

The Chemical Modification of Protease Enzyme Isolated from Locale Bacteria Isolate, *Bacillus Subtilis* ITBCCB148 with Cyanuric Chloride-Polyethylenglycol

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Abstract

The objective of the research was to study the effect of chemical modification on the stability of protease enzyme from a local bacteria isolate *Bacillus subtilis* ITBCCB148 with cyanuric chloride polyethylene glycol (CC-PEG). The results showed that the modified enzymes using CC-PEG with modification degrees of 24, 59, and 62% have the same optimum pH value 7.5 and active at pH ranges of 6.0-9.0, their optimum temperature was 60°C. The thermal stability values of the modified enzymes stored for 200 min at 60°C and pH 7.5 for each modification degree with CC-PEG were: 42% was $t_{1/2}$ 99 min, k_i 0.007 min^{-1} , ΔG_i 106.95 kJ mol^{-1} ; 59% was $t_{1/2}$ 173 min., k_i 0.004 min^{-1} , ΔG_i 108.5 kJ mol^{-1} ; and 62% was $t_{1/2}$ 173 min, k_i 0.004 min^{-1} , ΔG_i 108.5 kJ mol^{-1} . The residual activities of the modified enzymes with modification degrees of 42, 59, and 62% stored for 200 min at 60°C and pH 7.5 were 24, 36, and 44%, respectively. The chemical modifications used have successfully been able to increase the thermal and pH stabilities of the enzyme 2-3 times. All modified enzymes have working pH ranges of 6.0-9.0, which are broader than the purified enzymes.

Keywords: Protease, chemical modification, *Bacillus subtilis* ITBCCB148

1. Introduction

Protease is enzyme that breaks the peptide bond to produce amino acids and other simpler peptides. It can be isolated from a variety of sources such as plants, animals and microbia (fungi and bacteria). Its application is very broad and has been used in many fields for years, and is mainly used in food and detergent industries. The protease used in detergent industry must have a wide specificity spectrum toward a variety of substrates, as it must be able to clean up a variety of dirt left over such as food, blood and others. It must resist against the oxidizing and chelating agents which are other component of the detergent. Protease must also be stable and active at higher temperature and pH. The most important parameter of protease in order to be able to be used in detergent industry is the range of its pH value (Price and Stevens, 1996).

Mozaev *et al.* (1990) suggested that to obtain the stable enzyme, the chemical modification can be used. Some chemical modification researches which have been done previously have shown that the modified enzymes have increased their stability against pH and temperature compare to that without modification (Morand and Biellmann, 1991; Kobayashi and Takatsu, 1994; Yang *et al.*, 1996).

Based on the results previously reported, in this research, the chemical modification using CC-PEG to increase the stability of protease which is produced, isolated and purified from a local bacteria isolate of *Bacillus subtilis* ITBCCB148 is chosen (Yandri *et al.*, 2007) This is done based on the hypothesis that chemical modification using CC-PEG will increase the protease enzyme thermal and pH stabilities.

2. Materials and Methods

2.1. Materials

Polyethylene glycol activated with cyanuric chloride (CC-PEG) and other chemical used were purchased from Sigma Aldrich and used without further purification. Local bacteria isolate *Bacillus subtilis* ITBCCB148 was obtained from Microbiology Laboratory, Chemical Engineering Department, Bandung Institute of Technology, Bandung, Indonesia. Buffer pH was measured at the temperature of use, and the pH reported is that at the temperature of the incubation.

2.2. The Production of Protease Enzyme

The production stage of the protease enzyme includes: determination of incubation time to get enzyme with the highest activity, determination of optimum temperature and the optimum pH of fermentation media. The fermentation media used are the mixture of peptone 5%, yeast extract 0.15%, glucose 0.036% and NaCl 0.25% (Yandri *et al.*, 2007).

2.3. The Isolation and Purification of Protease Enzyme

The Isolation and purification of protease enzyme are carried in a few step experiments: the separation of the liquid of enzyme from the cell with cold centrifugation to obtain the raw enzyme extract, precipitation with ammonium sulphate in a variety of super saturated solutions, ion exchange column chromatography and molecule filtration column chromatography (Yandri *et al.*, 2007).

