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

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Abstract

Many studies have been done on various species of insects to investigate their potential use in industries. This is because insects have high protein content which could be further manipulated. Due to its eating habit, *Zophobas morio* larvae, also known as super mealworm has been shown to have high amylase activity. In this study, amylase from super mealworm has been immobilized via Cross-Linked Enzyme Aggregates (CLEA) technique and its kinetic performance, evaluated. CLEA is one of the best immobilization method with respect to enzyme stability and reusability. Kinetic performance of both free and CLEA-amylase were evaluated based on the Michaelis-Menten model. Results obtained based on Hanes-Woolf, Lineweaver-Burk, Eadie-Hofstee and Hyperbolic Regression plots showed that the kinetic parameters, V_{max} and K_M, changed upon immobilization. For CLEA-amylase, Hanes-Woolf plot showed the best-fitted model based on R^2 with V_{max}= 1.068 mM/min and K_M= 0.182 mM, however, Lineweaver-Burk plot was used to obtain the kinetic parameters for free amylase, with V_{max} and K_M of 17.230 mM/min and 2.470 mM, respectively. Thus it is observed that upon immobilization, V_{max} for amylase dropped appreciably, however, much lower substrate concentration is needed to saturate the enzymatic sites to reach its maximum catalytic efficiency. The result from this study might open the new path in discovering the potential use of insects in industrial applications, for example, making use of the recovered enzymes in the detergent industry.

Introduction

Body of insects contains proteins and the protein content is generally depending on the variability of the insect species as well as the life stages of the particular insects. For example, several insect species were studied at variable stages of life to determine their protein level (Veldkamp *et al*., 2012). Common housefly pupae have recorded the highest median crude protein composition with 65.7% dry matter. In contrast, the lowest level was determined in Black soldier fly larvae (38.9%) and its' pupae stages (37.4%). The larvae stage of *Zophobas morio,* a darkling beetle, is known as super mealworm, because it is mainly reared to be used as food for pets and fish. According to Finke and Winn (2004), the crude protein composition of the super mealworm was 19% suggesting a high enzymatic activity. This is due to the fact that enzyme was made up from protein.

Enzyme is important to help insects in digesting foods they consumed so that they will have sufficient nutrient for growing and biosynthesis (Liu *et al*., 2010). To meet the demand for their growth, larvae would normally feed on the carbohydrate foods (Kaur *et al*., 2014). Based on its eating habit, which consumed mostly starchy foods like wheat and carrot, this larva was expected to have high amylase activity, which has been shown by Easa and Yusof (2015).

Amylase is one of the hydrolases which plays vital role in various industries including beverages, brewing and detergent industries (de Souza and de Olieveira e Magalhaes, 2010). In fact, enzymes are often more superior than chemical catalyst due to several factors. For instance, they are more specific and could fasten the reactions by offering an alternative reaction pathway of lower activation energy. Besides that, even they facilitate in increasing the rate of reaction, the enzyme structure would remain unchanged at the end of each process (Gould, 2014). Enzymes also have high chemoselectivity, stereoselectivity and regioselectivity thus reducing the formation of undesirable by-products (Groger and Asano, 2012)

Despite of all the advantages offered by biocatalysts over their counterparts, they still have some drawbacks. For example, most of the industrial reactions nowadays are operated at harsh conditions

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such as extreme temperature and wide pH range. Moreover, the enzymes are not reusable and the recovery processes of the enzymes are often difficult, laborious and expensive.

Immobilization techniques like Cross-Linked Enzyme Aggregates (CLEA) have been introduced to increase enzymes stability and reusability targeting the industrial applications (Sheldon, 2007). CLEA is a very simple yet efficient method. For instance, Khanahmadi *et al.* (2014) reported that they have found significant improvement on CLEA-lipase activity where the residual activity of the CLEA had retained more than 60% of its initial activity after six cycles of reuse. They also reported that the optimal reaction temperature and pH value changed from 45°C at pH 8 of free lipase to 60°C at pH 8.2 for CLEA-lipase. Moreover, Talekar *et al.* (2012) who worked with *Bacillus amyloliquefaciens* have shown to retained 65% of original CLEA-amylase activity over four cycles. According to Lopez-Serrano *et al.* (2002), the cross-linked enzymes were prepared by simply precipitating the soluble protein before cross-linking the aggregates covalently. It combines two unit processes, which are immobilization and purification in one single operation hence it is more economical and labor effective (Sheldon, 2011).

