |
| EcoRI-HC_f3 | AAGAATTCCATCATCATCACCATCACCCACCATATGGCTACTAGGAGT | pSBL12 | MCH5 |
| NotI-HC_r4 | AGCGGCCGCTTAACGGATCAGTC | | |
| 9His-laLAAO2 | | | |
| EcoRI_Laccaria_for7 | AAGAATTCCATCATCATCACCATCACCCACCATATGGCTACTACCATCCCATACGACTGT | pMCH13 | MCH8 |
| NotI_Laccaria_rev8 | GCGGCCGCTTAACGGATCAGTC | | |
| hc_N54A_fwd1 | CTACCACCTTGGGTAGGAAAGCCATCTGTTCCCATCTT | | |
| hc_N54A_rev1 | TGGAGAGATGGAGAAGGAGATGGGCTTCTCACCC | | |
| hc_N193A_fwd2 | TCCAGCTTGGTGATCGCCCTTCCTCGTGA | | |
| hc_N193A_rev2 | GATGTCAATCAAGGAGGAGGCGATACCCAAAGCT | | |
| hc_N331A_fwd3 | GGTAACGCTTTCGTTATGGCCGCTTCCAGGT | | |
| hc_N331A_rev3 | ATAGCAGTAACGGAAGGAGGCGATACCCAAAGCT | | |
| EcoRI_for1 | AAGAATTCCATCATCATCACCATCACCCACCATATGGCTACTAGGAGT | pSBL12 | MCH5 |
| NotI-HH_r2 | AAGGCGGCCGCTTAACGGATCAGTC | | |

Notes: Forward primers: underlined- EcoRI site, bold- 9His-tag.
Reverse primers: underlined- NotI site, bold- stop codon.
| Substrates                        | Relative activity (%) |
|----------------------------------|-----------------------|
| **Hydrophobic amino acids**      |                       |
| L-alanine                        | 70                    |
| 1-alanine                        | 0                     |
| L-isoleucine                     | 4                     |
| L-leucine                        | 90                    |
| L-tert-leucine                   | 0                     |
| L-norleucine                     | 99                    |
| L-methionine                     | 64                    |
| L-phenylalanine                  | 65                    |
| L-phenylglycine                  | 0                     |
| rac-ß-phenylalanine              | 0                     |
| L-proline                        | 0                     |
| L-tryptophan                     | 33                    |
| L-valine                         | 3                     |
| **Polar amino acids**            |                       |
| L-asparagine                     | 50                    |
| L-cysteine                       | 0                     |
| L-glutamine                      | 100                   |
| L-serine                         | 5                     |
| L-threonine                      | 0                     |
| L-tyrosine³                      | 53                    |
| **Basic amino acids**            |                       |
| L-arginine                       | 37                    |
| L-histidine                      | 17                    |
| L-lysine                         | 41                    |
| L-ornithine                      | 53                    |
| **Acidic amino acids**           |                       |
| L-aspartic acid                  | 3                     |
| L-glutamic acid                  | 21                    |
| **Amino acid derivates**         |                       |
| L-alanine ethyl ester            | 16                    |
| L-glutamic acid dimethyl ester   | 33                    |
| L-leucine methyl ester           | 55                    |
| L-methionine methyl ester        | 45                    |
| L-phenylalanine methyl ester     | 32                    |
| L-tyrosine methyl ester          | 11                    |
| L-threonine methyl ester         | 0                     |
| L-leucine ethyl ester            | 35                    |
| L-ß-alanine ethyl ester          | 0                     |
| L-DOPA³                          | 8                     |
| DL-homophenylalanine³            | 77                    |
| 4-chloro-DL-phenylalanine⁴       | 55                    |

