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Immobilization of thermostable α-amylase from *Bacillus licheniformis* by cross-linked enzyme aggregates method using calcium and sodium ions as additives

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ABSTRACT

Cross-linked enzyme aggregates (CLEAs) of thermostable α-amylase from *Bacillus licheniformis* have been prepared for starch liquefaction. Among the six different precipitants, *tert*-butanol performed the best with an aggregation efficiency of 99.54% for starch hydrolysis. The optimal conditions for the immobilization process required 5 mM glutaraldehyde, 0.96 mg/mL enzyme, 1:2 ratio (0.5 ratio) of enzyme/bovine serum albumin (BSA), and 12 h crosslinking at 2–3 °C. Starch was used as the main substrate in enzyme assay. Immobilization did not affect pH (5.5) and temperature (95 °C) optima of free α-amylase thus, these values remained constant for produced CLEAs. Different concentrations of calcium and sodium ions were added to the enzyme during the aggregation process. It was observed that simultaneous addition of both calcium and sodium (1200 ppm of calcium and 400 ppm of sodium ions, in a ratio of 3:1) significantly improved the catalytic efficiency (k_{cat}/K_m), of CLEAs–BSA–CN from 3.91×10^5 to 4.57×10^5 (1.2-folds) compared with free enzyme. Although immobilization did not significantly affect V_{max} (5.35–5.25), substrate affinity of the enzyme increased (1.34–1.12) after addition of mixed ions to the prepared CLEAs. Moreover, compared with free α-amylase, enzyme half-life ($t_{1/2}$) of CLEAs–BSA–CN increased from 43.31 to 115 min (about 3-folds) at 110 °C. The CLEAs–BSA–CN retained 76% residual activity even after 10 cycles of reuse.

1. Introduction

Amylases have a broad range of applications in liquefaction and saccharification of starch. These enzymes are increasingly used in food, pharmaceutical, paper, textile, and detergent industries [1]. Among the starch-hydrolyzing enzymes, thermostable α-amylases are industrially important enzymes that are used for large-scale liquefaction of starch into malto-oligosaccharides. α-Amylase (α-1,4 glucan-4-glucanohydrolase, EC 3.2.1.1) is an amylolytic endo enzyme, which acts on the internal α-1,4-glycosidic linkages in amylose and amylopectin. Despite the strong sequence similarity between the thermophile *Bacillus licheniformis* (BLA) and other α-amylase producing bacilli, the α-amylase from *B. licheniformis* exhibits more thermostability than those from the other amylase producers [2,3]. Because of its considerably higher thermal resistance, this α-amylase is preferred and significantly used in starch liquefaction processes [4]. The optimal temperature of BLA is about 90–95 °C and requires the addition of Ca^{2+} ions for maintenance of optimal thermostability [5].

Enzyme immobilization is a highly advantageous method for protecting the enzyme from harsh conditions and conserving the functional stability of the enzyme during industrial applications. This leading technique promotes several beneficial features of the enzyme such as offering greater stability and improvement of activity, resistance to inhibition, selectivity or specificity, higher catalytic performance, and reusability [6]. Basically, enzymes are immobilized through three main ways that are binding to a carrier, encapsulation or entrapment, cross-linking (carrier free) [7]. Enhancement of enzyme stability is the most important effect of enzyme immobilization. Improvement of rigidity in enzyme

Abbreviations: CLEAs, cross-linked enzyme aggregates; BSA, bovine serum albumin; V_{max}, maximum velocity; K_m, Michaelis constant; k_{cat}, catalytic constant; k_{cat}/K_m, catalytic efficiency; k_i, inactivation constant; $t_{1/2}$, enzyme half-life; CLEAs–BSA, CLEAs with BSA; CLEAs–BSA–Na, CLEAs with BSA and sodium ion; CLEAs–BSA–Ca, CLEAs with BSA and calcium ion; CLEAs–BSA–CN, CLEAs with BSA and calcium and sodium ions.

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conformation is a reason for prevention of unfolding of the enzyme structure [8,9]. Moreover, enzyme immobilization makes possible reusability of the biocatalyst as well as their use in continuous operations, so reduces the amount of enzyme used and product costs significantly [8]. Another factors that may affect enzyme activity are substrate and/or product inhibition. It is reported that, in special cases immobilization can avoid or reduce enzyme inhibition [9]. Cross-linked enzyme aggregates (CLEAs) is a new generation of biocatalyst that can produces by physical aggregation without denaturation in an ionic, water-miscible organic solvents, or nonionic polymers environment. Cross-linking makes an insoluble aggregates together with preserved superstructure and catalytic activity of the enzyme [7].

This approach has significant benefits, such as the elimination of additional carriers, achieving highly concentrated enzyme activity, and higher enzyme stability [10].

One of the main advantages is that the CLEAs can be produced without extensive protein purification because it combines both purification and immobilization [11,12], into a single unit operation that does not require a highly pure enzyme [7,13].

The present work, introduces a novel two-step method for the preparation of CLEAs through the aggregation and crosslinking of thermostable α-amylase enzyme in a calcium and sodium ion-enriched non-aqueous environment. To the best of our knowledge, thus far, there have been no studies published on the preparation of thermostable α-amylase CLEAs using the cation-assisted strategy. To this end, we have optimized conditions for immobilizing enzyme using Liquozyme Supra, and characterized the properties of the enzyme such as optimum temperature and pH, thermal inactivation and half-life, reusability, and kinetic parameters of the resultant biocatalysts. Here, we present the use of the industrially important thermostable α-amylase from *B. licheniformis* for CLEA preparation and its enzymatic characterizations.

2. Materials and methods

2.1. Chemicals

Thermostable α-amylase (EC 3.2.1.1) from *B. licheniformis* (Liquozyme Supra, 135 KNU/g) was obtained from Novozymes. Glutaraldehyde (25% v/v in water), bovine serum albumin (BSA), and starch (in granule size from 2.83 to 289 μm), were purchased from Merck. All of the other reagents and solvents used were of analytical grade and obtained from Merck and Sigma–Aldrich.

