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Biochemical and Structural Characterization of Cross-Linked Enzyme Aggregates (CLEAs) of Organic Solvent Tolerant Protease

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Abstract: Cross-linked enzyme aggregates (CLEAs) is an immobilization technique that can be used to customize enzymes under an optimized condition. Structural analysis on any enzyme treated with a CLEA remains elusive and has been less explored. In the present work, a method for preparing an organic solvent tolerant protease using a CLEA is disclosed and optimized for better biochemical properties, followed by an analysis of the structure of this CLEA-treated protease. The said organic solvent tolerant protease is a metalloprotease known as elastase strain K in which activity of the metalloprotease is measured by a biochemical interaction with azocasein. Results showed that when a glutaraldehyde of 0.02% (*v*/*v*) was used under a 2 h treatment, the amount of recovered activity in CLEA-elastase was highest. The recovered activity of CLEA-elastase and CLEA-elastase-SB (which was a CLEA co-aggregated with starch and bovine serum albumin (BSA)) were at an approximate 60% and 80%, respectively. The CLEA immobilization of elastase strain K allowed the stability of the enzyme to be enhanced at high temperature and at a broader pH. Both CLEA-elastase and CLEA-elastase-SB end-products were able to maintain up to 67% enzyme activity at 60 °C and exhibiting an enhanced stability within pH 5–9 with up to 90% recovering activity. By implementing a CLEA on the organic solvent tolerant protease, the characteristics of the organic solvent tolerant were preserved and enhanced with the presence of 25% (*v*/*v*) acetonitrile, ethanol, and benzene at 165%, 173%, and 153% relative activity. Structural analysis through SEM and dynamic light scattering (DLS) showed that CLEA-elastase had a random aggregate morphology with an average diameter of 1497 nm.

Keywords: CLEA; solvent-tolerant protease; glutaraldehyde; immobilization

1. Introduction

Enzymes are considered one of the most notable biocatalysts and are extensively used in the industry considering its wide applications and advantages. Generally, the use of enzymes in industrial bioprocesses is desirable as enzymes offer a greener alternative to chemical catalysts when taking into consideration biodegradability, cost-effectiveness and the production of non-toxic byproducts [1]. The enzymes in this group, which include protease, lipase, and amylase, have been commonly used as biocatalysts in the industry for many years. More particularly, an enzyme such as protease is known

for its application in detergent, mostly in removing protein-based stains, leather industry that involves hair removal, and various food productions. However, there is a major shortfall in using enzymes as biocatalysts which is related to their tendency to denature under any extreme conditions. For example, protease is susceptible to oxidation and autolysis when kept over a period of time [2–4]. Other extreme conditions that may affect the stability of protease include extreme temperature, pH, and organic solvents which may be present in the reaction media [5,6]. Organic solvents are used in the reaction media to shift the catalytic reaction towards the completion of synthesis in a one-phase or two-phase system. Organic solvents can be classified according to their log $P_{o/w}$ value, where any organic solvent showing log $P_{o/w} < 2$ is a polar organic solvent and any organic solvent showing log $P_{o/w} > 4$ is a non-polar organic solvent [7–9].

The presence of an organic solvent in the reaction media is detrimental to enzymes because organic solvents alter the hydrophobicity of the medium and affect the bonding and conformational structure of the enzyme. The changes in conformational structure of the enzyme can lead to inactivation. Nevertheless, there are enzymes which are known to be tolerant and stable in organic solvent. Most of these enzymes are secreted by bacterial strains isolated from organic–solvent–contaminated sites such as *Pseudomonas* sp., *Bacillus* sp., *Rhizopus oryzae*, *Halobacterium* sp., and *Natrialba magadi*. There are also reports on thermostable enzyme that is able to tolerate the presence of 25% (*v*/*v*) organic solvents and is said to have an enhanced catalytic activity [10–13]. The effect of organic solvent on enzymes may accentuate the activity of the enzyme in this situation; the organic solvent molecules interact with the enzyme at different polarities and organic solvent concentrations which improve the conformation of the enzyme. Elastase strain K is an organic solvent tolerant protease that is isolated from *Pseudomonas aeruginosa* strain K, wherein the organic solvent tolerant protease exhibits stability and enhanced activity in a wide range of polar organic solvents. In Rahman et al., recombinant elastase strain K had previously shown stability in 25% (*v*/*v*) polar organic solvents such as ethanol, methanol, 1–propanol, and DMSO [14–16]. The ability of elastase strain K to withstand a broad range of organic solvents allows it to be applied in the enzyme industry and to be run through extreme processes. Although it shows stability in organic solvents, there are still limitations regarding the wavering stability of this enzyme under other extreme conditions which outweigh the value and usefulness of the enzyme for industrial application.

To overcome pitfalls in enzyme stability, there is a technique which involves the immobilization of the enzyme. There are many techniques of enzyme immobilization that are commonly used which include adsorption, entrapment, covalent binding, and cross–linking. In general, any immobilization of an enzyme can be achieved via physical or chemical approaches. The physical approach to enzyme immobilization involves formation of linkages between enzyme molecules and the carrier, whereas when using a chemical approach, a stable linkages between enzyme molecules is achieved through the use of a cross–linking agent [17]. Both adsorption and entrapment are examples of physical immobilization, whereas covalent binding and cross–linking are examples of chemical immobilization. At the industrial level, enzyme immobilization acts to improve the mechanical stability of the enzyme, which makes the enzyme more resilient towards surfactants, chemicals, inhibitors, and reactors used. In addition, enzyme immobilization reduces contamination in the reaction medium and modifies the purity of the enzyme, which makes it increasingly selective and specific [18,19].

