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Racemic Resolution of some DL-Amino Acids using *Aspergillus fumigatus* L-Amino Acid Oxidase

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Abstract The ability of *Aspergillus fumigatus* L-amino acid oxidase (L-aao) to cause the resolution of racemic mixtures of DL-amino acids was investigated with DL-alanine, DL-phenylalanine, DL-tyrosine, and DL-aspartic acid. A chiral column, Crownpak CR+ was used for the analysis of the amino acids. The enzyme was able to cause the resolution of the three DL-amino acids resulting in the production of optically pure D-alanine (100% resolution), D-phenylalanine (80.2%), and D-tyrosine (84.1%), respectively. The optically pure D-amino acids have many uses and thus can be exploited industrially. This is the first report of the use of *A. fumigatus* L-amino acid oxidase for racemic resolution of DL-amino acids.

Introduction

Amino acids and keto acids are important compounds, widely used in various industries. The numerous applications of optically pure D- and L-amino acids include additives in feed stocks, intermediates in semi-synthetic antibiotic synthesis [12] and starting materials for peptide hormone synthesis [8]. Several methods have been reported for production of optically active amino acids [14]. But chemical synthesis is unsuitable because of its high cost and low yields [15]. Biotransformation methods are the most advantageous because of the optical purity and high productivity of the process [1].

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L-amino acid oxidases (L-amino acid: O_2 oxidoreductase, EC 1.4.3.2) (L-aaos) are useful enzymes in this aspect and have advantages as catalysts. L-aaos are flavoenzymes that catalyze the oxidative deamination of L-amino acid substrate into an α-keto acid with the production of ammonia and hydrogen peroxide [13]. They accept broad range of substrates [5] and are, therefore, applicable as catalysts for many syntheses. But these enzymes are not much used industrially and there are very few that are utilized for racemate resolution [4, 10]. Thus, these enzymes can be employed not only for the production of enantiomerically pure D-amino acids from the corresponding racemate, but they can also yield the corresponding α-keto acid simultaneously, which in turn have many applications as starting material for various organic syntheses [2] and in the pharmaceutical company. The α-keto acid and the amino acid obtained from a racemic resolution reaction can be easily separated by various methods like chromatography, extraction, and α-keto acid derivatization [3].

In the present communication, the resolution of some racemic DL-amino acids was studied with the help of an L-aao isolated from a fungal strain, *Aspergillus fumigatus*. The *A. fumigatus* L-aao causes the resolution of DL-amino acids, yielding optically pure D-amino acids. Although the resolution of DL-alanine was reported earlier [13], this is the first report of the use of *A. fumigatus* enzyme for racemate resolution of DL-phenylalanine and DL-tyrosine.

Materials and Methods

Chemicals

The chemicals used were commercially available and of reagent grade. The chemicals were purchased from Qualigens, Merck, and HiMedia.

Microrganisms and Culture Condition

Aspergillus fumigatus NEIST P13 (GenBank Accession No: FJ765414) was cultivated in a medium containing (gl^{-1}): glucose 10; Na_2HPO_4 2.5; KH_2PO_4 2; $(NH_4)_2SO_4$ 4; yeast extract 4; and metal salts; $MgSO_4{\cdot}7H_2O$ 0.5; $CaCl_2{\cdot}2H_2O$. 0.5; H_3BO_3 0.05; Na_2MoO_4 0.04; $ZnSO_4{\cdot}7H_2O$ 0.04; $CuSO_4{\cdot}7H_2O$ 0.02; $FeSO_4{\cdot}7H_2O$ 0.02 and DL-alanine 20 mM as inducer, pH 7.0 at 30°C, in a shaking speed of 200 rpm. The strain was taken from the microbial repository of NEIST, Jorhat, Assam, India.

Isolation of L-aao Crude Enzyme

Aspergillus fumigatus cells (96 h) were harvested by centrifugation at 9,400×*g* and 10°C, for 10 min (Sigma Centrifuge 30 K). The cells were washed with 50 mM of sodium phosphate buffer, pH 7.2. The cells (38.4 g, wet weight) were then homogenized in a French pressure cell press (Thermo spectronic, USA) at 1,500 psi for 5 min in ice cold condition. The cell suspension obtained, was centrifuged at 13,600×*g* for 15 min at 5°C. The cell debris was discarded and the clear supernatant was taken as the crude enzyme.