2.2. Protein assay

Protein concentration was determined according to the Bradford method using bovine serum albumin (BSA) as the standard protein [14].

2.3. Enzyme assay

The activity of thermostable α-amylase was estimated by measuring the amount of reducing sugars released from starch using the 3,5-dinitrosalicylic acid (DNS) method. Soluble starch (1% w/v) in 100 mM sodium acetate buffer (pH 5.5) was used as a substrate [15]. The assay mixture contained 10 μL (0.096 mg) of thermostable α-amylase (Liquozyme supra), 500 μL of 1% starch suspension, 9500 μL of 100 mM sodium acetate buffer (pH 5.5), and 10 μL calcium chloride (Ca^{2+} 15 ppm). The mixture was incubated at 95 °C for 0–120 min. The reaction was stopped by adding 500 μL of DNS reagent to 500 μL of hydrolyzed mixture and incubating the mixture at 97–98 °C for 10 min. The absorbance was read at 575 nm using a Perkin Elmer, Lambda 25 UV/VIS spectrophotometer in cells with 1-cm path length. One unit of α-amylase was defined as the amount of enzyme that liberated 1 μmol of glucose per minute under the assay conditions. To ensure reproducibility of the results, each assay was repeated at least three times [16].

2.4. Preparation of CLEAs

The thermostable α-amylase was aggregated and cross-linked using a two-step procedure, according to a previously described method [17]. The first step, aggregation, was accomplished by adding 100 μL enzyme solution containing 0.961 mg α-amylase, and different concentrations (0.25–50 mg) bovine serum albumin (BSA) to 900 μL of precipitant (isopropanol, acetone, *tert*-butanol, acetonitrile, ethanol, and saturated ammonium sulfate). The suspension of α-amylase aggregates was thoroughly mixed and kept in ice for 20 min. The second step, cross-linking, was performed by addition of a certain amount of glutaraldehyde 25% v/v in water (cross-linker) after the precipitation step, followed by overnight incubation at 2–3 °C. Subsequently, the CLEAs were separated from the supernatant by centrifuging (Sigma, 2K 15) at 13,308 × *g* for 10 min at 4 °C. In order to removing the excess residual glutaraldehyde, the produced CLEAs were re-suspended in 1 mL 50 mM phosphate buffer, pH 7.0 centrifuged at 13,308 × *g* for 10 min, and washed using phosphate buffer, pH 7.0 for three times. Next, the CLEAs were resuspended in 100 μL of 0.1 M sodium acetate buffer of pH 5.5 by thorough mixing. A sample was withdrawn from the resulting suspension, which contains both CLEAs and the residual free enzyme, and was evaluated for α-amylase activity. Then, the CLEAs were separated by centrifugation, and the supernatant containing only the free enzyme was resampled for activity measurements.

The activity of CLEAs was calculated as the difference in α-amylase activity between the sample containing CLEAs and the sample with only the free enzyme. This is a precise and easy method to determine the exact activity of the CLEAs.

2.5. Effect of pH on free and immobilized α-amylase activity

The optimum pH for α-amylase activity was studied by preincubating the enzyme for 30 min at 95 °C with various buffer over a pH range of 5–9 using starch suspension (1% w/v) as substrate to determine the activity of free as well as immobilized enzyme. In this case, sodium acetate buffer 100 mM (pH 5.0–6.5), and phosphate buffer 100 mM (pH 7.0–9.0) were used to determine enzyme activity by DNS method.

2.6. Effect of temperature on free and immobilized α-amylase activity

The effect of temperature on activity of free and immobilized enzyme was determined by carrying out starch hydrolysis at different temperatures ranging from 60 to 110 °C and pH 5.5 for 30 min. Enzyme activity was determined by DNS method as described earlier.

2.7. Effect of cations on the activity and stability of CLEAs

To determine the stability of α-amylase CLEAs and its dependence on both Na^+ and Ca^{2+} ions, concentrations of 0–2200 ppm of Ca^{2+} in form of $CaCl_2$, and 0–800 ppm of Na^+ in form of NaCl were added separately to 100 μL liquid α-amylase (0.96 mg) and aggregated by adding 900 μL of precipitant (isopropanol, acetone, *tert*-butanol, acetonitrile, ethanol 96°, saturated ammonium sulfate) to the enzyme mixture. The suspension was entirely mixed and kept in ice for 20 min. Next, specified amounts of glutaraldehyde 25% v/v in water (cross-linker) was added, and the samples were incubated in the fridge (2–3 °C) overnight. The CLEAs

were separated from the supernatant (refrigerated centrifuge, at 13,308 × *g* for 10 min at 4 °C) and washed thrice with phosphate buffer of pH 7.0. The collected CLEAs were dissolved in 100 μL of sodium acetate buffer of pH 5.5, and were used to assess the effect of cations on the activity and stability of produced α-amylase CLEAs.

2.8. Thermal stability

Solutions containing CLEAs–BSA–CN, CLEAs–BSA, or free α-amylase (0.961 mg/100 μL liquid α-amylase) and soluble starch (1% w/v) in 100 mM sodium acetate buffer (pH 5.5) as substrate were incubated at 110 °C for 120 min. Aliquots were withdrawn and the residual activity (RA%) was measured at different incubation times (0–120 min) using the standard DNS method. Half-life was defined as the time, in minute, required for the residual enzymatic activity in the sample reaches one half of its initial value [18].