A cross–linked enzyme aggregate (CLEA) is a carrier–free enzyme immobilization technique whereby a cross–linking agent is used for the formation of linkages between aggregated enzyme molecules that are insoluble. The CLEA is arranged in a manner similar to a good porous support. Precipitating the free enzyme can be done by adding salts, non–ionic polymers, or water–miscible organic solvents at appropriate proportions to form aggregates. Additional bifunctional reagents such as glutaraldehyde can also form linkages through the reaction of glutaraldehyde and the active group, and in particular with the amine group of lysine residue on the surface of the enzyme. The presence of a non–catalytic carrier within the immobilized enzyme matrix further reduces the volumetric catalyst [20–22]. Eliminating the need to incorporate any solid support as a carrier maximizes the

catalytic performance and reduces the cost of preparing the CLEA since the cross–linkers are relatively small and inexpensive [23,24].

There are some issues on CLEAs such as diffusional problems when a large substrate is used, low number of lysine residue on enzyme limits the formation of linkages with glutaraldehyde, and having a delicate nature when produced on a large scale make CLEAs more difficult to handle in industrial operation. Means to improve the preparation of CLEAs have been initiated and implemented over the years. Some of the improvements include the addition of co–aggregants such as bovine serum albumin (BSA), which acts as a protein feeder and magnetic particles for better CLEA separation [25,26]. While CLEAs have gained recognition over the years, studies regarding the organization and conformational structure of CLEAs remains elusive [27]. It has been hypothesized that a deep understanding of the structure of CLEAs would enable scientists to engineer CLEAs which have a maximum recovered activity.

Taking into consideration the lack of structural conformity of CLEAs, there is a need to firstly address the consequences of factors affecting aggregation and cross–linking processes of CLEAs, which include various incubation times, the concentration of glutaraldehyde as a bifunctional reagent, and the effect of co–aggregants. The optimization of these parameters has been aimed at capitalizing on the recovered activity of CLEA–elastase in order to reduce the limiting factors of CLEAs. Secondly, the biochemical and biophysical aspects of CLEA derivatives have been compared to the free enzyme. Biophysical analysis focuses on the structural organization, which comprises the primary morphology, diameter, and particle distribution in aqueous media, of CLEAs.

2. Results

2.1. Effect of Glutaraldehyde Concentration on CLEA–Elastase Formation

Glutaraldehyde is a highly reactive protein cross–linking compound acting even at very low concentrations in mixtures. Figure 1 shows the recovered activity of CLEA–elastase treated with different concentrations of glutaraldehyde. The maximum recovered activity of CLEA–elastase was recorded when 0.2% (*v*/*v*) of glutaraldehyde was used. The recovered activity of CLEA depends on the amount of linkages formed between the molecules of glutaraldehyde and the active amino group of the enzyme molecules. By applying a low concentration of glutaraldehyde, there will be an insufficient number of linkages formed, contributing to a greater loss of activity because the unimmobilized enzymes are prone to being decanted during the washing process. On the other hand, increasing the concentration of glutaraldehyde would cause the enzyme to be subjected to the formation of multipoint linkages. The excessive linkages will lead to formation of a CLEA with tiny pores, thus limiting the diffusion of a large substrate to the active site of the enzyme [21]. The highest recovered activity of CLEA–elastase was recorded at 60% based on Figure 1. The 40% loss in enzyme activity may be attributed to the washing process or to steric hindrance.

2.2. Effect of Glutaraldehyde Modification Time on CLEA–Elastase Formation

Glutaraldehyde modification time is important to forming a functional CLEA with the highest recovery activity. While glutaraldehyde is known to be highly reactive, sufficient modification time is required to form enough linkages in the CLEA. Glutaraldehyde modification time showed the highest recovered activity of approximately 70% at 2 h according to Figure 2. A shorter glutaraldehyde modification time would result in insufficient linkages between enzyme molecules, therefore increasing the probability of loss of the enzyme during the washing process. Conversely, a prolonged modification time would cause formation of multipoint linkages thus limiting the diffusion of the substrate into CLEAs.

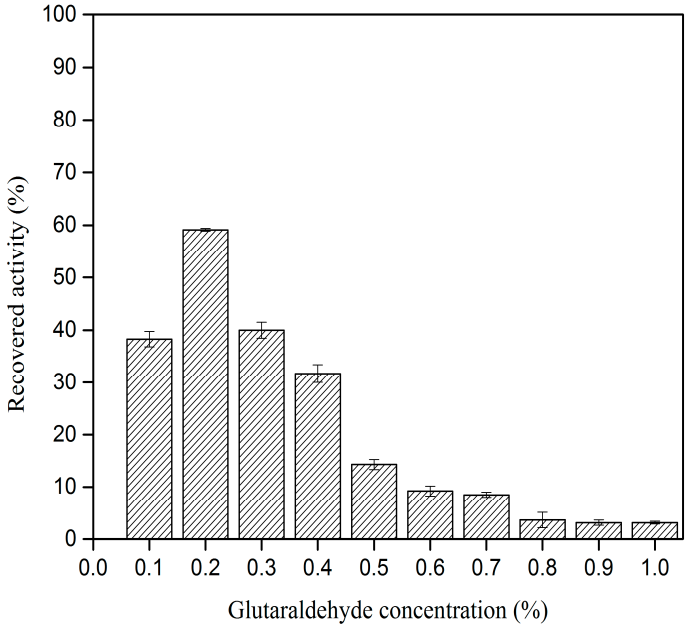


Figure 1. Effect of glutaraldehyde concentration on the recovered activity of cross–linked enzyme aggregate (CLEA)–elastase. Different concentrations of glutaraldehyde were added into aliquots of aggregated free elastase prior to incubation at 4 °C for 2 h with a rotating motion. The experiment was conducted in triplicate and the recovered activities are presented as mean values ± standard error.

