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

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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

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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

and 70 °C in a shaking water bath (Julabo Ltd.). The samples were withdrawn at regular time interval, rapidly cooled to 37 °C and immediately analyzed for the residual aminoacylase activity. The thermal deactivation rate constant (k_d) was determined from the Arrhenius equation (Eq. (3)) [23].

$$\ln k_d = \ln A - \left(\frac{E_d}{RT}\right) \quad (3)$$

where E_d is the deactivation energy and R is the universal gas constant (8.314×10^{-3} kJ kmol^{-1} K^{-1}). Natural logarithm of percentile residual activities of different temperatures was plotted against time wherein the slope of lines indicates the values of deactivation rate constant, k_d at the corresponding temperature [24]. Half-life of free aminoacylase and AP-CLEA for each temperature was calculated from their respective linear trend line equations. Further, the Arrhenius plot, i.e. plot of natural logarithm of k_d ($\ln k_d$) versus reciprocal of temperature in Kelvin scale ($1/T$) was obtained wherein slope of line indicates the deactivation energy (E_d). The difference in the deactivation energies (ΔE_d) of free aminoacylase and AP-CLEA was calculated to quantify the thermal stability before and after cross-linking of the enzyme.

2.4.5. Storage stability of AP-CLEA

Free aminoacylase and AP-CLEA were incubated in phosphate buffer (50 mM, pH 8) at 4 °C and their activities were determined as described elsewhere in the manuscript at regular time intervals up to 168 h. The storage stability of free enzyme and AP-CLEA was compared from the plot of log residual activity against storage time.

2.5. AP-CLEA catalyzed chiral resolution of unnatural amino acid derivatives

2.5.1. General procedure for chiral resolution

The biotransformation reaction was conducted in 25 mL stoppered flasks. Racemic substrate (0.01 g) was dissolved in 10 mL phosphate buffer (50 mM phosphate buffer, pH 8). Co^{+2} ions act as cofactor for aminoacylase enzyme therefore $CoCl_2$ (1.19 mg) was added in the reaction mixture. The biotransformation reaction was initiated by adding 0.05 g of AP-CLEA. The flask was incubated on oscillatory shaker at 30 °C at 120 rpm. The samples were periodically withdrawn and analyzed by HPLC.

2.5.2. Chiral resolution of (RS)-homophenylalanine amide in stirred cell reactor

The biotransformation reaction was carried out in a laboratory scale Millipore stirred cell reactor (XFUF04701, Millipore Inc., USA). A schematic diagram of stirred cell reactor assembly is shown in Fig. 1. A macroporous filtration fabric cloth (47 mm diameter) was placed on the membrane support. The reactor was charged with 0.1 g of (*RS*)-homophenylalanine amide and 25 mL of 50 mM of phosphate buffer (pH 7) containing 2.98 mg of $CoCl_2$. The biotransformation reaction was initiated by addition of 0.1 g of AP-CLEA. The temperature of the reaction mixture within the reactor was maintained at 30 °C under constant stirring at 200 rpm. The samples were periodically withdrawn and analyzed by HPLC. After about 45% conversion, the reaction was stopped by separating the reaction mixture. The reactor was thoroughly washed twice with phosphate buffer (50 mM, pH 7.0) to ensure the complete removal of product and unreacted substrate. The washings and reaction mixture were collected together and the pH of the resultant solution was adjusted to 9 with aqueous 2 M NaOH solution. The unreacted substrate, i.e. (*R*)-homophenylalanine amide was extracted with dichloromethane (3× 15 mL). The remaining aqueous solution was acidified (to pH 6.3) with 2 M HCl solution and maintained at 0–2 °C. After ~2 h, the precipitate of (*S*)-HPA was collected, washed thrice with cold acidified water and dried at 60 °C under vacuum.