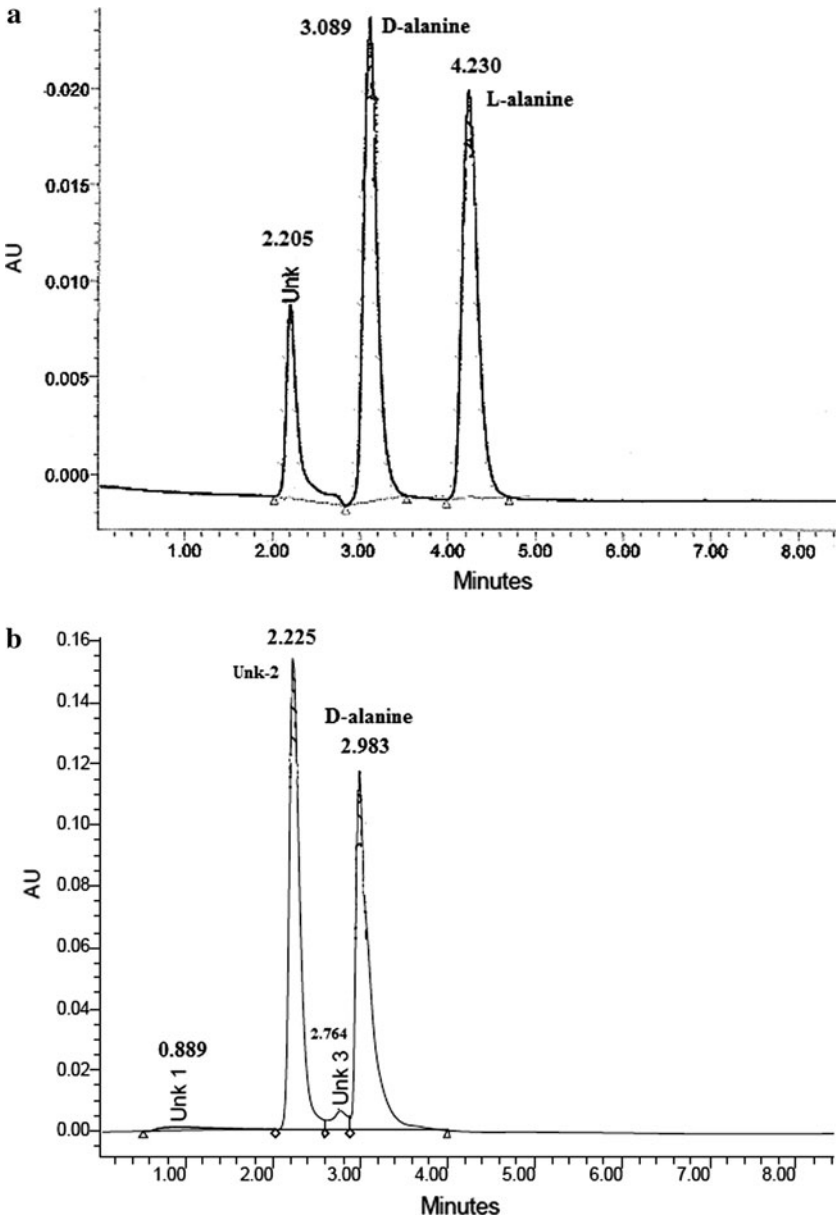

Fig. 1 **a** HPLC separation of standard DL-alanine (50 mM in HPLC grade water). *Peaks 2 & 3* represent D-alanine and L-alanine, respectively. **b** HPLC profile of fraction obtained after DL-alanine reaction. D-alanine is obtained as a single peak

L-aao Assay

The assay of L-aao activity was carried out with the crude enzyme by measuring the formation of keto acid according to the method of Jian Yu et al. [7] with slight modifications.

