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Characterization of Cross-Linked Enzyme Aggregates (CLEA)-amylase from *Zophobas morio*

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Abstract

Cross-Linked Enzyme Aggregates (CLEA) is known as one of the best enzyme immobilization technique nowadays. In this study, amylase extract from *Zophobas morio* (super mealworm) larvae was immobilized using acetone as the precipitant, glutaraldehyde as the cross-linker and bovine serum albumin as the additive. The characteristics of the produced CLEA were compared to the free soluble amylase, in terms of pH and temperature optimum and stabilities. The results displayed that CLEA and free amylase achieved an optimum temperature at 55°C and 45°C, respectively. CLEA-amylase also had showed greater stability against high temperature as compared to a free enzyme which had lost most of its activity when the temperature was set beyond 45°C. In comparison, at 65°C, CLEA-amylase still retained 73.2% of its activity. Results also revealed that CLEA-amylase has a pH optimum at 11, while it is pH 7 for free enzyme. Similarly, CLEA-amylase was more stable than the free form, over a wider range of pH, particularly at higher pH of 9, 10 and 11. Recyclability study showed that CLEA-amylase could retain 14.9% of its residual activity after 6 times of repeated uses. Since it is reusable, future works might include the evaluations of using CLEA-amylase at the industrial level, remarkably in detergent applications.

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Introduction

Enzyme or biocatalyst has been used widely in many applications since thousand years ago. It has been used traditionally in making food products such as cheese, beer, wine and vinegar in addition to several commodities like leather and linen productions (Kirk *et al.*, 2002). The demand for enzyme has been rising every year due to its intrinsic value as compared to the chemical catalyst. For instance, most of the enzymatic reactions could be performed under mild conditions (ambient temperature and pressure at physiological pH) (Sheldon, 2011). Besides that, the enzyme is regarded as 'biodegradable catalyst' which could be derived from manifold renewable resources. Even the end products are always in higher purity, the enzyme is generally less energy-demanding and produce less waste as compared to the chemical catalyst.

Amylase is one of the hydrolases enzymes which have been used most extensively in industries. Amylase plays vital roles in numerous enzymatic activities including beverages, brewing and detergent industries (de Souza and de Oliveira Magalhães, 2010). Up to date, a lot of studies has been made on amylases, but most of these amylases have been sourced from microbes (Talekar *et al*, 2012; Dahiya and Rathi, 2015) which require expensive downstream processing.

In an earlier study by Easa and Yusof (2015), amylase containing crude protein has been extracted from *Zophobas morio*, also known as super mealworm larvae under optimized conditions. Super mealworm is actually the larva stage of darkling beetle insects, which has been studied numerously for their high protein composition. For instance, studied by Veldkamp *et al*. (2012), they have found 45% of crude protein from the dry matter composition of this super mealworm larvae. Since it encompasses high protein content, high enzyme level also could be predicted. The result from the earlier study has shown that super mealworm contains 534.2 U/ ml or 31.4 U/ mg amylase activity.

Despite of all advantages offered by the enzymes, they also encounter several drawbacks. For instance, enzymes are often hindered by a lack of operational and storage stability, laborious recovery and they are non-reusable (Sheldon, 2007). This is due to the fact that most of the industrial processes require harsh conditions like high temperature and a wide range of pH. Studies have been carried out and affirmed that enzyme immobilization method could overcome this problem. One of the most promising immobilization techniques is via Cross-Linked Enzyme Aggregates

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(CLEA) which has been recognized to increase enzymes stability and reusability targeting the industrial applications (Sheldon, 2007).

In this study, amylase was extracted from super mealworm larvae before being immobilized via CLEA technique, using method by Easa and Yusof (2015). The produced CLEA-amylase was then tested for its optimum temperature and pH, thermal and pH stability as well as its reusability.

Materials and methods

Materials

Zophobas morio, at the larval stage, was bought from a supplier at Kota Damansara, Selangor, Malaysia. Starch, dinitro-salicylic acid (DNS), acetone, glutaraldehyde and other chemicals used in this study were of analytical grades attained from different suppliers (Merck, System, Bio- Rad, Friendman-Schmidt, Sigma-Aldrich and several others).