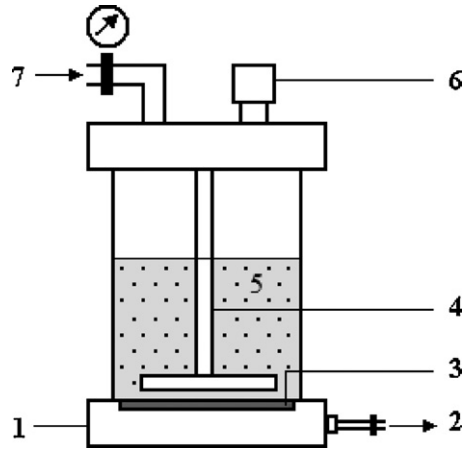

Fig. 1. Schematic representation of stirred cell reactor; 1: membrane support, 2: outlet, 3: membrane, 4: magnetic stirrer, 5: reaction mixture containing AP-CLEA, 6: sample port and 7: pressure gauze.

2.6. Analytical methods

2.6.1. Aminoacylase activity assay

The activity of aminoacylase was measured using the standard hydrolytic assay of N-acetyl L-methionine as described earlier [13]. One unit (U) is defined as amount of aminoacylase necessary to liberate 1 μmol of L-methionine per hour at 37 °C at pH 8. The activity of AP-CLEA was determined using 0.1 g of CLEA. The activity of AP-CLEA is expressed in terms of aminoacylase units per gram of AP-CLEA.

2.6.2. HPLC analysis

All reaction profiles were monitored by HPLC (Thermo Separation Products, Fremont, CA, USA). The quantitative analysis of different amino acids and their esters was carried out using a reverse phase C-18 column (125 mm × 4 mm, prepacked column supplemented with a 4 mm × 4 mm guard column procured from LiChrospher®, Merck, Darmstadt, Germany) eluted isocratically using acetonitrile–water mobile phase (30:70, v/v) at a flow rate of 0.6–0.8 mL/min. The enantiomeric excess (e.e.) of the product was determined by using a chiral CHIROBIOTIC® column (250 mm × 4.6 mm prepacked column supplemented with 20 mm × 4 mm guard column procured from Astec Inc., USA) eluted isocratically using water–methanol mobile phase (40:60, v/v) at a flow rate of 0.6–0.8 mL/min. Analytes were detected using an UV detector at 215 nm.

2.6.3. Determination of enantiomeric ratio

Enantioselectivity of AP-CLEA was calculated in terms of enantiomeric ratio (E) by using Chen's formula as described earlier [25]. The value of E mentioned in the present manuscript represents an average value of triplicate observations where the standard error was not more than 5%.

3. Results and discussion

3.1. Optimization of process parameters

'Extent of cross-linking' is the key parameter which decides an activity of CLEA (calculated in terms of activity recovery) and an extent of enzyme aggregation (calculated in terms of aggregation yield). Hence the 'extent of cross-linking' in CLEA should

Table 1
Effect of enzyme:PEI ratio.

Enzyme:PEI ratio	Activity recovery (%)	Aggregation yield (%)
1:3	23.3	68.0
1:2	33.1	62.6
1:1	50.4	60.4
2:1	44.0	42.8
3:1	37.5	34.6
1:0	6.9	11.3

Table 3
Effect of cross-linking time.

Cross-linking time (h)	Activity recovery (%)	Aggregation yield (%)
1	45.0	51.2
6	58.2	74.2
12	65.5	79.8
24	74.9	81.2
36	74.5	81.6
48	70.9	82.3

be critically controlled. Adequate cross-linking is essential to form stable enzyme aggregates. Excessive cross-linking however, inactivates enzyme as well as lowers the porosity of CLEA consequently decreases the activity recovery [15]. To summarize, up to the optimum point, the activity recovery and the aggregation yield increase with extent of cross-linking. However, additional cross-linking leads to decrease in the activity recovery. The aggregation yield may increase or remain constant beyond the optimum. The extent of cross-linking in the present study was controlled by optimizing three process variables *namely* enzyme to PEI ratio, concentration of glutaraldehyde and time of glutaraldehyde treatment.

3.1.1. Effect of enzyme:PEI ratio

To evaluate the effect of enzyme–PEI ratio, aminoacylase and PEI solutions (25 mg/mL each) were mixed in different concentrations (1:3, 1:2, 1:1, 2:1 and 3:1) and the resultant AP-CLEA were analyzed for the activity recovery and aggregation yield as described elsewhere in the manuscript. Table 1 gives the effect of enzyme:PEI ratio. Among the different enzyme–PEI ratios studied, 1:1 enzyme to PEI ratio was found to be optimum and gave maximum for activity recovery (50.4%) and therefore 1:1 enzyme to PEI ratio was used in all further experiments. It is interesting to note that, in the absence of PEI poor values of activity recovery and aggregation yield (i.e. 6.9% and 11.3% respectively) were obtained.