0.5 ml of the crude enzyme was allowed to react with 0.5 ml of 100 mM of substrate (DL-alanine, unless otherwise stated) in 50 mM of sodium phosphate buffer, pH 7.2 for 1 h at 30°C, 200 rpm. After the reaction, a total of 0.5 ml of the reaction mixture was diluted five times with distilled water and reacted immediately with 0.4 ml of 2,4-dinitrophenylhydrazine (0.2% saturated in 2 N HCl) for 10 min. To this 1.5 ml of 3 M NaOH was added and absorbance at 550 nm was recorded after 15 min. Respective blanks and controls were also taken during the assay. One unit of L-aao activity is defined as the amount of

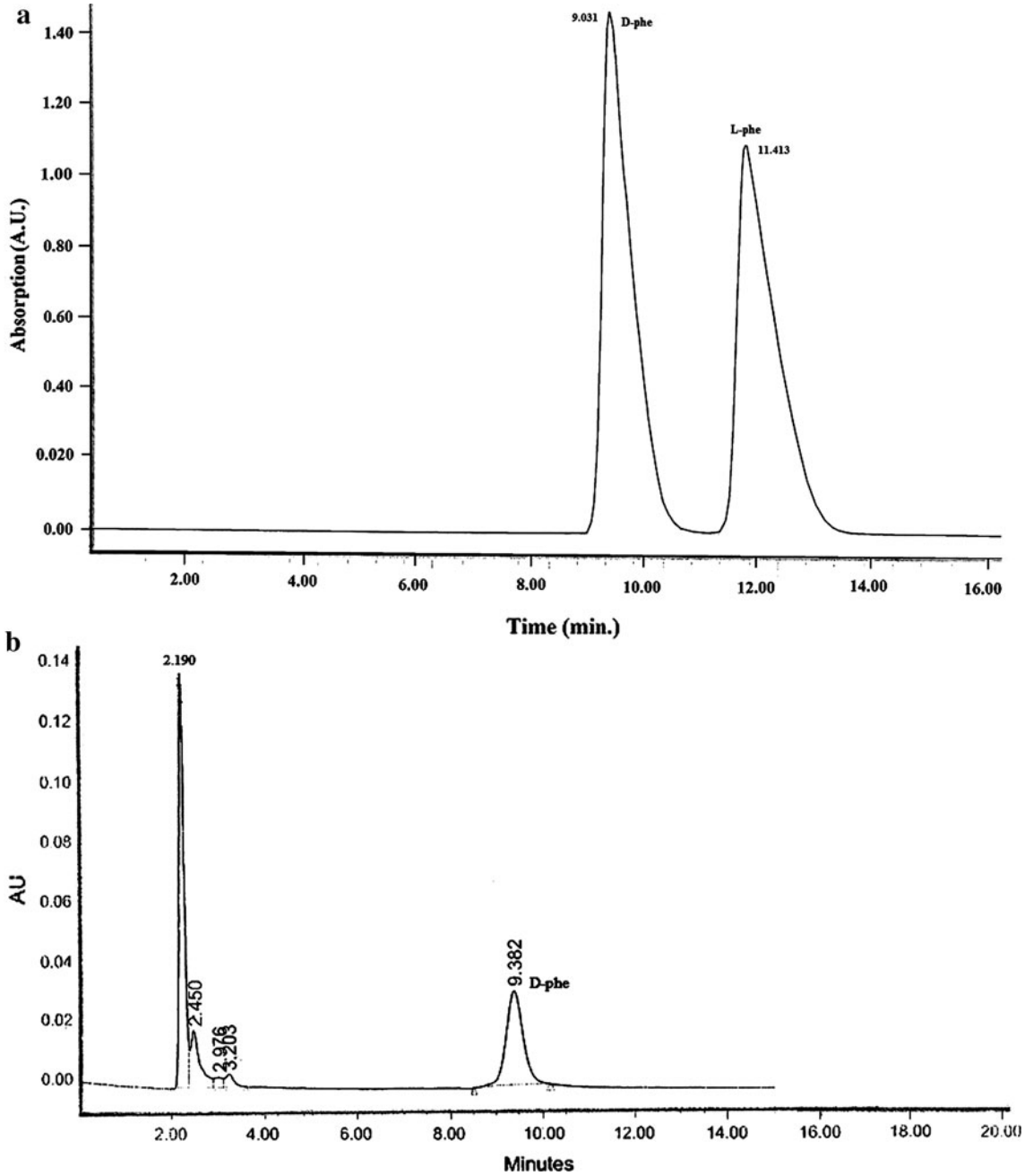

Fig. 2 **a** HPLC separation of standard DL-phenylalanine (50 mM in HPLC grade water). DL-phenylalanine separates into two peaks, D- & L-phenylalanine, with retention times of 9.03 and 11.4 min, respectively. **b** HPLC profile of fraction obtained after DL-phenylalanine reaction. The L-isomer is deaminated completely and the D-phenylalanine is obtained

enzyme that produces 1 μmol of pyruvate/minute/ml of enzyme, under the conditions described above.