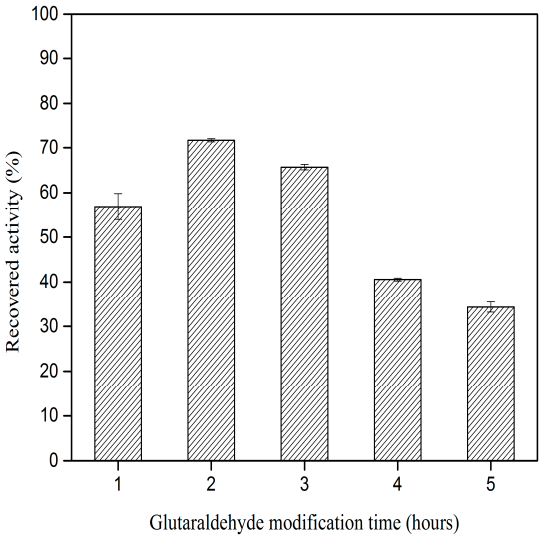


Figure 2. Effect of glutaraldehyde modification time on the recovered activity of CLEA–elastase. Glutaraldehyde with a concentration of 0.2% (*v*/*v*) was added to aliquots of aggregated free elastase prior to incubation at 4 °C with rotating motion. CLEA–elastase was collected every hour for 5 h. The experiment was conducted in triplicate and recovered activities are presented as mean values ± standard error.

2.3. Effect of Different Co–Aggregants on CLEA–Elastase Formation

Co–aggregates such as BSA and starch can be incorporated into the enzyme mixture to enhance the recovered activity of the elastase. BSA is a lysine–rich protein which acts as a spacer and protein feeder in the CLEA. This lysine–rich surface prevents excessive cross–linking between enzyme molecules, thus reducing the diffusional limitation of the substrate caused by narrow CLEA pores. Starch, on the other hand, does not provide a lysine–rich surface able to be a protein feeder, but some studies have incorporated starch into the enzyme mixture to form a porous CLEA [28,29]. CLEA formed

separately with BSA or starch is known as CLEA–B and CLEA–S, respectively, while a mixture of BSA and starch in CLEA is identified as CLEA–SB. The incorporation of a single co–aggregant in our work enhanced the recovered activity of CLEA–elastase by 70% compared to the native CLEA, as shown in Figure 3. A maximum recovered activity of 80% was measured after both co–aggregants were mixed in the enzyme mixture. The interactions between co–aggregants regulate the extent of linkages in CLEA–elastase–SB and initiate the formation of porous CLEA which further aid the substrate diffusion. The interactions of co–aggregants with elastase contributed to the rising activity of CLEA–elastase–SB when compared to CLEA–elastase, CLEA–elastase–S, and CLEA–elastase–B [30]. Because CLEA–elastase–SB exhibited the highest recovery, among other interactions, a characterization of CLEA–elastase–SB was conducted for which the results were compared to CLEA–elastase.

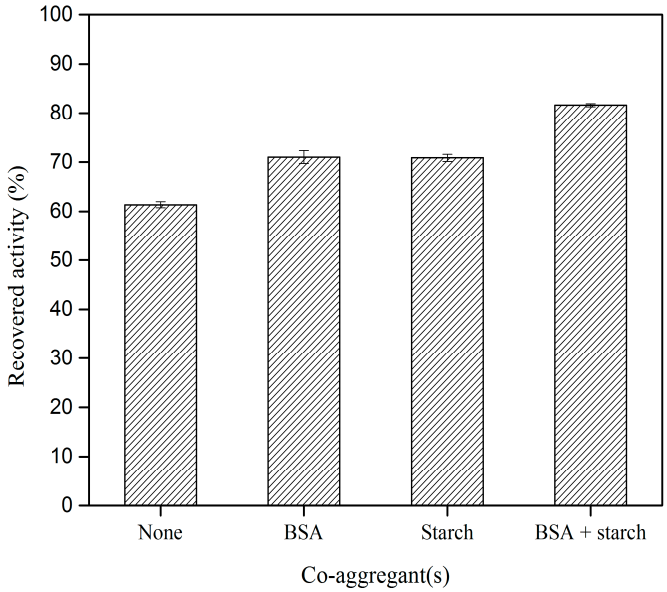


Figure 3. Effect of different co–aggregants on the recovered activity of CLEA–elastase. Ten mg/mL of each co–aggregant was incorporated into the aggregated enzyme prior to addition of 0.2% (*v*/*v*) glutaraldehyde. The suspension underwent incubation at 4 °C for 1 h with rotating motion. The experiment was conducted in triplicate and recovered activities are presented as mean values ± standard error. Legend: BSA, bovine serum albumin.

2.4. Effect of Temperature on Proteolytic Activity of CLEA–Elastase

The temperature profile of CLEA–elastase was compared to the free elastase. Studies by Rahman et al. have revealed that elastase strain K can be harvested from a recombinant *Escherichia coli* and exhibits optimum activity at 40 °C [15]. CLEA–elastase derivatives, on the other hand, exhibit an optimum temperature at 50 °C, as shown in Figure 4a. The thermal stability of CLEA–elastase was measured by pre–incubating the samples at different temperatures for 30 min before subjecting them to a proteolytic assay at the optimal temperature. The thermal stability profile as shown in Figure 4b reveals that the CLEA–elastase derivatives exhibit better tolerance and activity at higher temperature. The improvement of elastase activity can be observed at 50 °C for both CLEA–elastase and CLEA–elastase–SB. The immobilized elastase for CLEA–elastase and CLEA–elastase–SB at 60 °C achieved a relatively high recovered activity, being 67.7% and 84.6%, respectively. By contrast, free elastase exhibited a decline in protease activity at the said temperature. The presence of linkages between enzyme molecules in CLEA–elastase provides an additional stability and rigidity to CLEA–elastase which causes the interaction to be more resilient to high temperature. Nonetheless, at 70 °C, all samples exhibited a loss in activity as a result of the rupturing of the bonds within the enzyme, with the bonds that were ruptured having been essential in maintaining the catalytic conformation of the enzyme [31,32].