Based on the data captured by thermal stability study, the inactivation rate constant (k_i) was determined and the apparent half-life ($t_{1/2}$) was estimated by using Eq. (1) [19,20]

$$t_{1/2} = \frac{\ln 2}{k_i} \quad (1)$$

2.9. Recycling of immobilized α-amylase

The reusability of CLEAs was measured by frequent use of the immobilized α-amylase in liquefaction of starch. Final CLEAs that was prepared by adding of 400 ppm Na^+ and 1200 ppm Ca^{2+} were retrieved from the reaction media through filtration using a Whatman paper with a pore size of 0.2 mm. The activity of α-amylase CLEAs (0.96 mg) were analyzed using the DNS method, after repeated application of the same biocatalyst for starch hydrolysis. For biocatalyst reuse, after performing the enzymatic reaction for 120 min at 95 °C and 250 rpm shaking, the mixture was filtered and the recovered immobilized enzyme was washed thrice with freshly prepared 50 mM sodium phosphate buffer at pH 7.0, and used for another conversion cycle.

2.10. Determination of kinetic parameters

K_m and V_{max} values of the free and immobilized α-amylase were determined by Lineweaver–Burk method using various substrate (starch) concentrations (0.25–5 mg/mL) in sodium acetate buffer (0.1 M, pH 5.5) at 93–95 °C. All the experiments were carried out at least in triplicate, and the results were presented as their mean value. Experimental error seldom exceeded 5%.

2.11. Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) of free α-amylase and CLEAs was performed with a MIRA/TESCAN scanning electron microscope (USA). To record SEM images, a sample was dried with anhydrous acetone and then placed on a carbon tape over a microscope slide to coat with gold under vacuum. Representative micrographs were obtained for each sample at magnifications of 500× and 1500×.

2.12. Statistical analysis

All the values expressed were mean ± standard deviation of three replicate experiments.

3. Results and discussion

CLEAs were produced by enzyme precipitation by adding a precipitant such as salt or an organic solvent [21], followed by cross-linking using a bifunctional reagent such as glutaraldehyde, which reacts with the surface amino groups of enzymes and carriers [22].

3.1. Selection of compounds with best aggregation properties for enzyme precipitation

Aggregation of protein molecules into cumulative molecular structures without disrupting the original three-dimensional structures of the protein can be achieved by exposure to an environment with high ionic strength (salts), non-ionic polymers, or organic solvents [23]. Initially, we studied the effect of various precipitants on the specific activity of the aggregated α-amylase from *B. licheniformis*, prior to cross-linking. A nine-fold volume of precipitants was added to the enzyme cocktail, and specific activities of the resuspended enzyme aggregates were determined.

Saturated ammonium sulfate solution, ethanol, *tert*-butanol, acetone, acetonitrile, and isopropanol were used as precipitants [13]. The aggregation efficiency was evaluated by calculating the difference between the enzymatic activity of the resuspended aggregates combined with the remaining supernatant after centrifugation (13,308 × *g*, 10 min), and the activity of the free enzyme present only in the supernatant [11]. The specific activity of α-amylase is expressed in units per mg of total protein, and the results are shown in Table 1. The maximum specific activity and aggregation efficiency in the precipitation step was observed with *tert*-butanol. In contrast, α-amylase aggregates prepared in saturated ammonium sulfate showed lower activities under the same conditions. The control showed the activity of soluble enzyme with no addition of precipitant, at pH 5.5, which corresponds to the conditions usually applied in hydrolysis reactions. Since *tert*-butanol gave the best overall results, it was chosen as a suitable precipitant for CLEAs preparation in further experiments.

3.2. Effect of glutaraldehyde concentration and cross-linking time

Glutaraldehyde is one of the most widely used reagents in the design of biocatalysts [6]. The amino groups of lysine residues on the external surface of the enzymes involves in cross-linking [7].

Cross-linker concentration also had an important effect on the activity of CLEAs. In this case enzymes exhibit a lower catalytic performance (also cause by real effects on enzyme structure) [9]. For optimization of the cross-linking step, the effect of parameters such as the cross-linker amount and cross-linking time on the activity of the resultant CLEAs was studied. The results are shown in Fig. 1a. At this stage, different concentrations of glutaraldehyde (1–100 mM) were employed using *tert*-butanol as a precipitant for CLEAs preparation under storage conditions for overnight (about 12 h) to determine the optimum glutaraldehyde concentration required for stable cross-linking of α-amylase.

A glutaraldehyde concentration of 5 mM exhibited the highest activity. Thus, maximum enzyme activity was observed at a cross-linker to enzyme molar ratio of 3.05×10^5 (5 mM glutaraldehyde in CLEA formation environment) and in contrast to the free enzyme, the highest enzyme activity of CLEAs was observed when a 5 mM glutaraldehyde concentration was used.

At low glutaraldehyde concentrations (less than 5 mM), the activity of CLEAs was lower possibly due to insufficient crosslinking, leading to unstable aggregation and leaching of free enzyme into the aqueous phase. Similarly, at high glutaraldehyde concentrations (exceeding 5 mM), excessive cross-linking restrains the enzyme's flexibility, and limits mass transfer, leading to reduced enzymatic activity. Similarly, the activity also decreases with longer cross-linking times, probably due to the same reasons (Fig. 1b). In order to determine the optimal cross-linking time required to obtain a firm and stable α-amylase CLEAs, the enzymatic activity

Table 1
Selection of a precipitant optimal for enzyme aggregation.