Resolution of Racemic Mixtures

The ability of *A. fumigatus* L-aao for resolution of racemic mixtures of DL-amino acids was investigated for DL-alanine, DL-phenylalanine and DL-tyrosine, and DL-aspartic acid as substrates. 0.5 ml of the enzyme was allowed to react with 0.5 ml of 100 mM substrate (DL-alanine, DL-phenylalanine, DL-tyrosine, and DL-aspartic acid) in 50 mM of sodium phosphate buffer, pH 7.2 for 1 h at 30°C, 200 rpm. After the reaction, the solutions were deproteinized with 10% Trichloroacetic acid (TCA) for the purification of free amino acids.

Purification of Free Amino Acids for Determination by HPLC

The deproteinized reaction filtrates of L-aao reaction with the DL-amino acid substrates were passed through Dowex 50W-X 8 (20-50 US Mesh, BDH) (1.8 cm × 6 cm) for partial purification of free amino acids [9]. 20 fractions of 5 ml each were collected by elution with distilled water at

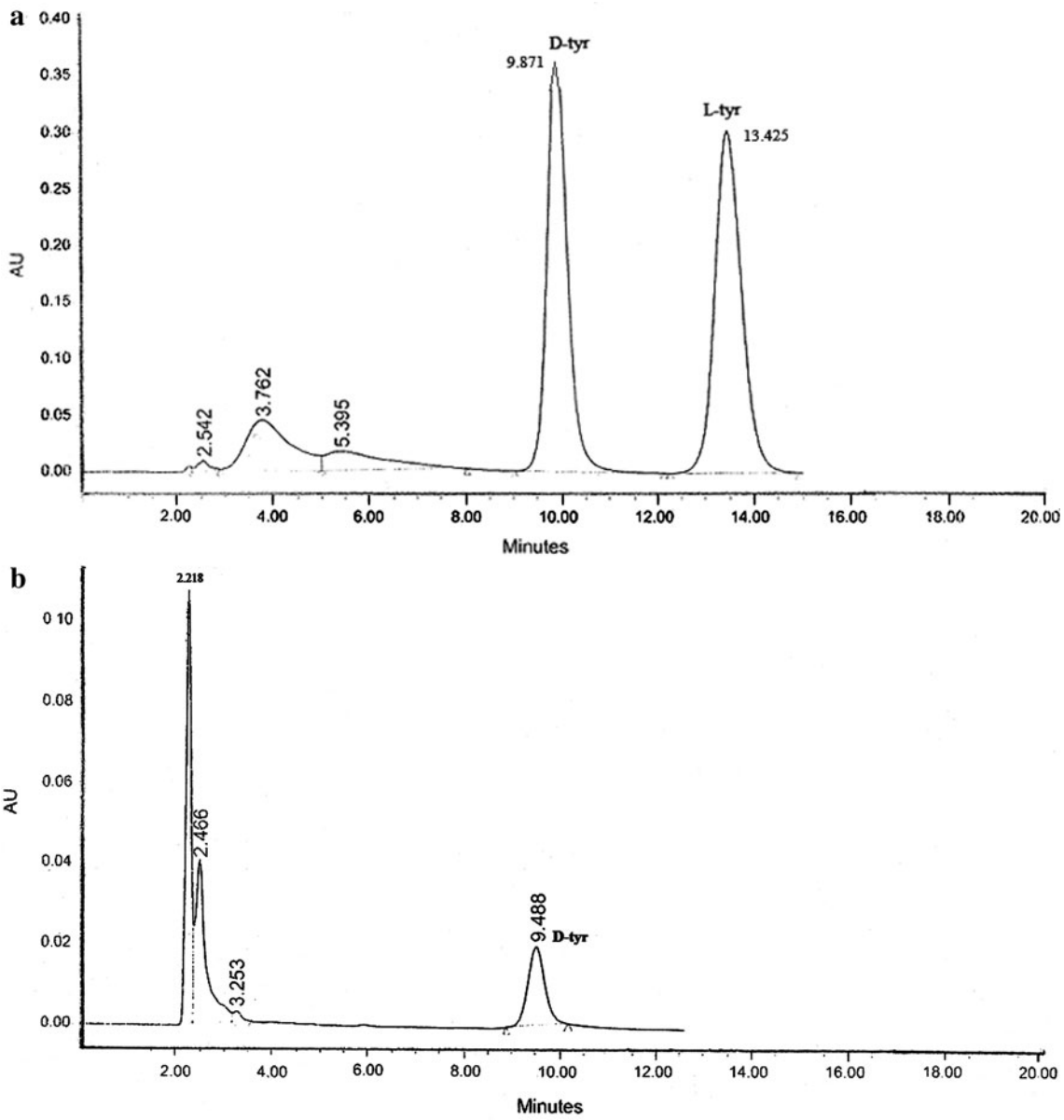

Fig. 3 **a** HPLC separation of standard DL-tyrosine (50 mM in HPLC grade water). Two distinct peaks of D- & L-tyrosine are seen with retention times of 9.8 and 13.4 min, respectively. **b** HPLC profile of fraction obtained after DL-tyrosine reaction. D-tyrosine is obtained as a distinct peak while the L-tyrosine is deaminated completely

a flow rate of 1.5 ml/min. The UV absorbances of the fractions were scanned in an UV–visible spectrophotometer (GB Spectral) from 190 to 350 nm. The fractions that showed a single peak in the range of 190–200 nm were checked by HPLC. A chiral column, CROWNPAK CR+ (150 mm × 4 mm I.D.) was used for analysis of D-/L-alanine formed during the reaction. Perchloric acid, pH 1.5 was used as the mobile phase. The UV detector was set at 200 nm.

Results

Racemic Resolution of DL-Amino Acids

The ability of the *A. fumigatus* L-aao for resolution of racemic mixtures of amino acids was measured for DL-alanine, DL-phenylalanine, DL-tyrosine, and DL-aspartic acid. The enzyme was able to cause the resolution of three DL-amino acids resulting in the production of D-alanine (Fig. 1a, b), D-phenylalanine (Fig. 2a, b), and D-tyrosine (Fig. 3a, b), respectively. The enzyme had no activity with DL-aspartic acid and hence no resolution occurred.

Quantification of the Resolution