Note: *n = 3.*

³2.5 mM.
## TABLE A3  Kinetic properties of 9His-cgLAAO1

| Substrates                     | $K_m$ [mM] | $v_{max}$ [U mg$^{-1}$] | $k_{cat}$ [s$^{-1}$] | $k_{cat}/K_m$ [s$^{-1}$ mM$^{-1}$] |
|--------------------------------|------------|-------------------------|---------------------|-----------------------------------|
| L-glutamine                    | 0.76 ± 0.1 | 12.04 ± 3.6             | 13.76               | 18.11                             |
| L-leucine                      | 1.20 ± 0.2 | 9.97 ± 1.0              | 11.40               | 9.50                              |
| L-leucine methyl ester         | 12.88 ± 0.3| 13.64 ± 0.6             | 15.59               | 1.21                              |
| L-phenylalanine                | 0.32 ± 0.03| 4.51 ± 0.2              | 5.15                | 16.11                             |
| L-phenylalanine methyl ester   | 3.97 ± 0.2 | 3.41 ± 0.2              | 3.90                | 0.98                              |
| L-methionine                   | 1.06 ± 0.5 | 9.84 ± 1.8              | 11.25               | 10.61                             |

Note: Data are means of three biological replicas ($n$ = 3).

## TABLE A4  Substrate spectrum of 9His-ncLAAO1

| Substrates                          | Rel. activity (0.1 mM) | Spec. activity (U mg$^{-1}$) (0.1 mM) | Spec. activity (U mg$^{-1}$) (10 mM) |
|-------------------------------------|------------------------|----------------------------------------|---------------------------------------|
| **Hydrophobic amino acids**         |                        |                                        |                                       |
| L-alanine                           | 15                     | 0.18 ± 0.006                           | 0.37 ± 0.06                           |
| L-isoleucine                        | 3                      | 0.03 ± 0.002                           | 0.33 ± 0.05                           |
| L-leucine                           | 95                     | 1.09 ± 0.02                            | 0.37 ± 0.07                           |
| L-methionine                        | 61                     | 0.70 ± 0.10                            | 0.14 ± 0.001                          |
| L-phenylalanine                     | 91                     | 1.05 ± 0.02                            | 0.53 ± 0.003                          |
| L-phenylglycine                     | 0                      | 0                                      | 0.12 ± 0.02                           |
| L-proline                           | 0                      | 0                                      | 0.02 ± 0.02                           |
| L-tryptophan                        | 84                     | 0.96 ± 0.11                            | 0.11 ± 0.06                           |
| L-valine                            | 0                      | 0                                      | 0.21 ± 0.04                           |
| **Polar amino acids**               |                        |                                        |                                       |
| L-asparagine                        | 65                     | 0.75 ± 0.06                            | 0.46 ± 0.05                           |
| L-cysteine                          | 0                      | 0                                      | 0                                      |
| L-glutamine                         | 63                     | 0.73 ± 0.146                           | 0.37 ± 0.04                           |
| L-serine                            | 0                      | 0                                      | 0.29 ± 0.02                           |
| L-threonine                         | 0                      | 0                                      | 0.24 ± 0.07                           |
| L-tyrosine                          | 67                     | 0.77 ± 0.10                            | 0.58 ± 0.23                           |
| **Basic amino acids**               |                        |                                        |                                       |
| L-arginine                          | 93                     | 1.07 ± 0.03                            | 0.13 ± 0.004                          |
| L-histidine                         | 75                     | 0.86 ± 0.02                            | 0.33 ± 0.06                           |
| L-lysine                            | 54                     | 0.62 ± 0.16                            | 0.08 ± 0.03                           |
| **Acidic amino acids**              |                        |                                        |                                       |
| L-aspartic acid                     | 0                      | 0                                      | 0.50 ± 0.02                           |
| L-glutamic acid                     | 0                      | 0                                      | 0.49 ± 0.02                           |
| **Amino acid derivates**            |                        |                                        |                                       |
| L-leucine methyl ester             | 20                     | 0.24 ± 0.01                            | 0.89 ± 0.08                           |
| L-phenylalanine methyl ester       | 17                     | 0.19 ± 0.003                           | 0.94 ± 0.17                           |
| L-leucine ethyl ester              | 7                      | 0.08 ± 0.007                           | 0.96 ± 0.07                           |
| N-α-acetyl-L-lysine                | 100                    | 1.15 ± 0.02                            | 0.24 ± 0.05                           |