2.4. The Protease Enzyme Activity Test and Protein Content Determination

The Protease enzyme activity test was done based on the modified Kunitz method (Yamaguchi *et al.*, 1982). Protein content determination was done based on the Lowry *et al.* (1951) method.

2.5. The Modification of Purified Enzyme with CC-PEG

The Modification of Purified Enzyme with CC-PEG was done based on the method used by Takahashi *et al.* (1985) and as following:

2 mL (0.15 mg/mL) of purified protease enzyme is placed in *wheaton* vial 8 mL containing 0.25 mg activated PEG in 2 mL of borax buffer (0.1 M, pH 9.0). The molar ratio used of PEG: purified enzyme in the experiments were 10:1; 20:1 and 30:1. The solution mixture was stirred for 3 hours at room temperature, and then dialyzed with phosphate buffer (20 mM, pH 6.0) for 24 hours, and liophilized for 48 hours on freeze dryer.

2.6. Determination of Modification Degree

Determination of modification degree was done based on the method used by Synder and Sobocinski (1975).

2.7. Characterization of the Enzyme Before and After the Modification

The enzyme characterizations before and after the modification include: determination of optimum pH and temperature, kinetics data, stability toward pH and temperature.

2.7.1. Determination of optimum pH and temperature before and after the modification

To get the optimum pH of the enzyme before and after the modification, the acetate buffer was used with pH variations of 4.5; 5.0; 5.5; 6.0; 6.5; 7.0; 7.5; 8.0; 8.5; and 9.0. The temperature was kept constant at the determined optimum pH. To find the optimum temperature, the variations of temperature used were 45; 50; 55; 60; 65 and 70°C.

2.7.2. Determination of enzyme kinetics data before and after the modification

The Michaelis-Menten (K^m) constant and maximum reaction rate (V_{max}) of the enzyme before and after the modification were determined based on equation of Lineweaver-Burk.

3. Thermal stability test and stability of the enzyme pH before and after the modification

The enzyme thermal stability before and after the modification was done by measuring the residual activity of the enzyme after being incubated for a specific period at specific pH and temperature based on the method applied by Yang *et al.* (1996)

4. Determination of half life ($t^{1/2}$), k_i and ΔG_i

Determination of k_i value (rate constant of thermal inactivation) of purified enzyme and the modified enzyme was done using the first order of inactivation kinetics equation (Equation 1) (Yandri, 2007):

$$\ln(E_i / E_0) = -k_i t \quad (1)$$

The denaturation energy change (ΔG_i) of the purified and modified enzymes was done using the Equation (2) (Yandri, 2007):

$$\Delta G_i = -RT \ln (k_i h / k_B T) \quad (2)$$

3. Results and Discussion

3.1 Modification of the Purified Enzyme with CC-PEG: Determination of Modification Degree

Modification of the purified enzyme with CC-PEG were done in three variations of proteine content ratio between purified enzyme and CC-PEG, *i.e.* 1: 10; 1: 15, 1: 20. The result of determination of modification degree is shown in Table 1.

Table 1: Determination of modification degree Penentuan derajat modifikasi dengan menggunakan asam 2,4,6- trinitrobenzen sulfonat (Synder and Sobocinski, 1975)

Sample	ΔA_{420nm}	Modification (%)
Purified enzyme	0.731	0
CC-PEG 1: 10	0.422	42
CC-PEG 1: 15	0.299	59
CC-PEG 1: 20	0.278	62

3.2. The Characterization of the Modified Enzyme with CC-PEG

3.2.1. Determination of optimum pH of the enzyme before and after midification

The enzyme activity (%) before and after the modification at various pH can be seen in Figure 1.