3.1.2. Effect of glutaraldehyde concentration

Different concentrations of glutaraldehyde (100–600 mM) were used to determine the optimum quantity required for stable cross-linking of aminoacylase and PEI. The activity recovery and aggregation yield of AP-CLEA were found to increase initially with increase in glutaraldehyde concentration from 100 to 500 mM (Table 2). Further increase in glutaraldehyde concentration above 500 mM resulted in small decrease in the activity recovery with no significant change in the aggregation yield. The small decrease in the activity recovery could possibly be due to an excessive cross-linking of enzyme molecules making them catalytically inactive. Glutaraldehyde concentration of 500 mM gave maximum activity recovery (61.3%) and aggregation yield (74.8%) and hence used in all further experiments.

3.1.3. Effect of cross-linking time

The cross-linking time was studied from 1 h to 48 h for stable cross-linking of aminoacylase and PEI (Table 3). The increase in cross-linking time resulted in significant increase in the activity recovery of AP-CLEA. The cross-linking reached to optimum within 24 h where maximum activity recovery (74.9%) and aggregation yield (81.2%) were obtained. Beyond 24 h, small decrease in the activity recovery was observed while the aggregation yield remained almost unchanged.

Table 2
Effect of glutaraldehyde concentration.

Glutaraldehyde (mM)	Activity recovery (%)	Aggregation yield (%)
100	50.9	60.8
200	55.8	63.3
300	58.0	68.0
400	60.0	70.2
500	61.3	74.8
600	56.9	74.7

3.2. Characterization of AP-CLEA

3.2.1. Physical properties of AP-CLEA

Scanning electron micrographs of AP-CLEA are given in Fig. 2. The SEM of AP-CLEA shows the cross-linked aggregates of enzyme and PEI. The SEM also indicates the highly porous structure of the aggregate. The average particle size of AP-CLEA as estimated from SEM data was 50 ± 10 μm. The values of pore surface area and pore volume of AP-CLEA, as determined by mercury porosimetry, were 66 ± 0.5 m^2/g and 0.5 ± 0.01 cm^3/g respectively.