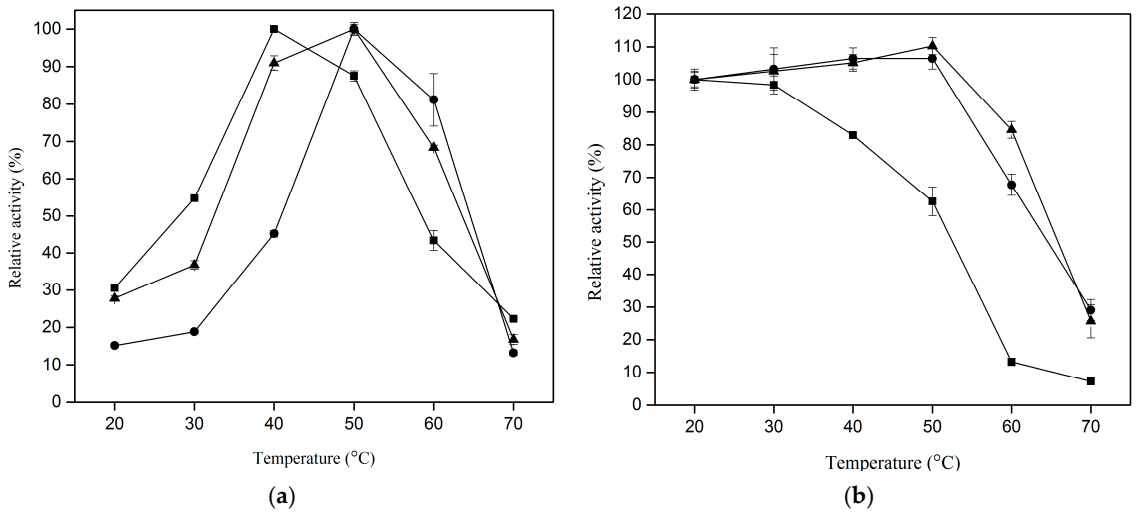


Figure 4. Temperature profile of elastase and its CLEA derivatives. (**a**) The effect of temperature on the optimum temperature of CLEA–elastase (●) and CLEA–elastase formed with starch and BSA (CLEA–elastase–SB) (▲) in comparison with free elastase (■); (**b**) thermal stability of CLEA–elastase (●) and CLEA–elastase–SB (▲) in comparison with free elastase (■). The experiment was conducted in triplicate and relative activities are presented as mean values ± standard error.

2.5. Effect of pH on Proteolytic Activity of CLEA–Elastase

The effect of pH on proteolytic activity of CLEA–elastase was studied. Samples containing the enzyme were subjected to pre–incubation in 50 mM of various buffer systems with pH ranging from 4 to 11. In furtherance to the assays at different pH levels, both free elastase and CLEA–elastase were shown to have the highest activity at pH 8 as shown in Figure 5, whereas the CLEA–elastase–SB showed a relatively high activity at pH 8, pH 9, and pH 10, having a relative activity of 100%, 96% and 80% at these pH levels, respectively. Changes in pH can alter the ionic charge of the protein residue, which corresponds to conformational changes in the catalytic region. Under a high pH, protein residues were deprotonated and the ionic charges reduced the relative activity of CLEA–elastase and CLEA–elastase–SB. A lower relative activity was found at the acidic point where the surroundings had a pH that was close to the isoelectric point of the substrate, which affects the activity of the enzyme [31].

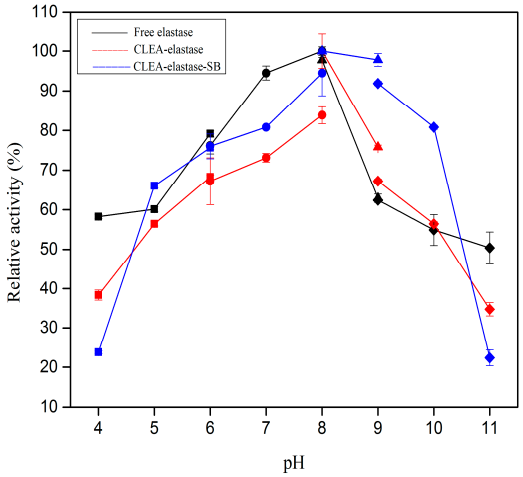


Figure 5. pH profile of elastase and its CLEA derivatives. For each pH range, 50 mM of different buffer systems was used, namely, sodium acetate (■), potassium phosphate (●), Tris–Cl, (▲) and glycine–OH (◆). The experiment was conducted in triplicate and relative activities are presented as mean values ± standard error.

2.6. pH Stability of CLEA–Elastase

Elastase strain K has a wide pH stability profile. The free elastase was found to retain proteolytic activity of up to 80% between pH 5 to pH 10 as shown in Figure 6. CLEA–elastase derivatives, on the other hand, retained up to 60% enzyme activity at pH 4, which increased the enzyme stability threefold compared to free elastase at a similar pH. Apart from this, the enzyme activities of CLEA–elastase and CLEA–elastase–SB were also preserved by up to 90% between pH 5 to pH 9. While the pH stability profile of CLEA–elastase and free elastase were homologous at pH 10 and pH 11, CLEA–elastase–SB showed stability at a similar range of pH with relative activities of 92% and 81%, respectively.