Precipitants	Specific activity (μmol min^{-1} mg^{-1})			
	Control	Aggregates	Supernatant	Aggregation efficiency (%)
Ammonium sulfate	–	1.707	0.489	56.10
Ethanol	–	1.818	0.354	67.43
Acetone	–	1.836	0.343	68.77
Acetonitrile	–	1.950	0.229	79.27
Isopropanol	–	2.021	0.156	85.90
tert-Butanol	–	2.167	0.006	99.54
Free enzyme	2.171	–	–	–

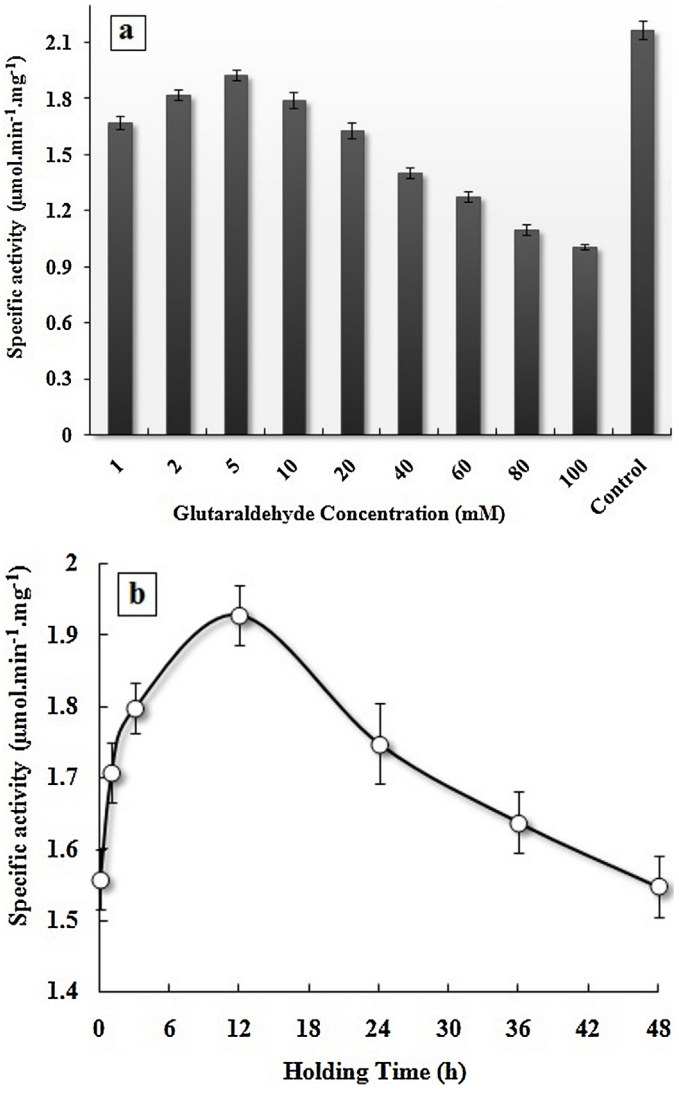

Fig. 1. (a) Effect of glutaraldehyde concentration on the activity of CLEAs. (b) The effect of cross-linking time on the enzymatic activity of the resultant CLEAs.

was assayed after subjecting the enzyme to cross-linking for various time intervals between 1 and 48 h at 2–3 °C. An increase in cross-linking time until 12 h, significantly increased the enzymatic activity, although cross-linking for longer time periods exceeding 12 h, resulted in reduced activity. Optimal cross-linking occurred within 12 h of incubation, where a maximum activity of 88.76% was recovered and hence followed in all further experiments.

Based on the possibility that an optimum number of free amino groups are present on the enzyme surface thus, if there are too few, insufficient cross-linking is occurred and improper CLEAs is produced. Unlike, extremely cross-linking results enzyme flexibility and activity reduction. Thus, for a given number of free amino groups one would expect there to be an optimum glutaraldehyde concentration more than that result in a loss of the minimum flexibility needed for activity. It is concluded that, based on resulted data the concentration of glutaraldehyde is important, and can influence the performance of CLEAs [24].

3.3. Effect of BSA on the quality and stability of α-amylase CLEAs

Despite the numerous benefits for using of CLEAs method for enzyme immobilization that are discussed, reduction in substrate and product diffusion rate is a limited factor in order to, formation of a quite small pore size in produce CLEAs. For that reason, reduction in activity of immobilized enzyme is observed. This problem (unwanted effect) can be overcomed by adding an inert protein during enzyme aggregation in CLEAs processing [25]. Addition of BSA to the enzyme solution (co-precipitation) increases the total protein content and increases the accessibility of highly reactive amino groups of lysine [6,26].

Subsequent addition of glutaraldehyde to this concentrated protein solution in the presence of adequate free amino groups facilitates the production of insoluble and active CLEA particles through efficient cross-linking, prevention of extensive cross-linking of the enzyme molecules, and preservation of structural integrity of the enzyme [27,26]. Fig. 2a shows that CLEAs that is prepared by co-precipitation with BSA exhibited about 6.2% higher conversion than the CLEAs that is formed without BSA addition. Therefore, BSA addition during CLEAs preparation improved the activity of α-amylase CLEAs. Moreover, selection of an optimal ratio between enzyme and BSA is so important in co-precipitation because, below this optimal ratio produced CLEAs has an unstable structure thus leach and losses during washing process while, addition of excessive amounts of BSA (higher than optimal ratio) prevents necessary cross-linking of α-amylase molecules, caused by competition between the free amino groups of BSA and those of the α-amylase [28]. To evaluate the effect of enzyme/BSA ratio on the activity of CLEAs, different α-amylase to BSA ratios (4, 2, 1, 0.5, 0.2, 0.1, 0.05, 0.03, 0.025, and 0.02) were prepared during the aggregation process, and the activity of resultant CLEAs were analyzed as described previously (Fig. 2b). Among the different enzyme/BSA ratios that were studied, an enzyme to BSA ratio of 0.5 was found to be optimal and led to the recovery of maximal activity (90.19%). Based on these results, a ratio of 0.5 α-amylase to BSA was used in all further experiments.

3.4. Effect of pH on the activity of α-amylase CLEAs

The effect of pH on both free α-amylase and CLEAs activities were determined in the pH range of 5–9. The pH optima of free and immobilized enzymes for starch hydrolysis were found to be 5.5. Therefore, based on resulted data, there was no significant change in the pH optimum of the enzyme after CLEAs preparation.