However, this immobilization technique might have some effects on the kinetic performances of the enzyme. The two most important kinetic properties of an enzyme are how quickly the enzyme becomes saturated with a particular substrate, and the maximum rate it can achieve. In this research, the kinetics of the amylase-catalyzed reaction, using starch as the substrate, has been measured via the rate of the production of reducing sugar, using a protocol developed by Bernfeld (1955). The kinetics reaction of CLEA-amylase was compared to the free form. Here, it is pertinent to evaluate the changes in the kinetic properties, knowing well that there was a physicochemical changes made from the free enzyme to the CLEA form. The enzyme kinetics was modelled according to the 'Michaelis–Menten' kinetics. The model takes the form of an equation describing the rate of enzymatic reactions, by relating the reaction rate V to [S], the concentration of a substrate, given as:

$$v=V_{max}[S]/(K_m+[S]) \quad (1)$$

The Michaelis constant, K_M is the substrate concentration at which the reaction rate is half of V_{max}. Enzymatic reactions involving a single substrate are often assumed to follow Michaelis–Menten kinetics, without regard to the model's underlying assumptions. In order to find accurately the K_M and the V_{max} values, few researchers had manipulated the Michaelis-Menten equation to gain a linearized function, such as:

i. The Lineweaver-Burk plot, where 1/V is a linear function of 1/S:

$$1/V=(K_m/V_{max})\ 1/([S]) + 1/V_{max} \quad (2)$$

ii. The Hanes plot, where [S]/V is a linear function of [S]:

$$[S]/V=[1]/V_{max}[S|+\ K_m/V_{max} \quad (3)$$

iii. The Eadie- Hofstee plot, where V is a linear function of v/[S]:

$$v= -K_m\ v/([S])+ V_{max} \quad (4)$$

In addition, K_M and V_{max}, was also determined using hyperbolic regression, making use of the Hyperbolic Regression Software (Hyper32; Version 3.95). The most accurate kinetic parameters (K_M and V_{max}) were chosen based on the best-fitted curve, described by the Coefficient of Determination, R^2.

Materials and methods

Materials

Zophobas morio, at larval stage was purchased from a supplier at Kota Damansara, Selangor, Malaysia. Starch, dinitrosalicylic acid (DNS) and other chemicals used in this study were of analytical grades obtained from different suppliers (Merck, Systerm and Sigma-Aldrich).

Extraction of amylase

Only super mealworms with weight above 600 mg each were used in this study. The worms were washed under tap water to remove dirt. Next, they were placed in -20°C freezer for 15 minutes to make them numb prior to weighing process. Extraction was conducted using the optimized procedures developed by Easa and Yusof (2015). All steps were carried out at 4°C to maintain enzyme stability. A 20 g of the super mealworm were blended in 72% (w/v) phosphate buffer of pH 6.11 for 30 seconds before being filtered by muslin cloth to separate the solid residue. The solution was then collected and centrifuged at 12000 rpm for 60 minutes at 4°C to remove lipids and fine solids. Salting out procedure was performed using 4M ammonium sulphate under slow stirring at 4°C.

It was centrifuged again at 10000 rpm for 60 minutes at 4°C and the pellet was collected prior to dissolved in minimal buffer and stored in -20°C (Mahmod *et al.*, 2014).

Amylase activity assay

Amylase activity assay was carried out following the method of Bernfeld (1955) where 1% starch (w/v) was used as the substrate. Enzyme extract (0.5 ml) was mixed with substrate (0.5 ml) in a tube and then incubated at 37°C for 15 minutes. Upon completion, 1 ml DNS reagent was added and boiled for 5 minutes. The tubes were left to cool at room temperature, 10 ml distilled water added. To ensure homogenous mixing, the tube was inverted several times. Finally, absorbance was read at 540 nm. Standard curve was prepared using a varying concentration of maltose stock solution. 1 unit of amylase is defined as the amount of enzyme which hydrolyze 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 CLEA- amylase was prepared fresh for each subsequent study, unless mentioned otherwise. Activities of CLEA-amylase are reported in terms of recovered activities when compared to free amylase. 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% (5)

CLEA-amylase kinetic studies

The study of kinetic parameters in both free and CLEA-amylase were carried out in 0.05 M phosphate buffer (pH 6.11) at 37°C. Varied concentration of substrate (starch), ranging from 0.2 mM to 2 mM were used in this kinetic study. For comparison between free and CLEA-amylase, 0.5 ml free crude enzyme was used directly, however for CLEA-amylase, 0.5.ml crude enzymes was first submitted to immobilization processes before it was used in the study. Bernfeld assay method (Bernfeld, 1955) was carried out to determine the amylase activities. In order to calculate the value of V_{max} and K_M, plots of Hanes-Woolf, Lineweaver-Burk, Eadie-Hofstee and Hyperbolic Regression were used.

Results and discussion

In order to evaluate Michaelis-Menten kinetic parameters, four model plots were used in this study which are Hanes-Woolf, Lineweaver-Burk, Eadie-Hofstee and Hyperbolic Regression. The highest coefficient of determination (R^2) was assumed to be the best fit model for that particular reaction in this study. The results are shown by Table 1.

The best-fitted models are evaluated from their R^2 values. The nearer the values are to 1, the better are the fit to the model equations. Based on the highest R^2 values, the best fitted model for free amylase was by the Lineweaver-Burk plot (0.997), whereas for CLEA-amylase was by the Hanes-Woolf plot (0.971). Results from Table 1 shows that both V_{max} and K_M values decreased upon immobilization. V_{max} values signify the maximum velocity achieved by the system and how fast the enzyme could hydrolyse a completely saturated substrate in a reaction. In contrast, K_M is the substrate concentration at which the reaction achieved half of the V_{max}, and is an inverse measure of the substrate's affinity for the enzyme, as a small K_M indicates high affinity, meaning that the rate will approach V_{max} with lower substrate concentration than those reactions with a larger K_M.