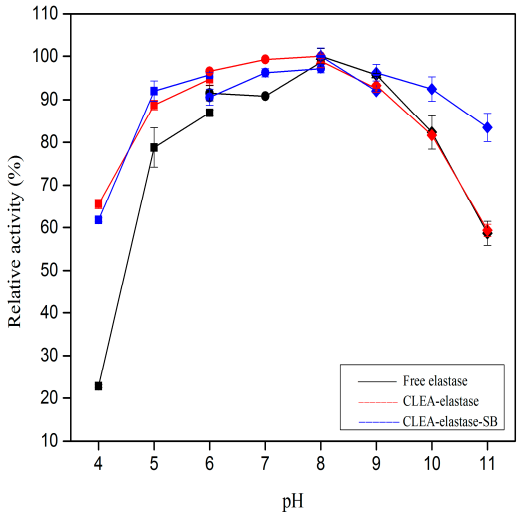


Figure 6. The pH stability profile of free elastase and CLEA–elastase derivatives. For every pH a different buffer system with a concentration of 50 mM was provided, and the buffer systems comprised sodium acetate (■), potassium phosphate (●), Tris–Cl (▲), and glycine–OH (◆). The experiment was conducted in triplicate and relative activities are presented as mean values ± standard error.

2.7. Stability of CLEA–Elastase in Organic Solvents

Elastase strain K is a known organic solvent tolerant enzyme for which, according to Table 1, the activity of the enzyme was preserved by up to 75% in polar and non–polar organic solvents. A significantly higher enzyme activity of CLEA–elastase in organic solvents indicates that the stability of the enzyme has changed in lieu of the formation of CLEA compared to free enzymes. Improvements in enzyme activity could be observed when CLEA–elastase was treated with organic solvents which had log $P_{o/w}$ values of −0.33 to 3.15. In particular, the presence of organic solvents such as acetonitrile, ethanol, benzene, and xylene was found to be the reason for which the activity became higher than that of the control, as shown by significantly high activities of 164%, 172%, 152%, and 133%, respectively. The improvements of CLEA–elastase activity could also be observed in 1–propanol. Compared to free elastase, which became detrimental after being treated with 25% (*v*/*v*) 1–propanol, CLEA–elastase was able squeeze off the effects by retaining an enzyme activity which was 11% higher than that of the control. With the exception of 25% (*v*/*v*) toluene, CLEA–elastase was able to maintain a relatively high enzyme activity within the rest of the organic solvents studied. The organic solvent treatment of CLEA–elastase–SB revealed that all samples retained their proteolytic activity except for DMSO. In 25% (*v*/*v*) DMSO, the relative activity of CLEA–elastase–SB plunged to 48% compared to the activity of the control. The massive decline in protease activity in DMSO was due to the fact that the polarity of DMSO took a toll on the co–aggregants, which further disrupted the overall stability of the CLEA. Although CLEA immobilization seeks to improve the stability of the enzyme, organic solvent has been shown to be the main factor that could pose a detrimental effect to the co–aggregants. This effect will prevent the co–aggregants from effectively enhancing the enzymatic performance of CLEA–elastase.

Table 1. Stability of free elastase and CLEA–elastase derivatives in 25% (*v*/*v*) organic solvents for 30 min represented using relative activity (%). Enzyme activity without organic solvent treatment was taken to be 100%. The experiment was conducted in triplicate and recovered activities are presented as mean values ± standard error.

Organic Solvents	Log $P_{o/w}$	Free Elastase	CLEA–Elastase	CLEA–Elastase–SB
Control	–	100	100	100
DMSO	−1.30	102.39 ± 1.64	104.55 ± 3.20	47.59 ± 2.06
Methanol	−0.78	87.29 ± 1.06	111.36 ± 3.18	101.87 ± 1.64
Acetonitrile	−0.33	82.81 ± 0.71	164.55 ± 1.15	100.80 ± 1.13
Ethanol	−0.24	79.22 ± 1.91	172.73 ± 3.57	101.34 ± 0.87
1–propanol	0.28	31.39 ± 1.21	111.36 ± 2.16	94.12 ± 5.81
Benzene	2.13	79.52 ± 1.74	152.73 ± 1.22	98.40 ± 4.57
Toluene	2.50	86.70 ± 0.77	96.36 ± 2.87	79.14 ± 0.34
Xylene	3.15	82.96 ± 0.62	133.18 ± 0.84	89.84 ± 0.59

2.8. Reusability of CLEA–Elastase

The benefits of immobilization via the CLEA method stand with the easy separation of the enzyme from the reaction medium where the cross–linking of the enzyme would render them insoluble. The reusability of CLEA–elastase was evaluated for up to 10 cycles with the first cycle being 100%. After the first cycle, the recovered activity of CLEA–elastase and CLEA–elastase–SB declined to 89% and 97%, respectively, as shown in Figure 7. In general, the presence of co–aggregants in the CLEA showed an improvement in recovery activity per cycle. The presence of BSA acts as a spacer and protein feeder which controls the degree of linkage and stabilizes the enzyme structure. The optimization parameter of glutaraldehyde used and the co–aggregants aided in the formation of a CLEA with an adequate rigidity to withstand numerous cycles and an adequate pore size for effective substrate diffusion [27]. The recovered activity of CLEA–elastase was found to be 50% when reaching the ninth cycle while CLEA–elastase–SB had a recovered activity of 61% at the tenth cycle.

