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Preparation of cross-linked enzyme aggregates of L-aminoacylase via co-aggregation with polyethyleneimine

Bhalchandra K. Vaidya*, Suyog S. Kuwar, Sandeep B. Golegaonkar, Sanjay N. Nene

Biochemical Engineering Department, Chemical Engineering and Process Development Division, National Chemical Laboratory, Dr. Homi Bhabha Road, Pune 411008, India

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ABSTRACT

L-Aminoacylase from *Aspergillus melleus* was co-aggregated with polyethyleneimine and subsequently cross-linked with glutaraldehyde to obtain aminoacylase–polyethyleneimine cross-linked enzyme aggregates (termed as AP-CLEA). Under the optimum conditions, AP-CLEA expressed 74.9% activity recovery and 81.2% aggregation yield. The said method of co-aggregation and cross-linking significantly improved the catalytic stability of L-aminoacylase with respect to temperature and storage. AP-CLEA were employed for enantioselective synthesis of three unnatural amino acids (*namely*: phenylglycine, homophenylalanine and 2-naphthylalanine) via chiral resolution of their ester-, amide- and N-acetyl derivatives. The enantioselectivity of AP-CLEA was the highest for hydrolysis of amino acid amides; was moderate for hydrolysis of N-acetyl amino acids and was the least for hydrolysis of amino acid esters. Furthermore, AP-CLEA were found to retain more than 92% of the initial activity after five consecutive batches of (*RS*)-homophenylalanine hydrolysis suggesting an adequate operational stability of the biocatalyst.

1. Introduction

L-Aminoacylase (N-acyl amino acid amidohydrolase or acylase-I; EC 3.5.1.14) has long been utilized for industrial production of enantiopure L-amino acids from N-acyl DL-amino acids [1]. The enzyme is widely distributed in variety of plants, animals and microorganisms. The most commonly used L-aminoacylases are those from hog kidney, porcine kidney, *Aspergillus oryzae* and *Aspergillus melleus*. Moreover, arecheal L-aminoacylases especially those from *Pyrococcus furiosus* and *Thermococcus litoralis* are receiving increasing attention due to their capability to operate at elevated temperatures [2,3]. L-Aminoacylases from *Aspergillus* sp. are well suited for large scale industrial biotransformations as they are inexpensive, readily available and more stable as compared to the others [4]. Aspergillus L-aminoacylases have been extensively utilized for the industrial production of natural amino acids (e.g. L-alanine, L-methionine, L-valine, etc.) as well as unnatural amino acids (L-α-amino butyric acid, L-norvaline, L-norleucine, etc.) [5].

The usefulness of crude L-aminoacylase of *Aspergillus* sp. in enantioselective hydrolysis of amino acid esters and amides is reported [6]. Furthermore, in the presence of organic medium, the enzyme can catalyze a number of synthetic transformations such as regioselective alcoholysis of carboxylic acid esters [7–9], acylation of primary and secondary alcohols [10–13], and acylation of amines [10,14]. Owing to higher enantioselectivity and broader substrate specificity, the commercial importance of Aspergillus L-aminoacylase is expected to expand from its current state in years to come.

The present work is aimed at developing a facile method for synthesizing catalytically active and stable cross-linked biocatalyst of *A. melleus* L-aminoacylase. Cross-linked enzyme technology in the past couple of decades, has emerged as an attractive alternative for enhancing stability of enzymes [15]. The cross-linking of enzymes by means of bi-functional cross-linking agents results in formation of stable heterogeneous biocatalyst which offers distinct benefits over conventionally immobilized enzyme. Immobilized enzymes are 'carrier-bound biocatalysts' and the presence of a large proportion of non-catalytic carrier (about 90–99% of total mass) causes dilution of their volumetric activity. On the other hand, cross-linked enzymes are referred as 'carrier-free biocatalysts' and express very high catalytic activity per unit volume thereby maximizing volumetric productivity and space-time yields [16].

The carrier-free cross-linked biocatalyst can be obtained by either of following three strategies *namely*: (i) direct cross-linking of free enzyme which gives Cross-Linked Enzymes (CLE); (ii) cross-linking of crystalline enzyme which yields Cross-Linked Enzyme Crystals (CLEC) and (iii) cross-linking of physically aggregated enzyme which yields Cross-Linked Enzyme Aggregates (CLEA) [15]. More often than not, CLE strategy suffers from several limitations such as low activity retention, poor reproducibility, poor mechanical stability and difficulty in handling the gelatinous CLE. Need of highly pure enzyme and crystallization protocols (which are often

* Corresponding author. Tel.: +91 20 25903057; fax: +91 20 25902612.
E-mail address: bk.vaidya@rediffmail.com (B.K. Vaidya).

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expensive, laborious and time consuming) are major limitations of CLEC strategy. Aforementioned limitations of CLE and CLEC can be eliminated by means of a strategy called as CLEA [17].

Synthesis of CLEA involves precipitation or aggregation of an enzyme (not necessarily in its pure form) followed by chemical cross-linking of the resulting enzyme aggregates [18,19]. Precipitation/aggregation is generally induced by addition of a precipitant (acid, salt, organic solvent or non-ionic polymer) to an aqueous solution of the enzyme. These physical aggregates are supramolecular structures held together by non-covalent bonding and re-dissolve when the precipitant is removed. Cross-linking of these aggregates gives CLEA which remain insoluble in the absence of the precipitant.

Only external amino groups (i.e. amino groups mostly of lysine which are available on the surface) of an enzyme can participate in the process of cross-linking. Hence, enzymes having sufficient number of external amino groups (typically electropositive enzymes) undergo effective cross-linking and form stable CLEA. On the other hand, enzymes having low number of external amino groups (typically electronegative enzymes such as L-aminoacylase) undergo inadequate cross-linking and form mechanically fragile CLEA that often release enzyme into the reaction medium during a biocatalytic reaction [20].