Extraction of amylase

Only super mealworms with weight more than 600 mg each were used for extraction. Extraction was conducted using the optimized procedures developed by Easa and Yusof (2015). The super mealworm was washed for several times under running tap water to remove dirt. They were placed in -20°C for 15 minutes to numb them before being weighed for a total of 20 g (Mohtar *et al*., 2014). All steps were done at 4°C to maintain enzyme stability. The larvae were blended in 72% (w/v) phosphate buffer of pH 6.11 for 30 seconds before filtered by muslin cloth to remove solid residue. Next, the solution was subjected to 12000 rpm centrifugation for 60 minutes at 4°C to remove excess fine solid and lipid. The supernatant was then collected and precipitated with 4 M ammonium sulphate under slow stirring for one hour for salting out the protein. After that, it was centrifuged again at 10000 rpm for 60 minutes at 4°C and the pellet was collected. The pellet was then dissolved in minimal phosphate buffer before stored in a freezer at -20°C (Mahmod *et al*., 2014).

Amylase activity assay

Bernfeld (1955) method was followed for amylase activity assay in which 1% starch (w/v) was used as the substrate. Enzyme extract (0.5 ml) was mixed with the starch substrate (0.5 ml) in a tube and then incubated at 37°C for 15 minutes. Next, 1 ml DNS reagent was added and the mixture was boiled for five minutes. Upon completion, the tubes were taken out and left to cool at room temperature before added with 10 ml distilled water. To ensure proper mixing, the tubes were inverted for several times. Finally, the absorbance reading was taken at 540 nm. The standard curve was prepared using a varying concentration of maltose stock solution. 1 unit of amylase is defined as the amount of enzyme which hydrolyzes 1 µmoles of maltose per minute under specified conditions.

Preparation of CLEA-amylase

CLEA-amylase was prepared according to Easa and Yusof (2015). A 0.5 ml crude sample (534.2 U/ml or 31.4 U/ mg amylase activity) was precipitated in 65% acetone, 90 mM glutaraldehyde and 1.60 mg/ml BSA in a total of 4 ml volume. After 16 hours of stirring at 37°C, the CLEA-amylase precipitate was separated from the supernatant by adding 3 ml acetone and centrifuging at 4000 rpm at 4°C for 30 minutes. Next, the CLEA was washed for two more times using 3 ml acetone, centrifuged and poured out to remove the excess chemicals. The recovery activity of the prepared CLEA- amylase was calculated by the following formula:

Recovery activity = (Total activity of CLEA)/(Total activity of free enzyme for CLEA preparation) x 100%

(1)

Optimum temperature and pH of CLEA-amylase

The optimum temperature for free and CLEA-amylase was studied from 25°C to 65°C. For each experimental run, the substrate (starch) were first incubated for 15 minutes at the selected temperature, followed by running the amylase activity assay at the same temperature, keeping the pH at 6 with phosphate buffer. Similarly, for pH optimum experiments, incubation of substrate at selected pH was conducted followed with amylase activity assay at the selected pH. In this case, pH was varied from 5 to 12, conducted at the optimum temperature found previously. Relative activity was calculated, based on the highest amylase activity, which was set at 100%.

Stabilities of CLEA-amylase

To conduct thermal stability experiments, both free and CLEA-amylase were pre-incubated in the substrate-free buffer for 30 minutes at varying temperatures (25°C to 65°C) in optimum pH found previously, before amylase activity assay was conducted. As for the pH stability experiments, both CLEA and free amylase were pre-incubated for 30 minutes in varying pH buffer (5-12), before normal amylase activity assay was conducted. Relative

activity was calculated by setting the highest activity as 100%.

Reusability of CLEA-amylase

The CLEA-amylase sample was kept in 3 ml acetone at 4°C to determine the reusability performance. The amylase activity assays were conducted up to six cycles. The first activity cycle was set at 100% and the residual activity was calculated based on it.

Results and discussion

Extraction of crude amylase and preparation of CLEA-amylase from super mealworm were conducted according to the optimized procedures reported by Easa and Yusof (2015). The optimum extraction parameters were done by using phosphate buffer (pH 6.11) with 72% sample to buffer ratio, whereas the optimum conditions for CLEA-amylase preparation were conducted using 65% acetone (precipitant), 90 mM glutaraldehyde (cross-linker) and 1.6 mg/ml BSA (additive). The highest recorded recovery activity after CLEA preparation was 27% of the free amylase activity.

Optimum activities of CLEA-amylase

As shown in Figure 1, the optimum temperature for free amylase was at 45°C, compared to the CLEA-amylase which achieved the highest activity at 55°C. One of the possible reason is that; denaturation of the soluble enzyme might occur at the high temperature (Yu *et al*., 2013). On the other hand, due to its rigid conformation, CLEA-amylase might be protected from the heat damage (Talekar *et al.*, 2012). Similar result pattern was reported in the previous study where the optimum temperature of soluble amylase was shifted from 45°C to 60°C upon CLEA formation (Talekar *et al*., 2012). In addition, Khanahmadi *et al.* (2014) who worked on CLEA-lipase from cocoa pod husk also reported that the optimum reaction temperature for free lipase was elevated from 45°C to 60°C after been immobilized.