The value of V_{max} dropped from 17.23 mM/min to 1.068 mM/ min after the CLEA immobilization process, which means that the CLEA-amylase require more substrate to convert a reactant to product per unit of time. The activity of CLEA-amylase in U/ml is lower than the free enzyme. As a result, the recovery activity of CLEA also is low since it is based on the total activity of the free enzyme. The result was common since cross-linking steps during the CLEA preparation might reduce accessibility of the substrate to CLEA's active site (Xu *et al*., 2011). Moreover, the reduction of V_{max} value also might be due to limited diffusion of the starch substrate. Since the CLEA enzyme was aggregated with acetone, it becomes solidified and precipitated. As a consequence, less active site might be accessible by the substrate for enzymatic reaction to take place as compared to the free enzyme. The result obtained here is similar in pattern with the results obtained by Gaur *et al.* (2006) who studied on CLEA-β-galactosidase. Similar results were also observed in CLEA-subtilisin (Sangeetha and Abraham, 2008), CLEA-tyrosinase (Xu *et al*., 2011) and CLEA-lipase

Table 1. Kinetic parameters of free and CLEA-amylase based on Michaelis- Menten model

Biocatalyst	Plots	V_{max} (mM/ min)	K_M (mM)	R^2
Free-amylase	Hanes-Woolf	20.30	3.075	0.988
	Lineweaver-Burk	17.23	2.470	0.997
	Eadie-Hofstee	18.26	2.657	0.979
	Hyperbolic Regression	19.38	2.893	0.990
CLEA-amylase	Hanes-Woolf	1.068	0.182	0.971
	Lineweaver-Burk	0.938	0.049	0.933
	Eadie-Hofstee	0.944	0.051	0.947
	Hyperbolic Regression	0.942	0.056	0.933

(Cui *et al*., 2016).

The value of K_M also decreased after immobilization, from 2.47 mM to 0.182 mM. Nevertheless, decreased in K_M value is an encouraging fact, since it indicates that the interaction between enzyme and substrate was stronger after the aggregation and the cross-linking process. According to Sangeetha and Abraham (2008) who worked on CLEA-subtilisin, the affinity of the CLEA enzyme might increase due to the presence of flexible glutaraldehyde arm which help the enzyme to preferably orient its active site towards the substrate.

Similar trend was found by Talekar *et al*. (2012), where they found the K_M of CLEA-amylase, prepared from *Bacillus amyloliquefaciens,* decreased from 2.748 mg/ml to 0.3245 mg/ ml after immobilization. Previous study by Yu *et al.* (2013) who worked on CLEA of mung bean epoxide hydrolases also found decreasing value of K_M after the immobilization process, from 16.89 mM to 5.05 mM.

From the previous studies, it was reported that there was exactly no definite changing trend in term of the kinetic parameters upon immobilization by CLEA techniques. As suggested by Xu *et al*. (2011), different responses of CLEA might occur in response to several factors, including the type of enzyme, the substrate, the reaction system, the procedures and the conditions which were used during the CLEA preparation. For instance, combi-CLEAs of lipase, α-amylase and phospholipase A2 had different patterns in V_{max} and K_M values, either they were unchanged, increased or decreased as compared to the free enzymes (Dalal *et al*., 2007).

Besides that, Mahmod *et al*. (2016) reported an increase of both V_{max} and K_M on protease extracted from viscera of channel fish *(Ictalurus punctatus)* upon CLEA immobilization. All these reports supported the fact that each enzyme act differently upon any particular immobilization techniques; the trend of changes in the kinetic parameters of enzymes should not be generalized and need to be worked out each time.

Table 2. Summary of kinetic performance of amylase

Amylase	Plots	K_M	V_{max}	Catalytic Efficiency (V_{max}/K_M)
Free	Lineweaver-Burk	2.47	17.2	6.96
CLEA	Hanes-Woolf	0.182	1.06	5.86

Table 2 shows the summary of the kinetic performances of amylase upon CLEA immobilization. The catalytic efficiency, which is denoted by V_{max}/K_M, of CLEA-amylase decreased a bit from 6.96 to 5.86 min^{-1}, when compared to the free enzyme. This result is parallel to result obtained by Khanahmadi *et al.* (2014) who worked on CLEA lipase where they found the catalytic efficiency of CLEA was lower compared to the lipase free enzyme (0.059 vs 0.388 min^{-1}).

Conclusion

In short, the kinetic parameters which are V_{max} and K_M were changed after immobilization. Different enzymes had shown different pattern of kinetic activities. The kinetic performances are largely depending on the type of enzyme being used, the substrate, the reaction system and the conditions used in preparing a particular CLEA. In this study, the kinetic parameters of CLEA-amylase, prepared from super mealworm were evaluated based the Michaelis-Menten equations, whereby the K_M and V_{max} values were estimated from Hanes-Wolf plot model, whereas the free enzyme was assessed using Lineweaver-Burk plot as reflected by the highest R^2

achieved.

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