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Adding an appropriate amino acid during crosslinking results in more stable crosslinked enzyme aggregates

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ABSTRACT

Carrier free immobilization, especially crosslinked enzyme aggregates (CLEAs), has become an important design for biocatalysis in several areas. Adding amino acids during formation of CLEAs was found to give biocatalysts more stable at 55 °C and in the presence of 60% acetonitrile. The half-lives of CLEAs prepared with and without Arg addition were 21 and 15 h (subtilisin) and 4 and 1.6 h (α-chymotrypsin) at 55 °C, respectively. The corresponding half-lives during acetonitrile presence were 4.1 and 3.0 h (subtilisin) and 39 and 22 min (α-chymotrypsin), respectively. CLEAs made with Arg had higher percentages of alpha helix. CLEAs made by adding Lys, Ala, or Asp also were more stable. In the case of *Thermomyces lanuginosus* lipase (TLL), CLEA with Ala was even more stable than CLEA with Arg. The addition of a suitable amino acid, thus, enhances CLEA stabilities. The results are discussed in the light of earlier results on chemical modification of proteins and the observation that the Arg/Lys ratio is invariably high in the case of enzymes from thermophiles.

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Enzyme stabilization for catalysis under harsh conditions, such as high temperature and the presence of organic solvents, continues to attract attention [1–3]. Carrier free immobilization, especially crosslinked enzyme aggregates (CLEAs) as immobilized enzyme forms, has been investigated extensively [4–6]. High volumetric activity, robust design, and a simple protocol that does not require the use of pure enzymes (as in the case of crosslinked enzyme crystals [7]) have made this approach one of the preferred methods of immobilization over the past few years.

The current work describes a simple variation of the protocol that results in CLEAs that are more stable than the original design. The approach is essentially based on two considerations. The first is that optimization of surface charges leads to greater stability of enzymes [8–12]. The second is that proteins from thermophilic organisms tend to have higher Arg/Lys ratios on their surfaces [13,14].

Based on the above, in addition to Arg, some other amino acids such as Ala (a hydrophobic amino acid), Asp (an acidic amino acid), and Lys (another basic amino acid) were tried as additives during the CLEA formation. Two major applications of CLEAs have been their use at high temperatures (to accelerate reactions) and their use in organic solvents (to enable higher synthesis/hydrolysis ratios with hydrolases) [1–7]. Hence, enzyme activities (employing standard assay conditions) under these conditions are generally measured to evaluate their stability under these conditions. The term "stability" is used in the restricted sense of "conformational stability." This is in line with the current practice while evaluating various CLEAs [1–7]. The resultant CLEAs were evaluated for their stability at high temperature and for catalysis in organic solvents.

The approach was tried with the proteases subtilisin and α-chymotrypsin as well as with a lipase. Both proteases and lipases represent two of the most often used classes of enzymes in biotechnology [15,16].

Materials and methods

Materials

α-Chymotrypsin (bovine pancreas) and subtilisin Carlsberg (from *Bacillus licheniformis*) proteases and the substrates benzoyl tyrosine ethyl ester (BTEE) and *p*-nitrophenyl palmitate were

Abbreviations used: CLEA, crosslinked enzyme aggregate; BTEE, benzoyl tyrosine ethyl ester; TLL, *Thermomyces lanuginosus* lipase; HPLC, high-performance liquid chromatography; SEM, scanning electron microscopy; FT-IR, Fourier transform infrared.

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purchased from Sigma (St. Louis, MO, USA). *Thermomyces lanuginosus* lipase (TLL) was a kind gift from Novozymes (Denmark). All amino acids (>99%) were purchased from Spectrochem (India). Glutaraldehyde (25% aqueous solution) was purchased from Merck (Hohenbrunn, Germany). Acetonitrile (HPLC grade, 99.9%) was bought from Sigma. Acetone (HPLC grade, 99.5%) was purchased from Qualigens (Mumbai, India).

Methods

Preparation of CLEAs

Briefly, 100 μl of subtilisin Carlsberg protease solution (10% [w/v] in 0.1 M Tris–HCl buffer, pH 7.8) was mixed with 90 μl of arginine solution (10 mg/100 μl in 0.1 M Tris–HCl buffer, pH 7.8). This resulted in enzyme solutions containing 90% (w/w) arginine (protease). Precipitation was carried out by adding these protease solutions dropwise into 900 μl of dry ice-cold acetone, followed by crosslinking by 40 mM glutaraldehyde solution (25%, Merck) at 4 °C with shaking at 200 rpm for 2 h. The CLEAs were recovered from the aqueous suspension by centrifugation at 8000 g, washed with ice-cold 50% ammonium sulfate solution, and stored in buffer. Similarly, α-chymotrypsin and TLL CLEAs were made by 0.1 M Tris–HCl buffer (pH 8.0). The amount of arginine added was 75 μl (10 mg/100 μl in 0.1 M Tris–HCl buffer, pH 8.0), and the amount of glutaraldehyde solution used was 100 mM. CLEAs from proteases without arginine, under the same conditions, were made as controls [4]. The glutaraldehyde concentration used was in the same range as that used in earlier works [4]. The concentration of arginine and the time period of crosslinking were optimized in the cases of subtilisin (see Fig. S1 in online supplementary material) and α-chymotrypsin (Fig. S2). The optimal concentrations were used for preparing CLEAs that were further studied. In the case of lipase, similar conditions as those for α-chymotrypsin were used. In the case of crosslinking in the presence of other amino acids, the same concentrations of those amino acids were used.