Figure 1: The optimum pH of modified enzyme (CC-PEG 42 %, CC-PEG 59 %, CC-PEG 62 %), and purified enzyme

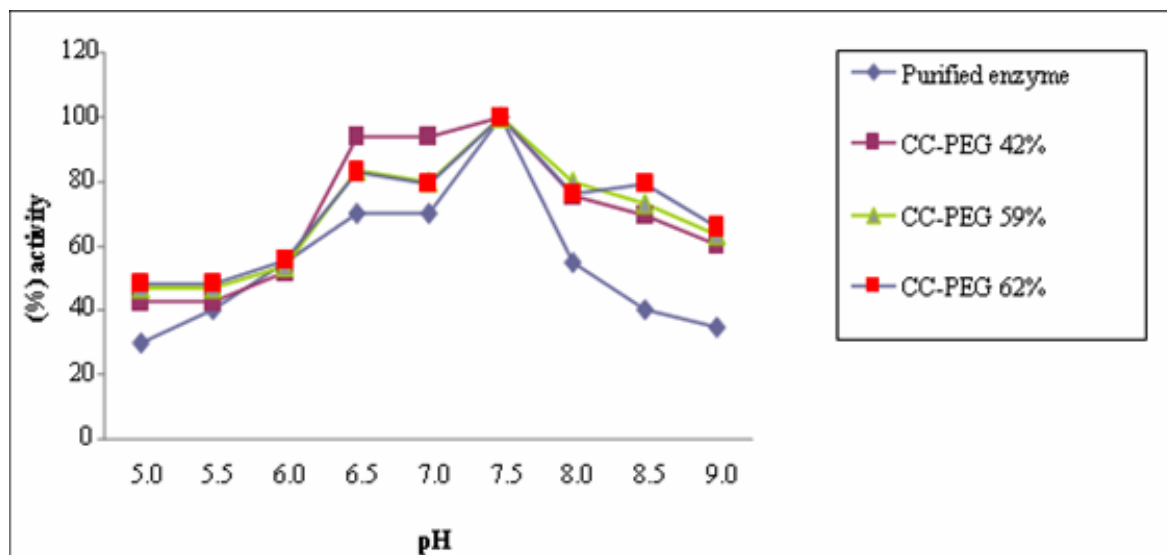


Figure 1 shows that the optimum pH of the purified enzyme is 7.5. Whereas the optimum pH of the modified enzymes with CC-PEG 42%, 59% dan 62% do not change their optimum pH and remain constant at 7.5. Figure 1 also shows that the purified enzyme is stable in the pH range of pH 6.0 – pH 8.0, in which the activity of purified enzyme at pH 6.0 and 8.0 are the same at 55 %. All modified enzymes increase their pH stability compared to that of the purified enzyme. The modified enzymes are stable on the pH range of 6.0 – 9.0. The percent activity of the modified enzymes of CC-PEG 42% at pH 6.0 is 52% and at pH 9.0 is 61%; CC-PEG 59% at pH 6.0 is 53%; and at pH 9.0 is 63%; CC-PEG 62% at pH 6.0 is 55%, and at pH 9.0 is 66%. This result indicated that the modified enzymes with CC-

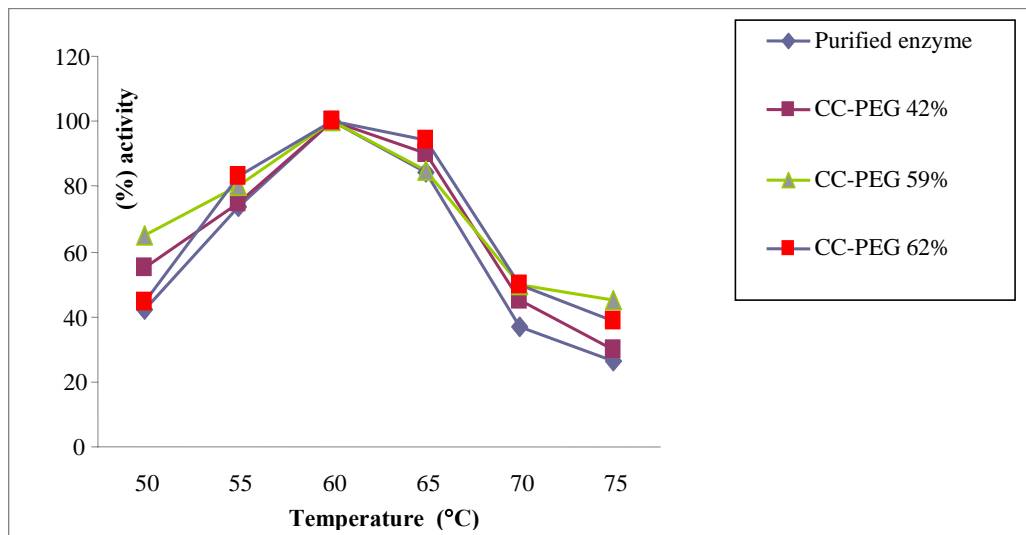
PEG are more stable toward acid and base than that of purified enzyme. The stability increased is more at basic condition.