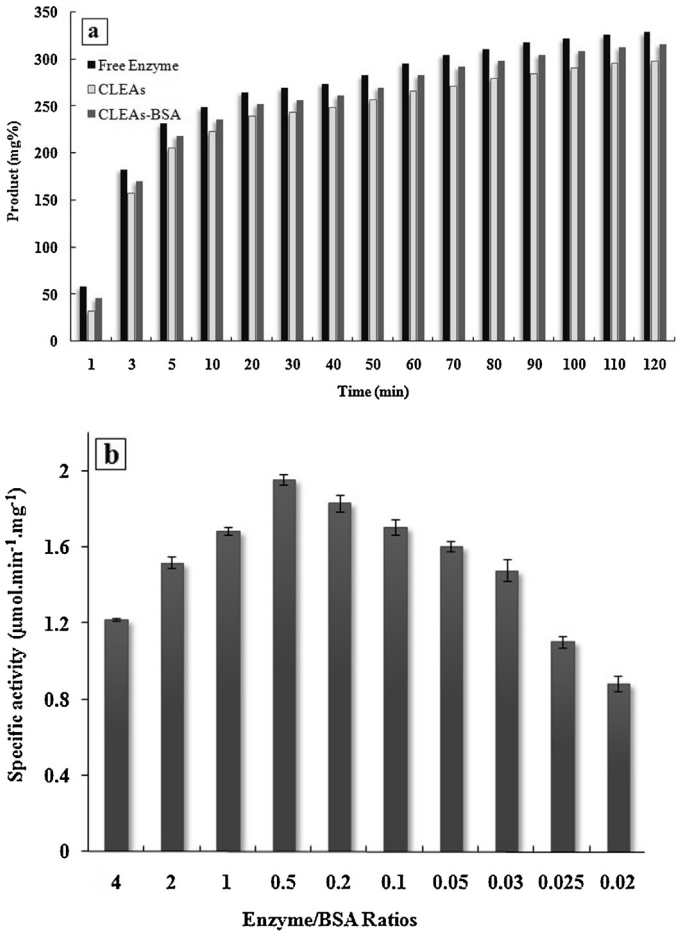

Fig. 2. (a) Effect of BSA on α-amylase CLEAs performance in starch hydrolysis. The figure shows higher percent value for starch conversion to product for CLEAs produced in the presence of BSA compared to CLEAs prepared without BSA and that of the free enzyme. (b) Effect of varying enzyme to BSA ratios on the activity of produced CLEAs.

3.5. Effect of temperature on the activity and stability of α-amylase CLEAs

The effect of temperature on the free and immobilized α-amylase CLEAs was also examined. The samples were subjected to different temperatures ranging from 60 to 110 °C at a pH of 5.5 for 60 min. At the end of the incubation period, the samples were cooled and their activity was assayed using the DNS method. The results signified that the α-amylase CLEAs reached their maximum activity at 95 °C and pH 5.5. However, the activity of CLEAs gradually decreased at higher temperatures exceeding 110 °C, as a result of enzyme denaturation (Fig. 3). It was also noted that the reduction in the activity of CLEAs at higher temperatures followed a more gentle slope compared with that of the free enzyme.

3.6. Effect of metal ions on CLEAs activity

A majority of amylases are recognized as metal ion-dependent enzymes, and depend on Ca^{2+} and Na^{+}, which are known to effectively increase the activity of *B. licheniformis* α-amylase [29,30]. The calcium ions assist desalting, expose the hydrophobic residues of the protein, resulting in the formation of a dense protein structure. This in turn renders structural integrity and thermostability to the protein [31]. It is recognized that the metal ions aid the substrate–enzyme interaction and maintain the structural

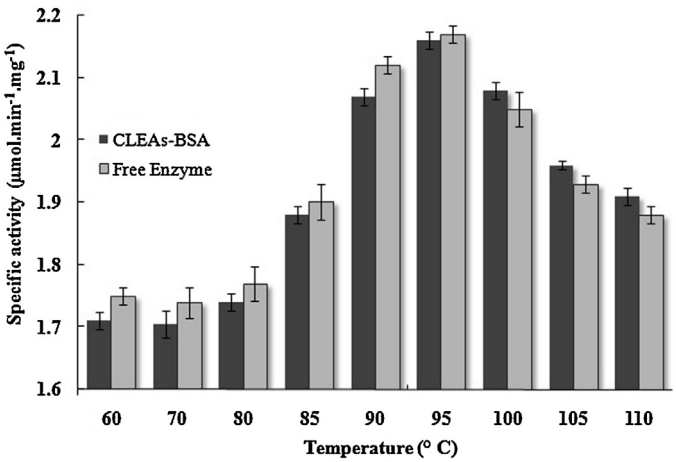

Fig. 3. Thermostability of CLEAs and free α-amylase. Soluble α-amylase represented as gray bars and CLEAs are represented by the corresponding black bars at 60–110 °C. The data points represent the mean values of three replicates with ±SD.

conformation of the enzyme–substrate complex at the active site of the enzyme.

3.6.1. Effect of Na^{+} ions on the stability of α-amylase CLEAs

The results revealed that, when α-amylase CLEAs were prepared in the presence of 0–800 ppm Na^{+} ions at 2–3 °C, the highest activity of CLEAs was observed at 400 ppm Na^{+} concentration (1.99 μmol min^{-1} mg^{-1}) compared to the activity of CLEAs produced in the absence of Na^{+} ions (1.95 μmol min^{-1} mg^{-1}) that indicated 2.1% increase in specific activity (Fig. 4a).