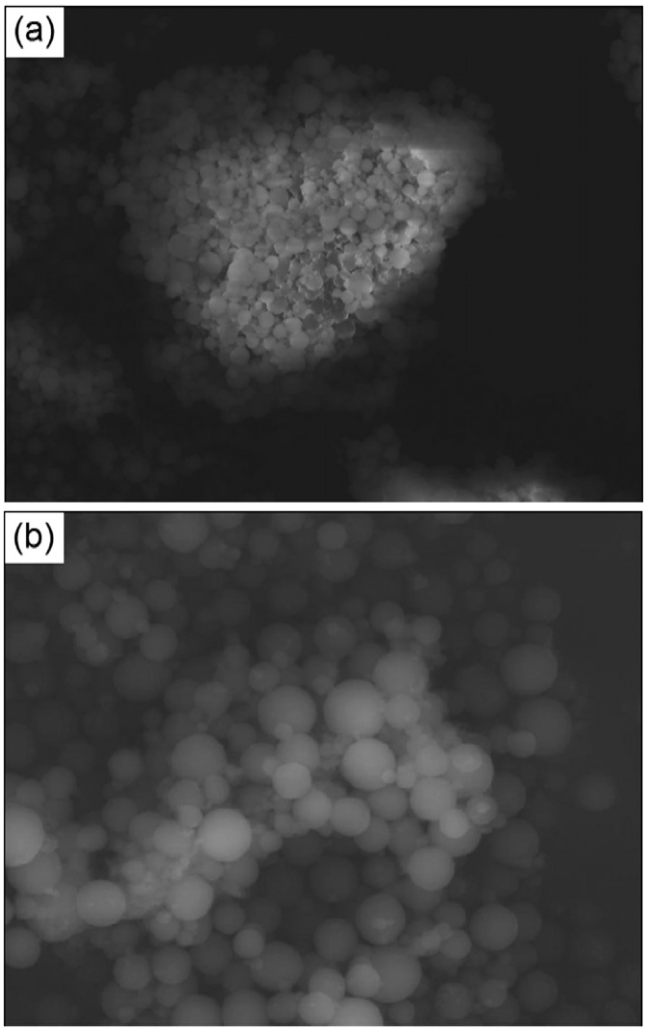

Fig. 2. SEM of AP-CLEA: (a) individual CLEA particle observed at lower magnification (1000×); (b) macroporous nature of CLEA observed at higher magnification (2500×).

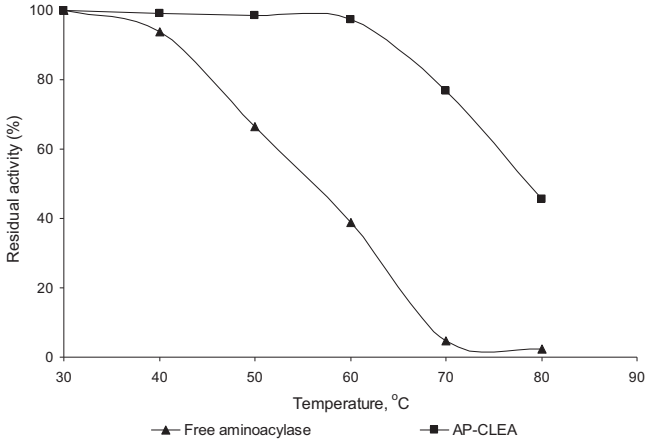

Fig. 3. Thermal stability of AP-CLEA.

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

Boiling of free enzyme with SDS causes dissociation of the protein into its polypeptide subunits. *A. melleus* aminoacylase is dimer and each subunit is having approximate molecular weight of 37 kDa [26]. The supernatant collected after boiling of free enzyme with SDS gave two distinct bands on the gel at around 37 kDa which is in agreement with the published data (results not shown). Boiling of AP-CLEA with SDS allows dissociation of non-covalently bound enzyme subunits (if any) from the aggregates into the supernatant solution. Interestingly, the supernatant of AP-CLEA did not give any band indeed indicate the absence of non-covalently bound enzyme subunits in AP-CLEA (results not shown). Moreover, these results highlight the potential of said method for stable and effective cross-linking of multimeric enzymes.

3.2.3. Thermal stability of AP-CLEA

The activity profiles of free aminoacylase and AP-CLEA at different temperatures are represented in Fig. 3. At 50 °C, the free enzyme retained only 66.4% residual activity while AP-CLEA 98.5% of its initial activity. Likewise, at 70 °C the free enzyme retained only 4.7% residual activity while AP-CLEA was found to retain 76.6% of its initial activity. Thus, the cross-linking of aminoacylase with PEI was observed to confer excellent thermal stability to the enzyme.

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

The temperature dependant loss of enzyme activity of free aminoacylase and CLEA is shown in Fig. 4(a) and (b) respectively. From these temperature dependant activity profiles, the Arrhenius plot was obtained. The Arrhenius plot for free aminoacylase and CLEA is shown in Fig. 5. The slope of Arrhenius plot indicates the deactivation energy (E_d). The deactivation energy of AP-CLEA and free aminoacylase were found to be $-8281.1\ \text{kJ kmol}^{-1}\ \text{K}^{-1}$ and $-9369.3\ \text{kJ kmol}^{-1}\ \text{K}^{-1}$. When an enzyme forms cross-linked aggregates, a large number of stabilizing linkages are formed between individual enzyme molecules. Energy must be put into the system in order to disrupt these stabilizing linkages. As a result, CLEA has higher deactivation energy than free enzyme [24]. Higher deactivation energy of AP-CLEA than that of free enzyme ($\Delta E_d = 1088.2\ \text{kJ kmol}^{-1}\ \text{K}^{-1}$) indicates the excellent thermal stabilization of the cross-linked enzyme. The half-life ($t_{1/2}$) and deactivation rate constants (k_d) of free aminoacylase and AP-CLEA are given in Table 4. On an average, upon cross-linking, there was 4.4 fold increase in the half-life of enzyme.