Thermal stability of aggregates

CLEAs were suspended in buffer solution (1 mg/ml) along with the free enzyme (in a different experimental set) and were incubated at 55 °C with constant shaking at 200 rpm in an orbital shaker. Aliquots were taken at different time intervals, cooled to the assay temperature, and checked for residual activity (the assay is described later). The initial activity of the enzyme formulations at zero time of incubation was taken as 100%.

Deactivation of CLEAs in organic cosolvent

CLEAs were suspended in different organic cosolvent mixtures (60% [v/v] in buffer) along with the control (free enzyme) and were incubated at 25 °C with constant shaking at 200 rpm in an orbital shaker. Aliquots at different time intervals were taken, and the residual hydrolytic activities were checked after assaying in the same cosolvent mixture. During assay, the identical denaturing condition (60% organic solvent with 40% water) was maintained to avoid the possibility of gaining some of its (enzyme's) lost activity in predominantly aqueous medium. The initial activity of each formulation at zero time of incubation was taken as 100%.

Assay for proteases and lipases

The esterase activities of subtilisin Carlsberg and α-chymotrypsin proteases were measured by the assay described by Walsh and Wilcox [17]. In a cuvette, 1.4 ml of BTEE solution (0.001 M in 50% [v/v] methanol) was added to 1.5 ml of Tris–HCl buffer (0.08 M, pH 7.8, containing 0.1 M calcium chloride). Hydrolysis was initiated by adding 0.1 ml of enzyme solution (0.1 mg/ml). The amount of product formed was followed by monitoring the absorption changes at 256 nm.

For lipases, the hydrolysis of *p*-nitrophenyl palmitate as described by Jain and coworkers [18] was followed.

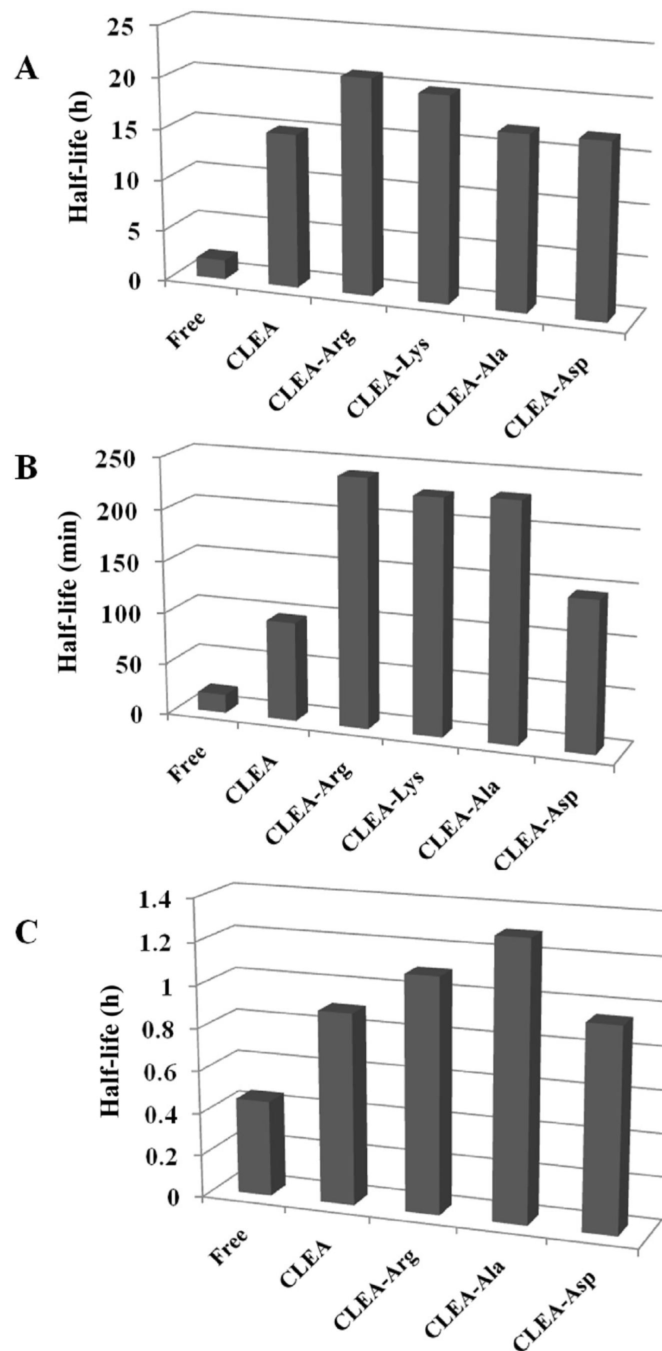

Fig.1. Thermal stability (half-life) of subtilisin (A), α-chymotrypsin (B), and TLL formulations (C) at 55 °C. CLEAs were suspended in buffer solution and incubated at 55 °C under constant shaking at 200 rpm in an orbital shaker. Aliquots were taken at different time intervals, cooled to the assay temperature, and checked for residual activity. The initial activity of the enzyme formulations at zero time of incubation was taken as 100%. Deviation among the three readings of a triplicate set was within 3%.

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