3.6.2. Effect of Ca^{2+} ions on the stability of α-amylase CLEAs

The effect of calcium ions at different concentrations (0–2200 ppm) during aggregation and formation of α-amylase CLEAs was evaluated. Swamy et al. [32] in their study revealed that Ca^{2+} enhances the thermostability and activity of α-amylases, when the temperature is increased. This effect indicates the protective nature of Ca^{2+} cation on amylase activity. In this study, we observed that α-amylase CLEAs formed in the presence of 1200 ppm Ca^{2+} exhibited the highest amylase activity (2.02 μmol min^{-1} mg^{-1}) compared to CLEAs synthesized in the absence of Ca^{2+} (about 4% increase). Moreover, a gradual decline was observed with further increases in Ca^{2+} concentrations between 1200 and 2200 ppm (Fig. 4b).

3.6.3. Effect of mixed ions (Ca^{2+} and Na^{+}) on the activity of α-amylase CLEAs

The previous experiments showed that the addition of calcium and sodium ions during the aggregation step improved the activity of CLEAs.

Therefore, the combined effect of the two ions on the CLEAs performance at 2–3 °C was studied. In this case, two cations were added simultaneously to the enzyme during aggregation process (Fig. 4c).

The results implied that CLEAs synthesized in the presence of cations mixture (Ca^{2+} 1200 ppm and Na^{+} 400 ppm) showed a 10.41% increase in activity unlike that in the CLEAs produced without the addition of mixed ions. Principally, all cognizant α-amylases have a preserved calcium ion in a particular location between the domains A and B, and are generally considered to be necessary for having an active enzyme with a constant structure. It has been proposed that the preserved calcium ion has structural significance, as it is very distant from the active site of the enzyme to participate directly in catalysis. Furthermore, it has been observed that in several amylase structures, more than one calcium ion exists.

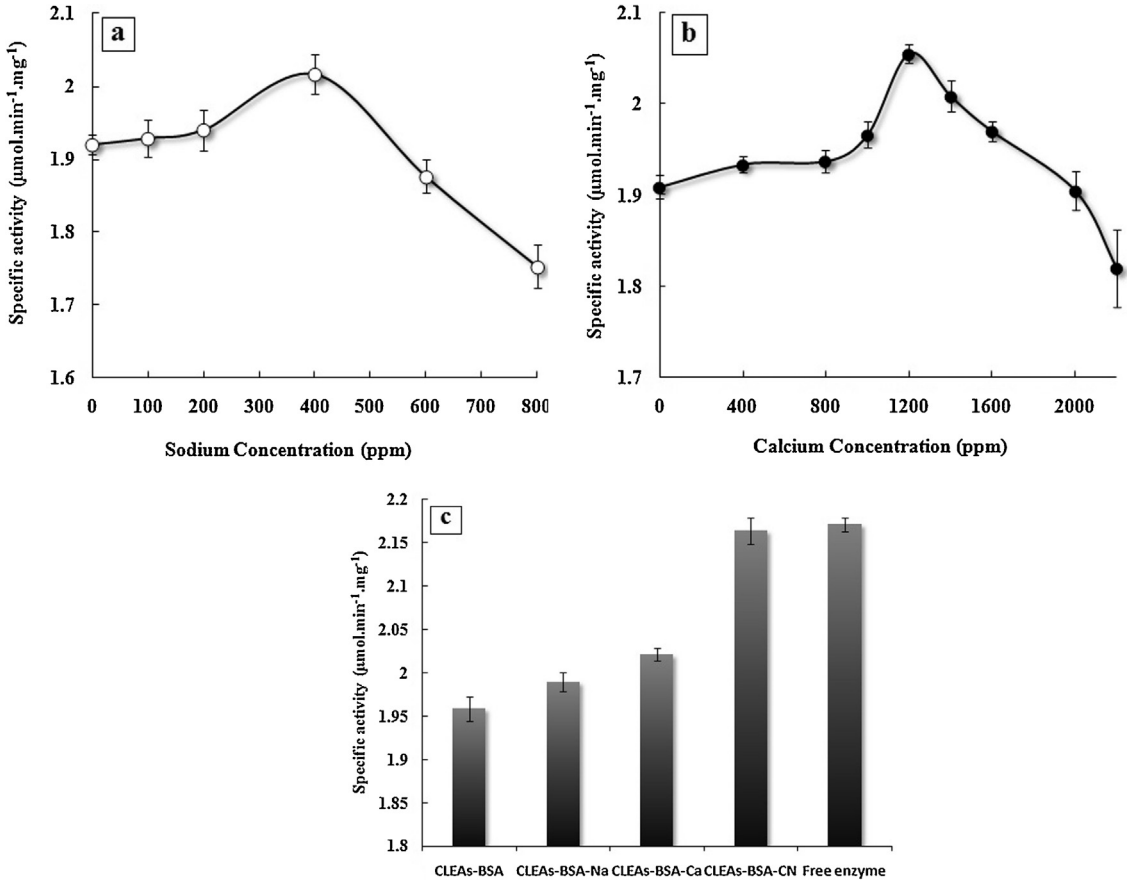

Fig. 4. (a) The effect of different concentration of sodium ions on the specific activity of α-amylase CLEAs. (b) The effect of different concentration of calcium ions on the specific activity of α-amylase CLEAs. (c) The effect of mixed cations ($3Ca^{2+}$:$1Na^{+}$) on the specific activity of CLEAs compared to the activity observed with the addition of calcium and sodium separately as well as CLEAs alone.

For example, a linear Ca–Na–Ca arrangement is established in *B. licheniformis* α-amylase [33].

In this case, we surprisingly found that the ideal ratio of Ca^{2+} to Na^{+} ions that is required for the formation of α-amylase CLEAs is exactly the same as the 3:1 ratio of Ca^{2+} to Na^{+} ions present in the molecular structure of α-amylase from *B. licheniformis*.

3.7. Reusability

Immobilization of enzymes provides an attractive opportunity for multiple use of the same biocatalyst.