Co-aggregation followed by cross-linking is known to confer better thermal stability to the enzyme than conventional immobilization procedures. The increased thermal stability of CLEA is mainly ascribed to inter- and intramolecular cross-links which maintain the catalytically active conformation of enzyme and also prevent the dissociation of multimeric enzyme into the individual subunits at the elevated temperatures [21,22].

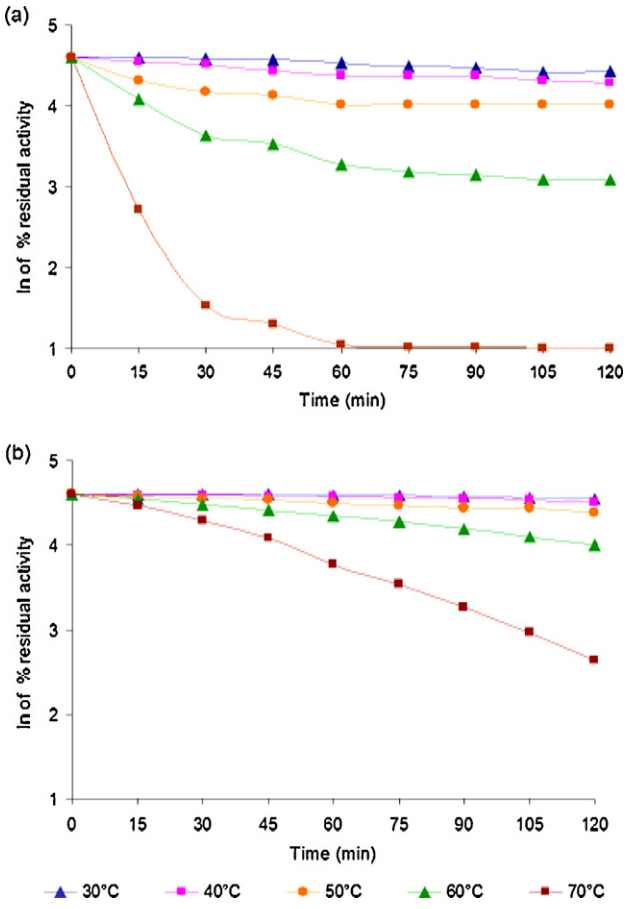

Fig. 4. Thermal deactivation of (a) free aminoacylase and (b) AP-CLEA in temperature range of 30–70 °C.

3.2.5. Storage stability of AP-CLEA

The storage stability of AP-CLEA was determined and compared with free aminoacylase. The plot of log residual activity verses storage time is shown in Fig. 6. The linear trend lines were drawn to the activity profiles of free aminoacylase and AP-CLEA. The storage half-life of each enzyme preparation was determined from its

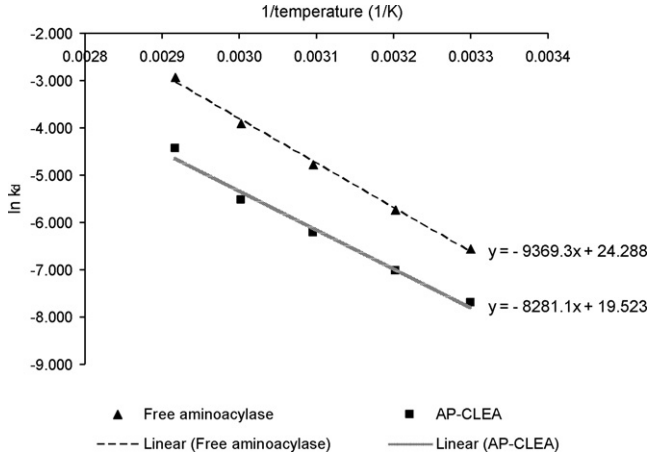

Fig. 5. Arrhenius plot for free aminoacylase and AP-CLEA.

Table 4
Thermal deactivation coefficient (k_d) and half-life ($t_{1/2}$) of free enzyme and AP-CLEA.