Figure 2 showed the optimum pH for free amylase and CLEA-amylase is pH 7 and 11, respectively. These results are in agreement with reports by Sangeetha and Abraham (2008), where they found an optimum pH of subtilisin changed from pH 7 to pH 9 upon immobilization by CLEA technique. Among the possible reasons was due to the secondary interactions between the enzyme and glutaraldehyde. They proposed that the glutaraldehyde might link all the available amino groups on the surface of the enzyme. This event would probably lead to the

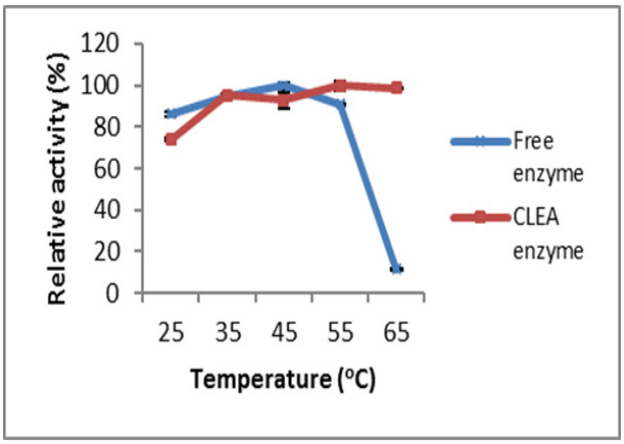

Figure 1. Temperature optimum test for CLEA and free amylase

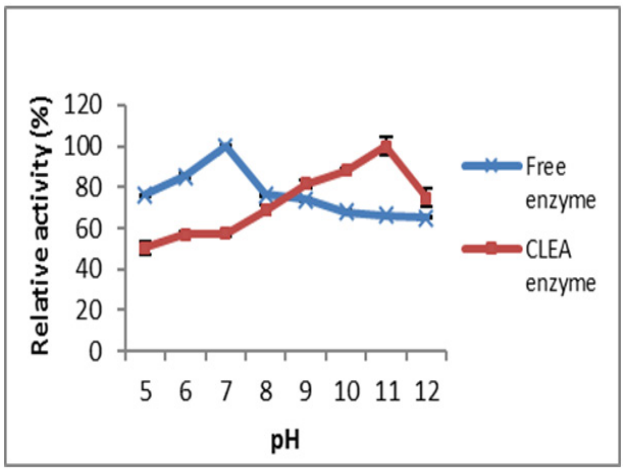

Figure 2. pH optimum test for CLEA and free amylase

donation of negative charge from the acidic groups on the enzyme surface to the enzyme protein, thus shifting of optimum pH towards alkaline side might have taken place. However, the level of increase in optimum temperature upon immobilization cannot be predicted for the amylases or any enzymes being studied; it is very dependent on the source of enzymes (whether they are from animals, plants, microbes or insects) and the method of immobilization used, including the types and the amount of precipitants, cross-linkers and additives used.

Stability of CLEA- amylase

Figure 3 shows that CLEA-amylase is more stable at a higher temperature compared to free amylase. At 65°C, CLEA-amylase still retained 73% of its relative activity as compared to the free enzyme which only has 14% activity remained. As suggested by Talekar *et al.* (2013), due to its thermal protection and more rigid conformation, much more energy was required to denature immobilized enzymes. In contrast, high temperature might interrupt tertiary structure of the proteins in free forms, which lead to denaturation (Gupta *et al*. 2015).