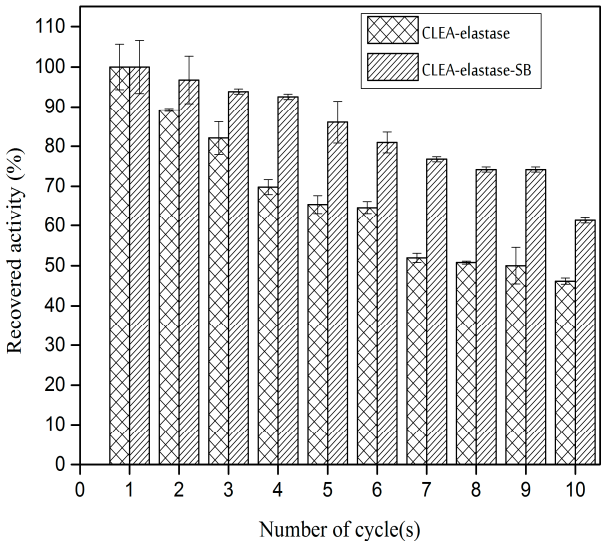
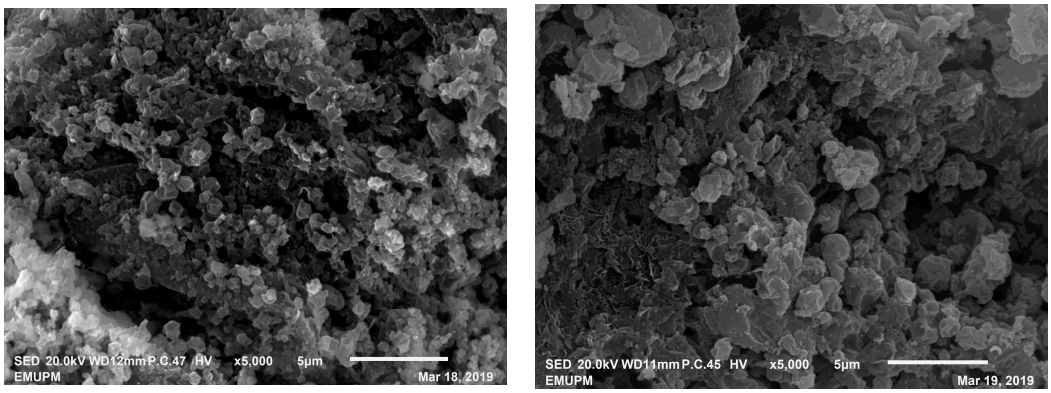


Figure 7. Reusability of CLEA–elastase and CLEA–elastase–SB. The experiment was conducted in triplicate and recovered activities are presented as mean values ± standard error.

2.9. Scanning Electron Microscopy of CLEA–Elastase

SEM analysis was conducted on dried samples of CLEA–elastase and CLEA–elastase–SB with the exception of the free elastase due to its solubility. The structure of the CLEA under an electron microscope could be classified into two types depending on the conformation of aggregates. The type

1 aggregate formed a ball–like structure while the type 2 aggregate formed a less defined and more random aggregation [27]. SEM images of CLEA–elastase and CLEA–elastase–SB revealed that the CLEAs majorly exhibited a type 2 aggregate. Although the type 2 aggregate was more random in its overall structure, there was a uniformity in the size of the molecules that made up the structure of the CLEA, as shown in Figure 8. While both CLEAs exhibited a type 2 aggregate structure, CLEA–elastase–SB was different in that the molecules that made up the aggregates were more robust since the co–aggregant present acted as a spacer and protein feeder to the enzyme molecules. The co–aggregants increased the surface area of the CLEA, allowing for more substrate binding and enhancing the enzyme activity.



(**a**) (**b**)

Figure 8. SEM images of CLEA–elastase (**a**) and CLEA–elastase–SB (**b**). Both images were taken under 5000× magnification.

2.10. Dynamic Light Scattering (DLS) of CLEA–Elastase

The particle sizes of free elastase strain K and CLEA–elastase were determined using DLS analysis. Based on Figure 9, the data from DLS revealed that free elastase, CLEA–elastase, and CLEA–elastase–SB showed diameters of 330 nm, 1109 nm, and 1497 nm, respectively. A major difference between free and immobilized elastase was attributed to the cross–linking of large aggregates in CLEA–elastase and CLEA–elastase–SB. The difference in the diameter of CLEA–elastase and CLEA–elastase–SB was coherent with the SEM images of both samples, in which CLEA–elastase–SB appeared morphologically larger and robust in size when compared to CLEA–elastase. The DLS analysis of CLEA–elastase also exhibited a small peak which may be attributed to the detection of a small aggregate present in the mixture. The small aggregate in CLEA–elastase occurred due to mechanical stress during physical separation. Hence, this finding supports reports on the delicate nature of native CLEAs [19,33].

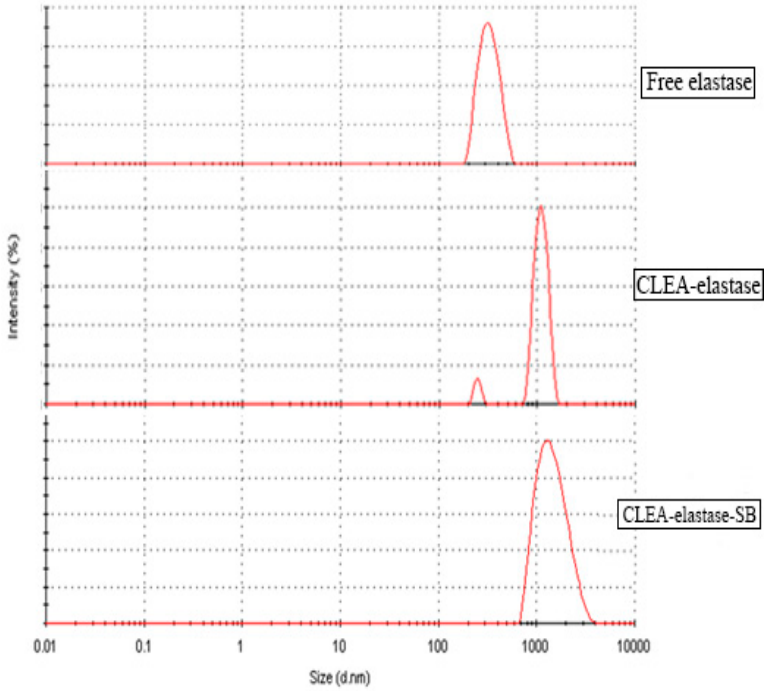


Figure 9. Dynamic light scattering (DLS) analysis of free elastase, CLEA–elastase, and CLEA–elastase–SB.