Temperature (°C)	k_d (min^{-1})		$t_{1/2}$ (min)		Fold increase in $t_{1/2}$
	Free enzyme	AP-CLEA	Free enzyme	AP-CLEA	
30	−0.00141	−0.00038	496.45	1842.11	3.7
40	−0.00319	−0.00081	219.43	864.19	3.9
50	−0.00840	−0.00189	83.33	370.37	4.4
60	−0.01979	−0.00404	35.37	173.26	4.9
70	−0.05262	−0.01015	13.30	68.96	5.1
Average of fold increase in the half-life over the range of 30–70 °C					4.4

respective trend line equation. The storage half-life of free aminoacylase at 4 °C was found to be ∼1 day while that of AP-CLEA was found to be ∼40 days. Thus, the present method of preparation of CLEA conferred extended storage life to the enzyme.

The cross-linking of aminoacylase has been achieved by different methods [26–29]. Bode et al. employed ethylene glycol dimethyl ether (as precipitant) and glutaraldehyde (as a cross-linking agent) to prepare aminoacylase CLEA. Interestingly this report also states that commercial L-aminoacylase is a mixture of several hydrolases [26]. Immobilization of thermophilic L-aminoacylase from *T. litoralis* was studied by Hickey et al. [27]. Here, two immobilization approaches were used and both involved cross-linking of L-aminoacylase. In the first approach, enzyme was attached to monoliths while in the second approach previously cross-linked enzyme was trapped using frits in the micro-fluidic channels. The cross-linked immobilized L-aminoacylase was successfully used in the form of miniaturized flow reactors wherein the enzyme exhibited excellent operational stability. Honda et al. reported a novel strategy wherein poly-L-lysine was used as a booster for attaining efficient cross-linking of aminoacylase [28]. Recently, Dong et al. have demonstrated that use of bovine serum albumin facilitates cross-linking of aminoacylase thereby allowing formation of catalytically stable CLEA of the enzyme [29]. To the best of our knowledge, PEI induced co-aggregation technique has not yet been studied for synthesizing aminoacylase CLEA.

The PEI induced co-aggregation technique for synthesizing CLEA is yet to be studied extensively. Till date, this technique of synthesizing CLEA has been studied for limited number of enzymes viz. glutaryl acylase [20], penicillin-G acylase [21], lipases [22,30] and *Pseudomonas fluorescens* nitrilase [31]. López-Gallego et al. prepared the CLEA of glutaryl acylase using the PEI induced co-aggregation technique wherein the use of PEI prevented the release of enzyme molecules from the aggregate. Besides this, the PEI induced CLEA showed enhanced thermal and storage stability [20]. Wilson et al. employed this co-aggregation technique for synthesizing CLEA of Penicillin-G acylase to enhance the stability of enzyme in organic medium. Here the CLEA prepared by PEI co-aggregation technique showed increased stability (∼25 fold higher) than the CLEA prepared by the conventional method [21]. The co-aggregation method has also been used to prepare stable CLEA of *Alcaligenes* sp. lipase and *Candida antarctica* B lipase. The catalytic properties of the lipase CLEA (such as specificity and enantioselectivity) were greatly modulated by the nature of co-aggregating polymers [22]. Pan et al. have recently reported the usefulness of PEI induced method of preparing CLEA for *Serratia marcescens* lipase. In this, CLEA showed enhanced thermal stability and excellent operational stability in repetitive use in aqueous-toluene biphasic system for asymmetric hydrolysis of trans-3-(4′-methoxyphenyl)glycidic acid methyl ester [30]. Mateo et al. prepared the co-aggregates of the nitrilases with high molecular weight PEI. Here, PEI induced co-aggregation technique increased the oxygen-resistance of nitrilase than free enzyme and conventionally cross-linked enzyme [31].