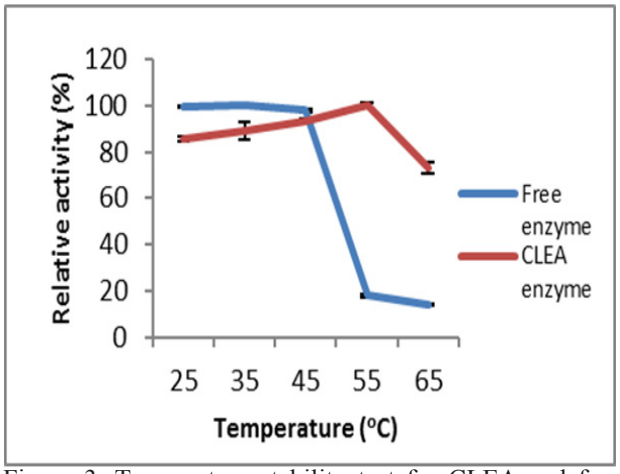

Figure 3. Temperature stability test for CLEA and free amylase

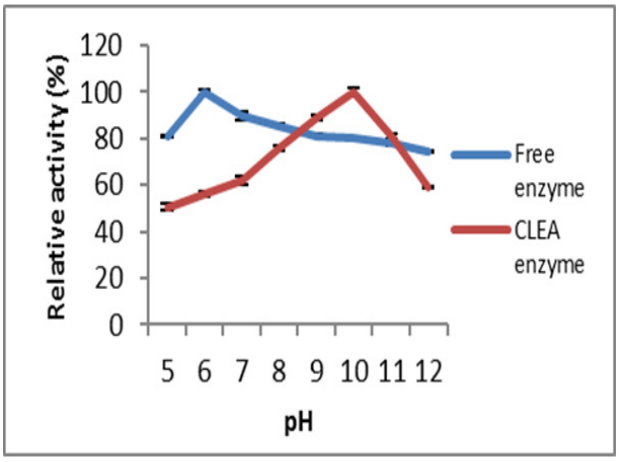

Figure 4. pH stability test for CLEA and free amylase

This result was supported by the previous study led by Khanahmadi *et al.* (2014) where they found CLEA-lipase had retained 70% of its residual activity as compared to the free lipase which was found to be only 1%. Figure 4 showed that CLEA-amylase displayed better pH stability from pH 9 to 11 when compared with its free form. Among the possible explanations was due to the cross-linking of the amino groups which might promote acidic condition on the enzyme surface. In this case, alkaline pH might be favorable to stabilize the enzyme by neutralizing the acidic groups.

A similar result was reported by Xu *et al*. (2011) where they found the better stability of CLEA-tyrosinase at higher pH value from pH 4 to 12. It is important to note that, during this experiment, CLEA and free amylase were incubated at their optimum temperature conditions, which were 65°C and 45°C, respectively. If this pH stability experiment was performed at a constant temperature, a greater difference in result could be expected between these two forms of enzymes (Khanahmadi *et al*., 2014).

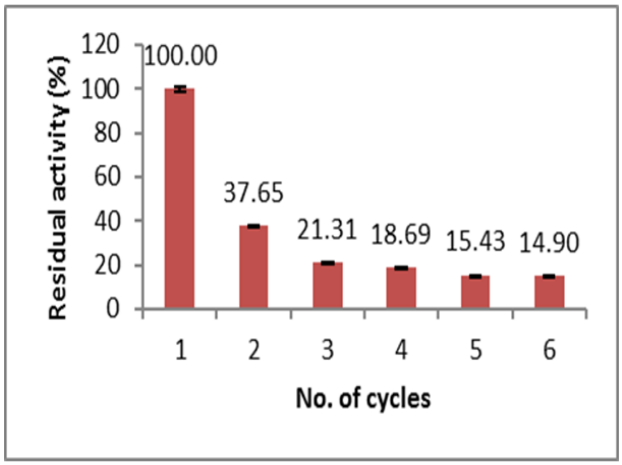

Figure 5. Reusability test for CLEA- amylase

Reusability of CLEA- amylase

The reusability test for CLEA-amylase was done for six cycles as shown in Figure 5. After the second cycle, the residual activity dropped significantly to 37.65%. The result obtained here was in agreement with Mahmod *et al*. (2015) where they found a major drop in lipase activity after the second cycle. After the sixth cycle, CLEA-amylase activity was recorded at 14.9%. However, it was in contradiction with several previous studies. For instance, CLEA-amylase which sourced from *Bacillus amyloliquefaciens* had shown 65% of its original activity over four cycles (Talekar *et al*., 2012).

The reasons for decreasing activity in this particular study might include the loss of precipitate and leaching of the enzyme during the washing and centrifugation processes in between the cycles. The centrifugal separation of CLEA from the reaction mixture which was applied in each cycle might cause clumping of CLEA, thus lead to activity reduction (Xu *et al.*, 2011). Another potential reason could be related to mechanical losses that were unavoidable during the process of pipetting the supernatant from the solid CLEA (van Pelt *et al*., 2008).

Conclusion

CLEA-amylase from super mealworm has been successfully prepared. As compared to the free enzyme, CLEA-amylase had displayed higher optimum values and better stabilities; in terms of pH and temperature. In addition, CLEA-amylase could also be recycled, resulting 14.9% residual activity after six cycles. Future works need to be carried out to increase the efficiency of this CLEA-amylase so that it could be used industrially.

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