3. Discussion

Organic solvent tolerant enzymes are valuable biocatalysts for industry. Despite their importance, only a few studies have been found to conduct immobilization on the enzymes and one of these used the organic solvent tolerant protease [34–36]. This study attempted to immobilize an organic solvent tolerant elastase strain K using the CLEA method and to determine whether this type of immobilization would affect the organic solvent tolerant property of this enzyme. Following the preservation of the organic solvent tolerant property of CLEA–elastase, the biophysical and biochemical properties of CLEA–elastase were studied.

Glutaraldehyde is used as the protein cross–linking reagent. The chemical reacts actively with the amino group of the enzyme forming a CLEA as a result of a Schiff base reaction. In fact, there are many proposed mechanisms of reactions of glutaraldehyde since the reaction in aqueous environment might be affected by the pH of solution, the presence of active amino groups, and the ability to form many conjugates. The reactive groups in glutaraldehyde are in equilibrium between its polymeric and monomeric configuration. Reactions under acidic or neutral conditions may involve the formation of a Schiff base and nucleophilic attack on the amino group of the protein. On the other hand, the reaction of glutaraldehyde under alkaline conditions has been found to undergo two mechanisms: the formation of a Schiff base and the Michael addition of C–C bonds forming the linkages [21,37]. The linkages formed by the reaction of glutaraldehyde and the amino group of the enzyme have been said to have good stability against high temperature and a wide range of pH values [30,31]. As glutaraldehyde is highly reactive, the formation of linkages in a CLEA could be achieved by using a small amount of glutaraldehyde and incubation for a short treatment time. An insufficient incubation time and amount of glutaraldehyde might lead to a low degree of cross–linking and increase the possibility of enzyme leaching while an excessive incubation time and amount of glutaraldehyde will cause steric hindrance and low substrate diffusion [38]. Nonetheless, enzymes immobilized using the CLEA method experience loss of activity which can be attributed to enzyme leaching. Leaching produces smaller CLEA aggregates which have a tendency to be lost during the washing process. Hence, incorporation of co–aggregants in the enzyme mixture could provide a support for the aggregates to link onto and form a more rigid CLEA structure. Following this, CLEA–elastase has been found

to exhibit an enhanced stability under high temperature when compared to free elastase, which is coherent with subsequent CLEA studies [39–41]. The enhanced tolerance of CLEA–elastase towards high temperature is a result of increased rigidity of the structure caused by additional linkage between enzyme molecules. More energy is needed to weaken the durable linkage of the CLEA that prolongs CLEA–elastase stability at high temperature. A shift in the optimum temperature by 10 °C has been found to be a result of increased rigidity of the CLEA structure because in this case more energy was used to bring about the optimum functional conformational state of CLEA–elastase to catalyze a reaction [42]. The optimal pH profile of CLEA–elastase and free elastase have not displayed a significant change. In one study, a regular shift in obtaining the optimal pH under a slight alkaline condition was observed, and there have also been cases where CLEA immobilization did not shift the optimum pH of the enzyme [43]. Regardless of the shifting of the optimum pH, it is clear that CLEAs improve the pH stability of the enzyme. The pH stability profile covers a wide pH range, suggesting that CLEAs contribute to enzyme stabilization against protonation and deprotonation.

Elastase strain K has been shown to possess stability in organic solvents. Immobilization of elastase strain K has been linked to enhancing the operational and storage stability of the enzyme and preserving or enhancing the organic solvent tolerant characteristic. In industry, organic solvents are often used to minimize the reverse reaction and to shift the reaction towards completion of synthesis. However, organic solvents are usually toxic to the enzyme and cause denaturation. The stability of CLEA–elastase in organic solvent is enhanced and yields better activity in organic solvents with low log $P_{o/w}$. Even in its free form, elastase strain K has been found to exhibit better stability in polar solvents due to the presence of a disulfide–bridge and the reaction of its aspartate residue with water molecules in the organic solvent [44]. Formation of linkages in CLEA–elastase holds the structural conformation of the enzyme which leads to the enhancement of enzyme activity in non–polar organic solvents. CLEA–elastase–SB has been found to exhibit lower yields compared to CLEA–elastase due to the effect of organic solvents on the co–aggregants. CLEA–elastase–SB appears to be more stable in terms of reusability compared to CLEA–elastase. The loss of enzyme activity after each cycle has been linked to the leaching of the enzyme or mechanical stress during centrifugation causing the CLEA pellet to clump [36]. Our SEM images showed that CLEA–elastase exhibits a type 2 aggregate and this coheres with studies suggesting that type 2 aggregates are often formed by hydrophilic enzymes [45]. The DLS analysis provided insight into the diameter size of the aggregate in aqueous media, where the diameter of CLEA–elastase appeared to be significantly larger than that of a free elastase.

4. Materials and Methods

4.1. Bacterial and Enzyme Source

Recombinant *E. coli* KRX/pCon2(3) harboring the organic solvent tolerant elastase strain K gene was obtained from the Enzyme and Microbial Technology (EMTech) Research Centre, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia. Overexpression of elastase strain K was conducted according to Rahman et al. [11]. The bacterial culture was then sonicated thrice with 30% duty cycle and 8 control output settings to harvest the enzyme.

4.2. Assay of Proteolytic Activity

Proteolytic activity of elastase strain K was measured using the azocasein method. The azocasein method was conducted based on Rahman et al. with slight modification [16]. Azocasein (0.5% (*w/v*)) was first dissolved in 0.1 M tris–Cl buffer at pH 7. One hundred microliters of enzyme sample was added into 1 mL of the azocasein substrate and incubated in a water bath shaker for 30 min at 37 °C and 150 rpm. The reaction was terminated by adding an equal amount of 10% (*w/v*) trichloroacetic acid (TCA). The mixture was further incubated at room temperature for another 30 min and was centrifuged at 13,000× *g* for 10 min afterwards. One hundred microliters of the supernatant was pipetted into a 96–well microplate and an equal amount of 1 M NaOH was added to yield a yellowish orange color.