The quantification of the resolution occurring was calculated from the area covered by the respective peaks. *A. fumigatus* L-aao causes complete deamination of DL-alanine leaving behind 100% of the product i.e. D-alanine. The enzyme causes deamination of DL-phenylalanine and DL-tyrosine to cause the formation of the products i.e. D-phenylalanine and D-tyrosine up to 80.2 and 84.1%, respectively.

Discussion

In the present work, we investigated the ability of *Aspergillus fumigatus* L-aao to cause the racemic resolution of DL-amino acids to produce the optically pure D-amino acids. The enzyme has greater specificity toward hydrophobic aromatic amino acids namely tyrosine and phenylalanine [13]. This enzyme causes the deamination of DL-alanine, DL-phenylalanine, and DL-tyrosine to produce D-alanine, D-phenylalanine, and D-tyrosine in optically pure form. For the determination of D, L-amino acids by HPLC, two methods have mainly been adopted [6]; diastereomer formation, followed by separation on achiral stationary phases or direct enantiomer separation on chiral stationary phases. Konno et al. [9] reported the analysis of the isomer composition of the urinary alanine by Marfey's method [11] in which the diastereomers of alanine obtained by derivatization with Marfey's reagent were analyzed on a reversed phase Nova-pak C18 column. We used the alternative method of analysis i.e. enantiomer separation on chiral stationary phase and used a chiral column Crownpak CR+. Effective separation of the standards DL-alanine, DL-phenylalanine, and DL-tyrosine was observed and also the products i.e. D-alanine, D-phenylalanine, and D-tyrosine gave distinct and significant peaks. This is a significant result since it allows the direct enantiomer separation and reduction of time and also the use of expensive derivatizing reagents can be avoided.

In the literature, there are only a few reports on the use of L-aaos for racemate resolution. Koyama [10] used an L-phenylalanine oxidase from *Pseudomonas* sp. while Faust et al. [4] used *Rhodococcus opacus* L-aao for this purpose. Thus, *A. fumigatus* L-aao is a potential enzyme that can be exploited industrially for production of optically pure D-amino acids which in turn have many applications.

In conclusion, it can be said that *Aspergillus fumigatus* produces a novel enzyme capable for racemate resolution and this property can be exploited industrially.

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