Note: $n$ = 3 ([S] = 0.1 mM/10 mM); *2.5 mM
TABLE A5  Kinetic properties of 9His-ncLAAO1

| Compound                        | $K_m$ [μM] | $v_{max}$ [U mg$^{-1}$ | $k_{cat}$ [s$^{-1}$] | $k_{cat}/K_m$ [s$^{-1}$ mM$^{-1}$] |
|--------------------------------|------------|------------------------|----------------------|-------------------------------------|
| L-leucine                      | 10 ± 8.6   | 1.11 ± 0.10            | 1.18 ± 0.10          | 120                                 |
| L-leucine methyl ester         | 610 ± 160  | 1.09 ± 0.03            | 1.17 ± 0.03          | 2                                   |
| L-leucine ethyl ester          | 70 ± 30    | 1.23 ± 0.04            | 1.32 ± 0.02          | 20                                  |
| L-phenylalanine                | 7.4 ± 4.7  | 1.06 ± 0.05            | 1.13 ± 0.05          | 150                                 |
| L-phenylalanine methyl ester   | 50 ± 20    | 1.07 ± 0.02            | 1.15 ± 0.02          | 23                                  |
| L-arginine                     | 5 ± 0.3    | 1.27 ± 0.07            | 1.35 ± 0.07          | 270                                 |

Note: Data are means of three biological replicas ($n = 3$).

TABLE A6  Effect of N-glycosylation on 9His-hcLAAO4 activity

|            | Untreated [U mg$^{-1}$] | Activated (pH 3) [U mg$^{-1}$] |
|------------|-------------------------|---------------------------------|
| Wt         | 14.1 ± 4.0              | 42.8 ± 4.0                       |
| +PNGaseF, 37°C | 14.5 ± 3.1            | 40.4 ± 1.5                      |
| N54A       | 4.9 ± 0.7               | 40.7 ± 3.7                      |
| N164A      | 13.4 ± 3.1              | 40.0 ± 1.6                      |
| N193A      | 13.1 ± 3.8              | 41.5 ± 9.6                      |
| N331A      | 13.1 ± 3.4              | 44.5 ± 4.7                      |
| N164A N193A N331A | 11.7 ± 2.2       | 39.6 ± 1.5                      |
| ΔN-Glyc.   | 2.4 ± 0.9               | 44.5 ± 6.2                      |
| E. coli Wt | 1.1 ± 0.1               | 33.0 ± 5.8                      |

Note: Data are means of three biological replicas ($n = 3$) ± standard deviations.

Figure A1  Expression of 6His-ncLAAO1 and MBP-cgLAAO1 in E. coli Arctic Express (DE3). (a) 6His-ncLAAO1 (64 kDa) was found only as insoluble protein in the pellet fraction (P) and not in the supernatant (S) after cell lysis. (b) MBP-cgLAAO1 was detected predominately in the pellet fraction. The low amounts of the soluble enzyme were inactive. Expression was induced with 0.05 mM IPTG for 18 h (6His-ncLAAO1) or the indicated periods (MBP-cgLAAO1) at 11°C. Samples were separated by SDS-PAGE and stained with Coomassie Brilliant Blue.
Figure A2  Concentration-dependent activity of 9His-ncLAAO1 toward different substrates. Initial activities of the purified enzyme were measured with different substrate concentrations (0.1 mM–15 mM) for (a) L-isoleucine, (b) L-phenylalanine and (c) L-arginine. (a) For L-isoleucine, 9His-ncLAAO1 showed regular enzyme kinetics and a $K_m$ value of 1.6 mM. (b) L-phenylalanine concentrations above the $K_m$ value of 7.4 µM did not have a big impact on the activity of 9His-ncLAAO1. (c) A strong reduction of activity could be measured with increasing L-arginine concentration starting already at 0.1 mM. The $K_m$ value was 5 µM for L-arginine. Data are means of three independent experiments; error bars represent standard deviations.

Figure A3  Effect of SDS and pH on the activity of 9His-ncLAAO1 and 9His-cgLAAO1. The purified LAAOs were preincubated with 1.5 mM SDS or different pH values (4.5, 7.0, or 9.0) for 10 min, and activity was determined with coupled peroxidase and o-dianisidine in TEA buffer. The data were normalized to the control incubated at standard conditions (pH 7.0). (a) 9His-ncLAAO1 was slightly activated by all tested conditions. (b) The pH treatment of 9His-cgLAAO1 showed no impact on activity while 1.5 mM SDS resulted in reduced activity. Data are means of three independent experiments; error bars represent standard deviations.