The absorbance was read at 450 nm using a microplate reader. A blank sample was prepared by replacing the enzyme sample with buffer under the same procedure.

4.3. Preparation of CLEA–Elastase

Preparation of CLEA involved two processes: aggregation of the enzyme and glutaraldehyde modification time. For enzyme aggregation, 60% (*w/v*) of ammonium sulfate was slowly added into crude elastase strain K. Once the ammonium sulfate was completely dissolved, the enzyme suspension was incubated at 4 °C with gentle stirring for 3 h. For the glutaraldehyde modification time, 0.2% (*v/v*) glutaraldehyde was added into the suspension and incubated for 2 h at 4 °C with gentle stirring. The enzyme suspension was then centrifuged at 13,000× *g* for 10 min. The CLEA–elastase pellet was washed with 50 mM tris–Cl buffer at pH 7 thrice to remove excess glutaraldehyde.

CLEA–elastase–SB was prepared by adding 10 mg/mL BSA and starch into the enzyme suspension prior to adding glutaraldehyde. The mixture was then incubated for 2 h at 4 °C with gentle stirring for the cross–linking process. The CLEA formed was then centrifuged at 13,000× *g* for 10 min and the pellet washed a few times to remove residual glutaraldehyde and co–aggregants. The recovered activity of CLEA–elastase was then calculated using Formula (1).

$$\text{Recovered activity } (\%) = \frac{\text{Proteolytic activity of free enzyme}}{\text{Proteolytic activity of CLEA}} \times 100\% \quad (1)$$

4.4. Optimum Temperature and Thermal Stability of CLEA–Elastase

The effects of temperature on free elastase and CLEA–elastase were determined from 20 °C to 70 °C using 10 °C increments. For determination of the optimum temperature, a mixture of the enzyme sample and the azocasein substrate were incubated at different temperatures for 30 min before the absorbance was taken to determine the enzyme activity. The highest enzyme activity for each sample was considered to be 100% and served as the control. The thermal stability of free elastase and CLEA–elastase were determined by pre–incubating the enzyme samples at different temperatures for 30 min prior to addition of the azocasein substrate and were assayed at the optimum temperature. Enzyme activity at 20 °C was considered to be 100% and served as the control.

4.5. Optimum pH and pH Stability of CLEA–Elastase

The effects of pH on free elastase and CLEA–elastase were determined using 50 mM different buffer systems, i.e., sodium acetate (pH 4–6), potassium phosphate (pH 6–8), Tris–Cl (pH 8–9), and glycine–OH (pH 9–11). Different buffer systems were used as each system has their own pH range. Determination of optimum pH was conducted by dissolving the azocasein substrate in the respective buffer system prior to addition of enzyme samples for the assay analysis. Determination of pH stability was conducted by re–suspending and pre–incubating the enzyme samples in different pH for 30 min at room temperature before proteolytic assays were undergone at the optimum temperature. The proteolytic assay in both tests was conducted at the optimum temperature measured earlier.

4.6. Stability of CLEA–Elastase in Organic Solvent

Organic solvent stability of free elastase and CLEA–elastase was determined by pre–incubating the enzyme samples with 25% (*v/v*) of different organic solvents at room temperature for 30 min. The samples were then assayed using azocasein at the optimum temperature measured earlier for another 30 min. Enzyme samples without organic solvent treatment were considered to be 100% and serve as the control. The selection of organic solvents was based on their boiling point not exceeding the optimum temperature used in the assay.

4.7. Reusability of CLEA–Elastase

The soluble free elastase could not be reused and thus reusability tests were conducted on the CLEA–elastase derivatives. The reusability test for CLEA–elastase and CLEA–elastase–SB was determined by assaying the enzyme samples for up to 10 cycles. The proteolytic assay was conducted using the azocasein substrate and incubated at the optimum temperature measured earlier. After a cycle of the proteolytic assay, the insoluble immobilized enzyme was separated from the reaction medium by centrifugation at 13,000× *g* for 10 min. A pellet of CLEA–elastase was washed and re–suspended in 50 mM Tris–Cl buffer to remove any residual azocasein substrate. Enzyme activity of the first cycle was considered to be 100% and served as the control.

4.8. Structural Analysis of CLEA–Elastase

Structural analysis and particle size distribution of the free elastase and CLEA–elastase derivatives were determined using JSM–IT100 In TouchScope scanning electron microscope (SEM) and Malvern ZetaSizer (UK) dynamic light scattering (DLS) respectively.

5. Conclusions

The data presented in this work have revealed that the organic solvent tolerant elastase strain K is able to be successfully immobilized using the CLEA method. While immobilization using the CLEA method has often been reported as causing a significant loss of enzyme activity, CLEA–elastase has been shown to retain up to 70% the enzyme activity. The recovered activity of CLEA–elastase was improved after a series of optimization measures of aggregation and cross–linking parameters including saturation of the precipitant, amount of glutaraldehyde, incubation time, and the effects of co–aggregants. Immobilization of elastase strain K using a CLEA also did not have any detrimental effects on the existing organic solvent tolerant characteristic of the enzyme. In fact, the presence of additional linkages in CLEAs has been shown to further enhance its tolerance and stability in a wide range of organic solvents. Despite the fact that a structural analysis of CLEAs still remains elusive, SEM and DLS analyses of CLEA–elastase revealed that the morphology and diameter of the aggregates were significantly larger compared to those measured with free elastase. The structural analysis of CLEAs is continuously becoming an interesting subject to consider.

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