The stability increase of the modified enzyme with CC-PEG toward pH may be due to the NH_2 free group on the main R group on the surface of enzyme is bound to CC-PEG, as a result the alteration of the charge on the enzyme structure is occurred. According to Zubay (1983) the effect of pH on the activity of some enzymes depended on their pK, the group ionization on the enzyme as well as the substrate involves in the reaction. The pH sensitivity of an enzyme is generally indicated by the group which can be ionized in the reactive site of the enzyme. The ionized group is the determining factor on the change of enzyme tertiary structure which affects the active site of the enzyme.

3.2.2. Determination of optimum temperature of enzyme before and after the modification

The activity (in %) of enzyme before and after the modification at various temperatures is shown in Figure 2. The optimum temperature of the purified enzyme based on Figure 2 was 60°C , the optimum temperature of the modified enzyme with CC-PEG were also 60°C . Although the optimum temperature of the modified enzyme did not increase significantly compare to that of the purified enzyme, however, the stability of the modified enzymes were increase in the temperature ranges of $65 - 70^\circ\text{C}$. The activity of the purified enzyme at 65°C was 84.0%, while the modified enzyme with CC-PEG 42%, 59%, and 62% were 85.0%; 90.0%; dan 94.0%, respectively. The activity of the purified enzyme at 70°C was 37.0%, while the modified enzymes with CC-PEG 42%, 59%, and 62% were 45.0%; 50.0%; and 50.0%, respectively. All modified enzymes have optimum temperature the same as the purified enzyme, *i.e.* 60°C .

Figure 2: The optimum temperature of the modified enzymes with CC-PEG of 42%, 59%, 62% and the purified enzyme