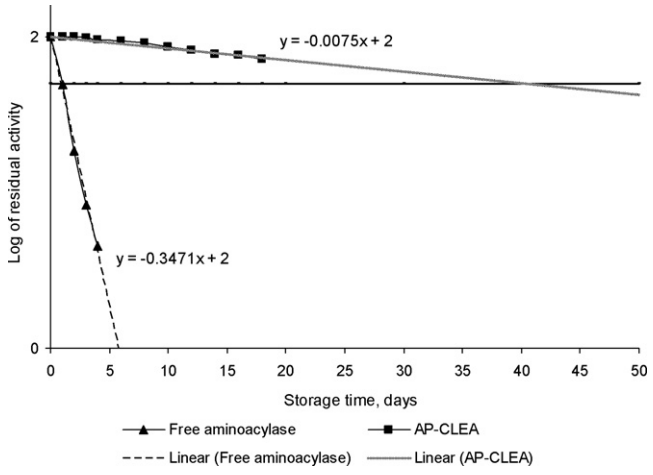

Fig. 6. Storage stability of AP-CLEA (Linear trend line equations were used to calculate the respective storage half-lives. The horizontal line (—) at log(50) is drawn merely for a graphical representation of the storage half-lives.).

In vitro stability of enzymes remains a critical issue in the field of biocatalysis. The practical usefulness of an enzyme is often decided by its storage and operational stability. Analogous to previous reports, the present study manifests that the PEI induced co-aggregation method of synthesizing CLEA confers great stability to the enzyme. In AP-CLEA, enzyme molecules are presumably held together by multipoint enzyme–PEI linkages which would have stabilized the native (and active) conformation of the enzyme against denaturizing forces. Besides this, the improved stability of AP-CLEA is complemented by the protein-protective nature of PEI. PEI in particular, is known to impede the process of oxidation of sulfhydryl groups of enzyme (which is a prevailing mechanism of enzyme inactivation during storage) [32].

3.3. AP-CLEA catalyzed chiral resolution

Aminoacylases have the ability to hydrolyze various amino acid derivatives (such as amino acid esters, amino acid amides and N-acetyl amino acids) in an enantioselective manner to yield enantiopure amino acids. 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. These unnatural amino acids are key chiral components of variety of pharmaceutical agents. For instance, phenylglycine is used in synthesis of ampicillin; (*S*)-homophenylalanine is a central chiral intermediate for synthesis of variety of antihypertensive agents (benzpril, enalapril, lisinopril, etc.); and (*S*)-2-naphthylalanine is a component of a peptide drug nafareline, which is used in symptomatic treatment of endometriosis [33].

Table 5
AP-CLEA catalyzed chiral resolutions.

Substrate	Preferred configuration	ee_p (%)	*C* (%)	*E*
(a) *Chiral resolution of amino acid esters*				
(*RS*)-Phenylglycine ethyl ester	*S*	64.7	35.2	6.5
(*RS*)-Homophenylalanine ethyl ester	*S*	82.4	27.9	14.1
(*RS*)-2-Naphthylalanine ethyl ester	*S*	67	41.7	8.1
(b) *Chiral resolution of amino acid amides*				
(*RS*)-Phenylglycine amide	*S*	98.5	44.2	>200
(*RS*)-Homophenylalanine amide	*S*	98.2	41.6	>200
(*RS*)-2-Naphthylalanine amide	*S*	96.4	43.9	124.4
(c) *Chiral resolution of N-acetyl amino acids*				
(*RS*)-N-acetyl-phenylglycine	*S*	92.5	43.3	54.3
(*RS*)-N-acetyl-homophenylalanine	*S*	93.5	41.8	60.0
(*RS*)-N-acetyl-2-naphthylalanine	*S*	87.4	32.9	22.6

Table 6
Summary of five repetitive batches of hydrolysis of *rac*-HPA-amide.

Batch number	ee_p (%)	*C* (%)	*E*	Yield of (*S*)-homophenylalanine (mg)	% Yield
1	98.5	44.3	>200	42.6	84.7
2	98.0	45.4	>200	43.7	86.9
3	98.6	44.1	>200	43.0	85.5
4	98.2	44.3	>200	42.4	84.3
5	98.3	44.0	>200	42.2	83.9
Total	98.3	–	–	213.9	85.0

3.3.1. Chiral resolution of amino acid esters

The results of AP-CLEA catalyzed enantioselective hydrolysis of amino acid esters are given in Table 5(a). AP-CLEA were observed to preferentially hydrolyze the *S*-enantiomer of amino acid esters. The enantiomeric ratios of AP-CLEA towards hydrolysis of phenylglycine ethyl ester, homophenylalanine ethyl ester and 2-naphthylalanine ethyl ester were 6.5, 14.1 and 8.1 respectively.