The major purpose for the synthesis of CLEAs is to ultimately design suitable reusable immobilized biocatalysts that can be easily removed from the reaction medium during downstream processing, with maximum recovery of enzymatic activity for improving production costs of industrial processes. To this end, the efficiency of reusing α-amylase CLEAs was evaluated up to 15 cycles (Fig. 5). The activity of α-amylase CLEAs remained 76.1% of its initial activity after 10 cycles, which suggests strong operational stability. However, the activity further decreased to approximately 63.75% of the original activity after 15 cycles.

3.8. Thermal stability and half lives of free enzyme and CLEAs

Comparative study on thermostability of the enzyme species including free α-amylase, CLEAs, and mixed ions–CLEAs were performed in the presence of substrate at 110° C for at least 120 min (Fig. 6). Fig. 6 reveals the natural logarithm of residual activities as a function of the incubation time in presence of starch as the enzyme substrate. Thermo-inactivation of the enzyme species (free and CLEAs with and without Ca^{2+} and Na^{+} ions) up to 120 min was analyzed to determine the inactivation rate constants or k_i. The half-lives corresponding to each sample were calculated using Eq. (1). The results indicated an improved half-life of the CLEAs with mixed ions (115.5 min) compared to the CLEAs without mixed ions (86.63) and free α-amylase (43.31) species. In fact, enzyme

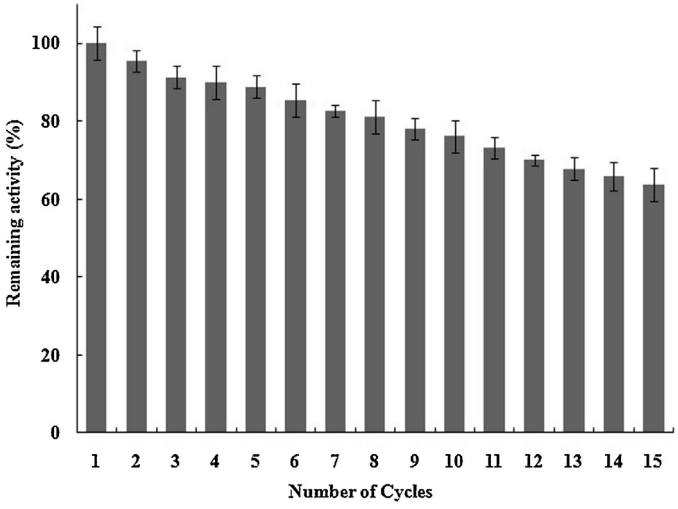

Fig. 5. The effect of the enzyme reuses on the activity of immobilized α-amylase CLEAs (enriched with $3Ca^{2+}$:$1Na^{+}$) during starch hydrolysis at pH 5.5 and 95 °C for 120 min. Each treatment was performed in triplicate.

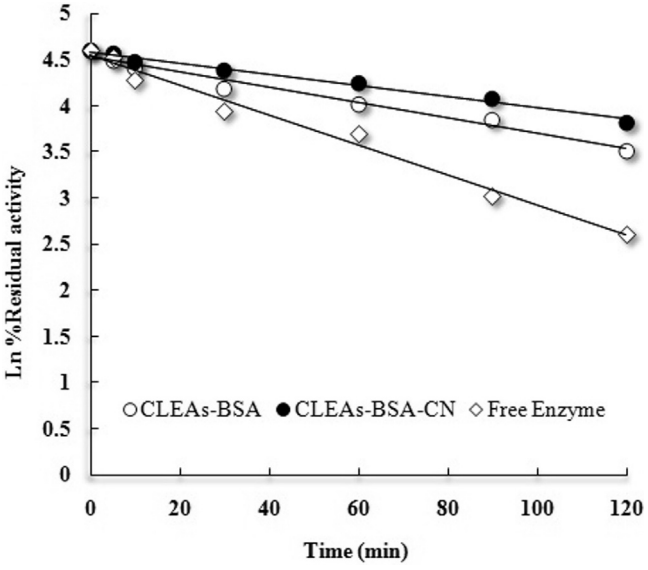

Fig. 6. Thermal inactivation of the free and immobilized α-amylase at 110 °C for enzyme half-life determination. The natural logarithm of residual activities of the free enzyme (◊), CLEAs without mixed ions (○), and CLEAs with mixed ions (●) as a function of the incubation time. All the data presented in this study are average values ± SD of three experiments.

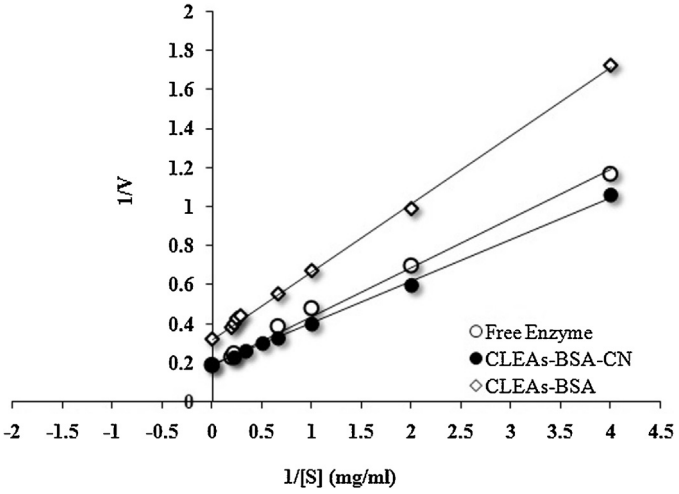

Fig. 7. Lineweaver–Burk plot for kinetic constant determination of free and immobilized enzymes. Free enzyme (○), CLEAs with BSA and mixed ion addition (●), and CLEAs with BSA (◊).

half-life of free α-amylase is increased 2.7-folds after mixed ions–CLEAs preparation.