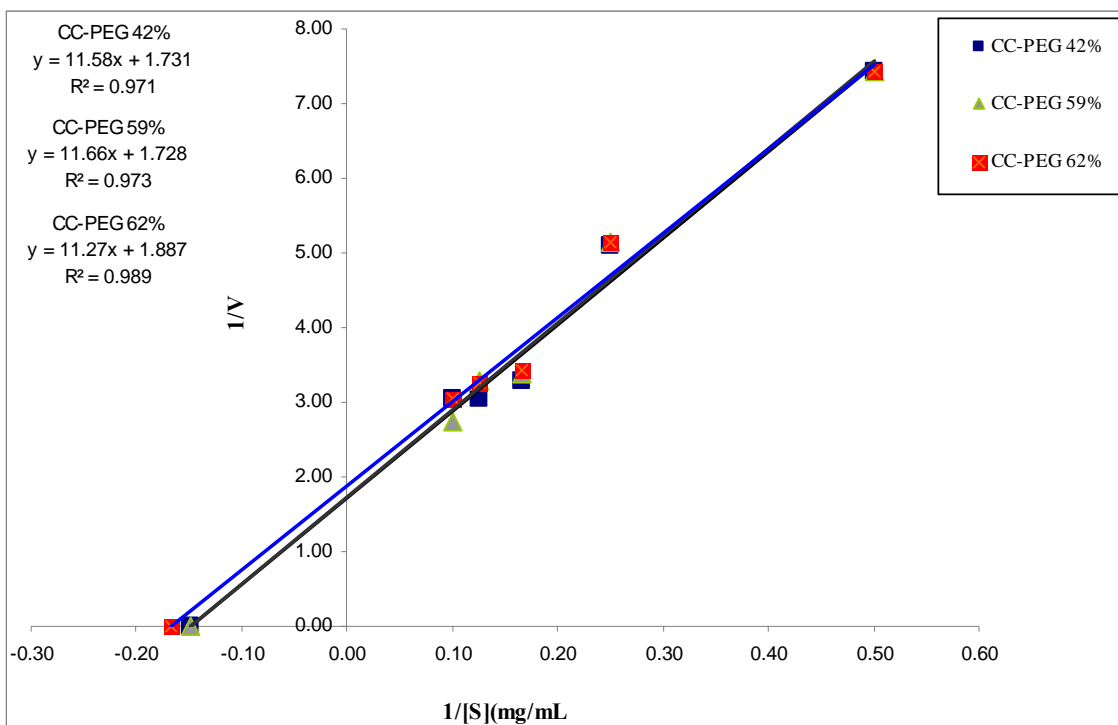
This phenomenon can be explained as the following due to the bulky size of PEG, it makes the PEG molecule can not insert to the space of the active site on the enzyme, and as a result the three dimension structure of enzyme does not significantly change, thus the activation energy does not change either. This condition causes the optimum temperature does not change. Kazan *et al.* (1997) and Francis *et al.* (1992) reported that the chemical modification was not always causing the temperature change on the modified enzyme. Although the optimum temperature change does not change, however, the activity of modified enzyme with CC-PEG at higher temperature shows better

result than that of purified enzyme. This condition occurred is perhaps the modification process causes the rigidity of the modified enzyme increases, so the modified enzyme is more tolerant against the temperature.

3.2.3. Determination of enzyme kinetics enzyme after the modification

The graph of determination of K_m and V_{max} values of the enzyme before and after the modification is shown in Figure 3. This figure shows V_{max} and K_m values of the modified enzyme as following: CC-PEG 42% $V_{max} = 0.58 \mu\text{mol mL}^{-1} \text{min}^{-1}$, $K_m = 6.7 \text{ mg mL}^{-1}$ substrate; CC-PEG 59% $V_{max} = 0.58 \mu\text{mol mL}^{-1} \text{min}^{-1}$, $K_m = 6.8 \text{ mg mL}^{-1}$ substrat; CC-PEG 62% $V_{max} = 0.53 \mu\text{mol mL}^{-1} \text{min}^{-1}$, $K_m = 6.0 \text{ mg mL}^{-1}$ substrate, while for the purified enzyme the values obtained as $V_{max} 3.26 \mu\text{mol mL}^{-1} \text{min}^{-1}$, and $K_m 6.48 \text{ mg/mL}$ substrate (Yandri *et al.*, 2007). The results obtained indicated that the K_m values of all modified enzymes did not change significantly. This is because there is no change on the active site of the enzyme structure, so the enzyme affinity toward the substrate did not change either. Whereas the V_{max} values of all modified enzymes with CC-PEG were decreased. This decrease is because the chemical modification with CC-PEG which has long chain on the surface of the enzyme molecule made the enzyme was not flexible in aqeous solution (Yang *et al.*, 1996). PEG also affects proximity and enzyme orientation. The enzyme without modification can normally interact easily with a substrate, whereas with the presence of long chain PEG causing the change on the structure of an enzyme, as a result the V_{max} decreases. Kazan *et al.* (1997) and Francis *et al.* (1992) reported that the chemical modification did not always cause the change of K_m and V_{max} of the modified enzyme.