Interestingly, Wilson et al. have demonstrated that if the co-aggregation of enzyme is induced with polyethyleneimine (PEI) then the cross-linking efficiency of the enzyme can be improved significantly [21]. PEI is water soluble, cationic polymer consisting of large number of terminal amino groups. The co-aggregation of enzyme with PEI allows the extension of polymer branches (having terminal amino groups) closer to some of the embedded amino groups of enzyme favouring cross-linking between them [20,22]. Thus, besides external amino groups, a few embedded amino groups (which are otherwise not accessible during conventional cross-linking procedures) can be utilized in the formation of stable intra- and inter-molecular cross-links. Thus, the PEI induced co-aggregation technique for synthesizing CLEA is highly advantageous strategy especially for electronegative enzymes like L-aminoacylase.

The present study explores the feasibility of the PEI induced co-aggregation technique for synthesizing CLEA of *A. melleus* L-aminoacylase. Herein, the enzyme was co-aggregated with polyethyleneimine and subsequently cross-linked with glutaraldehyde to obtain aminoacylase–polyethyleneimine cross-linked enzyme aggregates (termed as AP-CLEA). Furthermore, AP-CLEA were systematically characterized with respect to their physical properties, catalytic stability and enantioselectivity.

2. Materials and methods

2.1. Materials

L-Aminoacylase and N-acetyl-L-methionine were purchased from Fluka Chemicals, USA. PEI was purchased from Sigma–Aldrich, USA. Unnatural amino acids (*namely*: phenylglycine, homophenylalanine and 2-naphthylalanine) and derivatives thereof were purchased from Bachem Chemicals, Switzerland. All other chemicals were of analytical grade and purchased from Merck India Ltd.

2.2. Preparation of AP-CLEA

AP-CLEA were prepared by method described by López-Gallego et al. [20]. To 25 mL solution of PEI (750 kDa, 25 mg/mL) 25 mL of aminoacylase (25 mg/mL) solution was added under agitation. The mixture was left under gentle stirring for 10 min. After 10 min, 2.0 mL of glutaraldehyde solution (25%, v/v) was added to cross-link the enzyme precipitate and the mixture was kept under stirring for 1 h. Then the volume was doubled by adding 100 mM sodium bicarbonate buffer (pH 10) and a total amount of 75 mg of sodium borohydride powder was added to reduce the Schiff's bases formed. After 15 min, an additional 75 mg of sodium borohydride powder was added and allowed to react for 15 min. The resultant precipitate of AP-CLEA was repeatedly washed with sodium phosphate buffer (100 mM, pH 7) and centrifuged at 12,000 rpm for 15 min. Finally the CLEA were dried at 50 °C for 24 h using vacuum oven to remove residual moisture.

2.3. Optimization studies

Different process parameters such as enzyme–PEI ratio, glutaraldehyde concentration and cross-linking time were optimized on the basis on two assessment parameters: namely, activity recovery (Eq. (1)) and aggregation yield (Eq. (2)) as described earlier [22].

$$\text{Activity recovery} = \left(\frac{A_{\text{CLEA}}}{A_{\text{Free}} \times V_{\text{Free}}}\right) \times 100 \quad (1)$$

$$\text{Aggregation yield} = \left[100 - \left(\frac{A_{\text{Residual}} \times V_{\text{Residual}}}{A_{\text{Free}} \times V_{\text{Free}}}\right)\right] \times 100 \quad (2)$$

where A_{CLEA} is activity expressed by AP-CLEA; A_{Free} is activity of free enzyme (U/mL); V_{Free} is volume (mL) of free enzyme used for preparation of AP-CLEA; A_{Residual} is activity (U/mL) of residual enzyme solution; and V_{Residual} is volume (mL) of residual enzyme solution remained after formation of CLEA. All experiments were performed at least in triplicate and the results are presented as their mean value. Standard deviation of results never exceeded 5%.

2.4. Characterization of AP-CLEA

2.4.1. Physical properties of AP-CLEA

The surface morphology of AP-CLEA was studied by scanning electron microscopy (SEM). Micrographs were taken on a JEOL JSM-5200 SEM instrument. Pore size and pore volume of AP-CLEA were determined by mercury intrusion porosimetry using Autoscan 60 Mercury Porosimeter (Quantachrome, USA) in the range of 0–4000 kg/cm^2.

2.4.2. Study on release of enzyme subunit from AP-CLEA

The stability of AP-CLEA against release of enzyme subunit(s) from the aggregates was evaluated according to the method described earlier by López-Gallego et al. [20]. Both free aminoacylase and AP-CLEA were boiled separately (~95 °C) in 2 volumes of 2% sodium dodecyl sulfate (SDS). Then, supernatant of AP-CLEA and supernatant of free aminoacylase were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), the gel was stained with silver stain and analyzed by densitometry.

2.4.3. Thermal stability of AP-CLEA

Free aminoacylase and AP-CLEA were incubated in phosphate buffer (50 mM, pH 8) at different temperatures ranging between 20 and 90 °C and their activities were determined as described elsewhere in the manuscript. The residual activities were calculated as a percentile ratio of the activity of enzyme after incubation to the activity enzyme at the optimum temperature. A plot of residual activity against temperature was obtained to compare the thermal stability of AP-CLEA over that of free aminoacylase.

2.4.4. Thermal deactivation kinetics of aminoacylase before and after cross-linking

Kinetics of thermal deactivation of free aminoacylase and AP-CLEA were studied at different temperatures ranging between 30 °C