3.3.2. Chiral resolution of amino acid amides

The results of AP-CLEA catalyzed enantioselective hydrolysis of amino acid amides are given in Table 5(b). AP-CLEA were observed to preferentially hydrolyze the *S*-enantiomer of amino acid amides. The *E* values of hydrolysis of phenylglycine amide and homophenylalanine amide were >200 and that of hydrolysis of 2-naphthylalanine amide was >100 indicating the remarkable enantioselectivity of CLEA towards these hydrolytic reactions.

3.3.3. Chiral resolution of N-acetyl amino acids

The results of AP-CLEA catalyzed enantioselective hydrolysis of N-acetyl amino acids are given in Table 5(c). AP-CLEA were observed to preferentially hydrolyze the *S*-enantiomer of N-acetyl amino acids. CLEA exhibited higher enantioselectivity towards hydrolysis of N-acetyl-phenylglycine and N-acetyl-homophenylalanine as indicated from the higher *E* values (54.3 and 60.0 respectively) than that towards hydrolysis of N-acetyl-2-naphthylalanine ($E = 22.6$).

The process of co-aggregation followed by cross-linking occasionally alters the catalytic properties (e.g. substrate specificity, enantioselectivity) of an enzyme. For instance, drastic alterations in the enantioselectivity of lipase were observed when the enzyme was cross-linked by PEI-induced co-aggregation technique [22]. The extent of change in the catalytic properties of enzyme possibly depends on the nature of polymer, nature of enzyme and type of protein–polymer interactions. Considering the possibility of enantioselectivity modulation, we compared the enantiomeric ratios of free aminoacylase with that of AP-CLEA towards hydrolysis of unnatural amino acid amides. For the given amide substrate, no significant difference in the enantiomeric ratio of free enzyme and that of AP-CLEA was observed.

To summarize, enantiomeric ratios of the AP-CLEA were excellent ($E \approx 124$–200) towards hydrolysis of amides, moderate ($E \approx 23$–60) towards hydrolysis of N-acetyl derivatives and poor ($E < 15$) towards the hydrolysis of amino acid esters. Youshko et al. reported similar observations wherein free *A. melleus* L-aminoacylase enzyme gave higher enantiomeric ratios towards hydrolysis of phenylglycine amide and homophenylalanine amide ($E > 200$) than that towards hydrolysis of N-acetyl-phenylglycine and N-acetyl-homophenylalanine ($E = 96$ and 70 respectively) [6].

3.4. Operational stability of AP-CLEA

AP-CLEA catalyzed chiral resolution of (*RS*)-homophenylalanine amide was conducted in repetitive batch mode. The initial activity of AP-CLEA was 211.7 U g^{-1} and that after the fifth batch was 196.8 U g^{-1}. Thus, AP-CLEA were found to retain more than 92% of the initial activity after five consecutive batches which indicates an adequate operational stability of AP-CLEA. In the first batch, the maximum of 98.5% enantio-enrichment of the product (i.e. ee_p) was obtained at the reaction time of 70 min. In order to maintain the maximum possible enantio-enrichment of the product, the reaction time of all consecutive batches was carefully monitored. From five repeated batches, 213.9 mg of (*S*)-homophenylalanine having enantiomeric excess of 98.3% was obtained (Table 6).

4. Conclusions

Co-aggregation of enzyme with polyethyleneimine (PEI) facilitates formation of stable Cross-Linked Enzyme Aggregates (CLEA) of L-aminoacylase. The said method of co-aggregation and cross-linking confers significantly higher temperature and storage stability to the enzyme without affecting its enantioselectivity. Aminoacylase-PEI CLEA (termed as AP-CLEA) can catalyze enantioselective hydrolysis of amino acid amides, N-acetyl amino acids and amino acid esters. The enantioselectivity of AP-CLEA was remarkably high for hydrolysis of amino acid amides; was moderate for hydrolysis of N-acetyl amino acids and was poor for hydrolysis of amino acid esters.

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