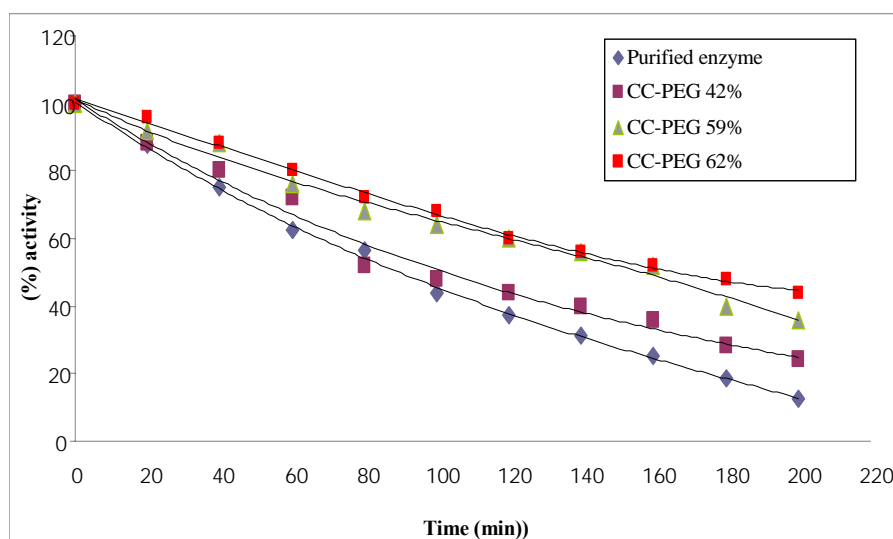
Figure 3: The Lineweaver-Burk graph of modified enzymes with CC-PEG 42%, 59%, 62%



3.2.4. Thermal and pH stabilities of the enzyme before and after the modification

The residual activities of the purified enzyme and the modified enzyme with CC-PEG toward temperature and pH are determined by incubating each enzyme at pH 7.5 and temperature of 60°C for 200 min. The curve of thermal and pH stabilities of the purified enzyme and the modified enzyme with CC-PEG is shown in Figure 4. From this figure, it can be seen that the residual activity of the purified enzyme was 13%, while those for the modified enzyme with CC-PEG 42%, CC-PEG 59%, CC-PEG 62% were 24%; 36%; dan 44.6%, respectively. This figure also tells us that the thermal and pH stabilities of the modified enzymes were increased compare to that of the purified enzyme. All modified enzymes with various degree of modification have higher residual activity after being incubated for 200 min compare to that of purified enzyme. The chemical modification on the purified protease enzyme of *B. subtilis* ITBCCB148 showed a better effect on the structure stability. The mechanism of structure stability occurred on the protease enzyme due to the chemical modification with activated PEG can be explained as the following: (1) the decrease of contact between the hydrofob group and the solvent; (2) the increase on the aromatic interaction; and (3) the addition of hydrogen bond (Vieille and Zeikus, 1996).

Figure 4: Thermal stability express as a graph between residual activity (%) of the purified enzyme and modified enzymes with CC-PEG 42%, CC-PEG 59%, CC-PEG 62%; at pH 7.5 and temperature of 60°C vs time



3.2.5. The energy change due to the denaturation (ΔG_i), half-life ($t_{1/2}$), and k_i value of the enzyme before and after modification

The data of energy change due to the denaturation (ΔG_i), half-life ($t_{1/2}$), and k_i value of the purified enzyme and the modified enzyme are shown in Table 2.

Table 2: The energy changes (ΔG_i), half life ($t_{1/2}$), and k_i value of the purified enzyme and the modified enzyme

Enzyme	k_i (min^{-1})	$t_{1/2}$ (min)	ΔG_i (kJ mol^{-1})
Purified	0.009	77	106.25
CC-PEG 42%	0.007	99	106.95
CC-PEG 59%	0.004	173	108.50
CC-PEG 62%	0.004	173	108.50

