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# Structural Characterization and Enzyme Engineering of a Fungal L-Amino Acid Oxidase

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## PURPOSE OF THE ABSTRACT

Introduction

L-amino acid oxidases (LAAOs, EC 1.4.3.2) catalyze the oxidative deamination of L-amino acids to the corresponding α-keto acids under formation of hydrogen peroxide and ammonium. LAAOs are of high interest for the synthesis of α-keto acids and the deracemization of enantiomeric mixtures of D-amino acids, which are both pharmaceutical building blocks. The fungal HcLAAO4 from Hebeloma cylindrosporum has a broad substrate spectrum, consisting of divergent L-amino acids and derivates, and can be expressed recombinantly with high yield in expression systems like E. coli or Pichia pastoris[1,2]. The HcLAAO4 was already utilized for the production of L-phenylpyruvate[1] and as part of an enzyme cascade with a catalase and transaminase[3].
As optimization by rational enzyme design and directed evolution requires detailed structural information about an enzyme and its active site, we aimed to crystallize the HcLAAO4 and determine its structure.

Materials and Methods

HcLAAO4 variants were recombinantly expressed in E. coli Arctic Express and purified by affinity chromatography and size exclusion chromatography. The enzyme was crystallized after limited proteolysis with trypsin. Crystals were harvested directly or soaked with different substrates prior to harvesting. Diffraction data were collected at beamlines at the ESRF[4]. The initial model for molecular replacement was created by using the ColabFold-Server[5]. Docking was carried out with AutoDock Tools 1.5.7[6]. The enzyme activity was monitored by a peroxidase coupled photometric assay.

Results

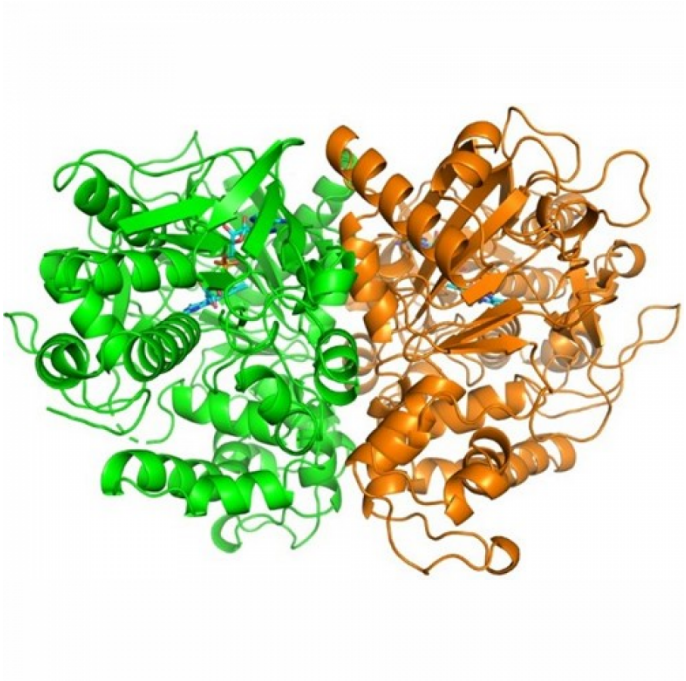
Crystallization of HcLAAO4 was achieved after removal of unstructured terminal peptides by limited proteolysis with trypsin and by mutating a cluster of hydrophilic residues at the protein surface to alanine, utilizing the surface entropy reduction (SER) strategy. The asymmetric unit (AU) consists of four HcLAAO4 molecules, forming a dimer of dimers. The positioning of the SER-mutation site in the interface between the dimers indicates that the SER-mutations are critical for the formation of this AU.
By soaking the crystals with different substrates, we also determined the structure of the substrate-bound enzyme with resolutions of about 2 Å and identified residues involved in substrate binding. Similar to other LAAOs the carboxylate moiety and the amine group of the L-amino acid substrate are coordinated by ionic interactions, hydrogen bridges and a main chain carbonyl group. The active site of HcLAAO4 is overall hydrophobic and mostly consists of aromatic site chains, which stabilize the aliphatic portion of a substrate. The preference for substrates with an aliphatic portion at the γ- and δ-C-atom is also explained by interactions with active site residues.
By utilizing a molecular docking model previously unknown, industrially interesting substrates were identified.

Finally, we analyzed the role of an active site residue on the substrate scope of HcLAAO4 by generating two point mutants. Both mutants showed an approximately 2-fold increased activity for L tryptophan, which is of high interest for the deracemization of racemic mixtures to the D enantiomer.

## Conclusion

Here we crystallized the HcLAAO4 and determined the structure of the apo enzyme and the substrate bound state. Residues interacting with the substrate in the active site were identified as potential targets for enzyme engineering approaches and by utilizing a molecular docking model previously unknown substrates were found. By site directed mutagenesis we generated mutants with increased activity for industrially interesting substrates, demonstrating the suitability of our structure model for future optimization of the enzyme.

## FIGURES



### FIGURE 1

Structure of HcLAAO4.

The biological dimer of HcLAAO4 is shown as cartoon with the bound FAD co-factor as sticks. The asymmetric unit contains a second dimer.

### FIGURE 2


## KEYWORDS

L-amino acid oxidase | enzyme engineering | protein crystallization | molecular docking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