3.9. Kinetic studies

Kinetic constants of free α-amylase compared to CLEAs produced with and without mixed ions ($3Ca^{2+}$:$1Na^{+}$ ratio) were evaluated by measuring initial reaction rates at various starch concentrations (0.25–5.0 mg/mL in 100 mM sodium acetate buffer pH 5.5) as a substrate at 95 °C for 120 min. Both free α-amylase and CLEAs exhibited Michaelis–Menten type kinetics behavior (Fig. 7).

The values of the kinetic constants were estimated from the Lineweaver–Burk plot (Fig. 7) based on the enzymatic starch hydrolysis activity of free and immobilized α-amylases. The resulted kinetic constants are displayed in Table 2. As expected and in accordance with the published literature, immobilization of α-amylase as CLEAs led to changes in the K_m and V_{max} values [34–36]. As it is shown in Table 2, the V_{max} value of free α-amylase is decreased from 5.35 to 3.12 μmol min^{-1} (1.7-folds) after CLEAs–BSA preparation as well as, K_m value decreased from 1.34 to 1.09 mg/mL (1.2-folds) respectively. Although immobilization did not significantly affect V_{max} of free enzyme compared to CLEAs–BSA–CN (5.35–5.25), substrate affinity of the enzyme increased (1.34–1.12) after addition of mixed ions to the prepared CLEAs.

Table 2
Kinetic constants of free α-amylase and prepared CLEAs with and without mixed ions.

Enzymes	V_{max} (μmol min^{-1})	K_m (mg/mL)	k_{cat} (S^{-1})	k_{cat}/K_m
Free α-amylase	5.35	1.34	5.24×10^5	3.91×10^5
CLEAs–BSA	3.12	1.09	3.06×10^5	2.81×10^5
CLEAs–BSA–CN	5.26	1.12	5.16×10^5	4.57×10^5

As indicated in Table 2, k_{cat}/K_m values of the CLEAs–BSA–CN were significantly improved by adding 3:1 ratios (1200 ppm of Ca^{2+} and 400 ppm Na^{+}) of mixed ions of during the aggregation process and formation of CLEAs.

These results imply that catalytic efficiency (k_{cat}/K_m) of free enzyme is decreased from 3.91×10^5 to 2.81×10^5 (about 1.4-folds) after CLEAs–BSA formation presumably due to the mass transfer limitations, reduction of substrate accessibility to the active site of the enzyme, and also due to the restricted conformational freedom-induced crosslinking and BSA addition [37].

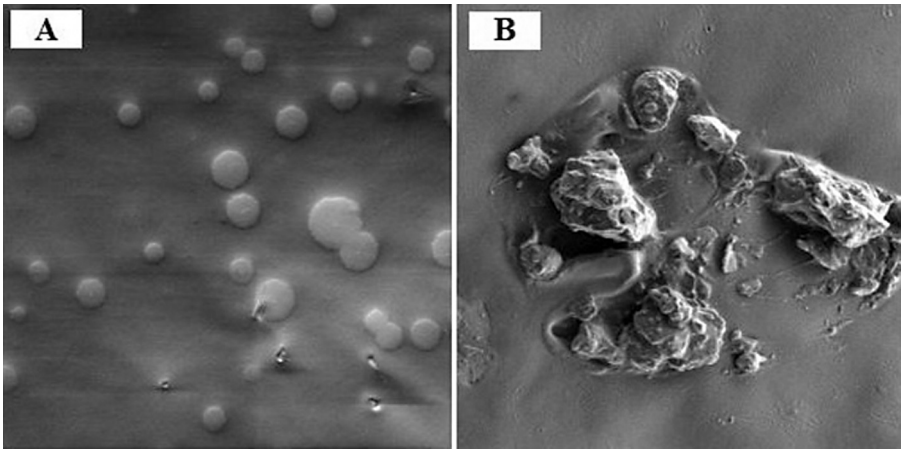

Fig. 8. (A) SEM image of free α-amylase (magnification 500×) and (B) SEM image of α-amylase CLEAs (magnification 1500×).

In contrast to the CLEAs–BSA, the resultant k_{cat}/K_m value of CLEAs–BSA–CN that was prepared in the presence of 1200 ppm of Ca^{2+} and 400 ppm Na^{+} exhibited higher value (4.57×10^5) about 1.2-folds enhancement compare to free enzyme.

The resultant k_{cat}/K_m values suggests that the biocatalytic efficiency of enriched mixed ions CLEAs represents 1.6-folds increase compared with that of the CLEAs–BSA, and therefore, the CLEAs–BSA–CN are more efficient for starch hydrolysis.

3.10. Scanning electron microscopy (SEM)

The photomicrographs of free α-amylase and CLEAs were observed by SEM as presented in Fig. 8. Free α-amylase molecules were visualized as spherical drops in shape with well-defined edges and smooth surface. The α-amylase CLEAs exhibited considerable alterations in morphology.

4. Conclusions

CLEAs were prepared from *B. licheniformis* α-amylase by precipitation and perfect aggregation of free enzyme molecules. It was observed that *tert*-butanol was an appropriate α-amylase aggregator, and the addition of optimal concentrations of bovine serum albumin and glutaraldehyde, appropriate cross-linking period, pH, and temperature had significant effects on CLEAs activity. The activity and stability of produced CLEAs was also significantly increased by adding an optimal ratio of calcium and sodium ions during enzyme aggregation and CLEA formation. Moreover, catalytic performance of the immobilized α-amylase were significantly improved with the application of CLEAs. Finally, the catalytic activity of the enzyme immobilized as α-amylase CLEAs was sustainable up to 10 cycles of reuse, without major changes in efficiency. α-Amylase CLEAs produced using this novel method were structurally stable and thermo-tolerant, which ensures their applicability in several industrial processes such as starch liquefaction process in a continuous bioreactor and in biosensor production.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2014.06.005.

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