Based on Table 2, all modified enzymes with CC-PEG exhibited a higher half-life than that of the purified one, and their half-lives were increased two- to three-fold. According to Stahl (1999) thermal and pH stability of an enzyme are determined by the half-life of the enzyme. The increased half-life of the modified enzymes reported here were similar to that previously reported by Hernaiz *et al.* (1999). The modified enzymes with modification degree of 62% has increased its half-life from 77 min to 173 min, and the enzyme activity recovery was 87,5%. Yandri *et al.* (2003, 2005) have also done chemical modification on α -amilase enzyme from local bacteria isolate *Bacillus subtilis* ITBCCB148 with activated-PEG (CC-PEG and NPC-PEG) and the results showed that the thermal stability of the modified enzymes were increased 2 to 4 times based on the decrease of k_i values and the working pH ranges are also increased. Thus result presented here agrees to those previously reported that the chemical modification using activated-PEG were able to increase not only thermal stability, but also the working pH range.

Table 2 shows the decrease of k_i value which is the rate of thermal inactivation constant for each of the modified enzyme. Based on those data the denaturation rate of the modified enzymes was decreased compare to that of the purified enzyme, accordingly the thermal stability of the modified enzymes was increased 2-3 times. The effect of chemical modification using CC-PEG on the decreased of k_i value in this research is assumed due to the formation of bonding between long chain PEG and the NH_2 group on the side change of lysine residue on the surface of enzyme. Due to this bonding, the enzyme is less flexible on the aqueous solution, therefore the protein unfolding becomes less too, accordingly the stability of the enzyme is increased (Yang *et al.*, 1996).

The ΔG_i of the modified enzyme with CC-PEG was increased compared to that of the purified enzyme. The higher the modification degree used, the higher the ΔG_i value obtained, this indicated that the modified enzyme was more rigid and less flexible, thus the energy required to denaturate the enzyme was getting higher. The lower ΔG_i value indicates that the enzyme is more flexible in the solution and it tends to be more easily denaturated, and vice versa. The bigger ΔG_i value causes the enzyme activity decrease as the enzyme flexibility gets less, whilst the smaller ΔG_i value shows that the enzyme is too flexible and is easily denaturated, although the activity is higher. These phenomena agree with the data obtained in this research that the higher the modification degree, the higher the ΔG_i value and as a result the enzyme stability is also increased.

Conclusions and Future Works

Conclusions

All modified enzymes using CC-PEG with modification degree of 42%, 59%, and 62% have optimum pH of 7.5, the enzyme optimum temperature was 60°C. The kinetics data of the modified enzymes were: CC-PEG (42%) K_m 6.7 mg/ mL substrate, V_{max} 0.58 U/mL; CC-PEG (59%) K_m 6.8 mg mL substrate, V_{max} 0.58 U/mL; CC-PEG (62%) K_m 6.0 mg mL substrate, V_{max} 0.53 U/mL. The modified enzymes were stable on the pH ranges of 6.0–9.0. The thermal stability test on the storage temperature of 60°C and pH 7.5 for 200 min showed the modified enzymes with CC-PEG 42% has residual activity of 24%; $t_{1/2} = 99$ min; $k_i = 0.009 \text{ min}^{-1}$; $\Delta G_i = 106.95 \text{ kJ mol}^{-1}$; CC-PEG 59% has residual activity of 36%; $t_{1/2} = 173$ min; $k_i = 0.004 \text{ min}^{-1}$; $\Delta G_i = 108.5 \text{ kJ mol}^{-1}$; CC-PEG 62% has residual activity of 44%; $t_{1/2} = 173$ min; $k_i = 0.004 \text{ min}^{-1}$; $\Delta G_i = 108.5 \text{ kJ mol}^{-1}$.

Chemical modification with CC-PEG has successfully increased not only the thermal stability of the enzyme in 2 to 3 times, but also broadened the working pH ranges to 6.0 - 9.0, which is far broader than that of the purified enzymes.

Future Works

To get higher increase on the stability of protease enzyme from local bacteria isolate *B. subtilis* ITBCCB148 a further study with other chemical modification agents might be tried. Besides using the modification agent which has bigger molecular weight than CC-PEG, such as NPC-PEG, the modification agent of smaller molecular weight, for instance dimethyl adipimidate might be worth to be tried and to be compared with the modification agent used in the current work.

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