Nature and Science, 2009;7(2), ISSN 1545-0740, http://www.sciencepub.net,
naturesciencej@gmail.com

The Effect of Chemical Modification on the Thermal Stability of Protease from Local Isolate Bacteria, *Bacillus subtilis* ITBCCB148

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Abstract: This research aims to study the effect of chemical modification on the thermal stability of protease enzyme from local bacteria isolate *Bacillus subtilis* ITBCCB148 which was modified using dimethyladipimidate (DMA). To approach this aims, the production, isolation and purification of the enzyme were done. The purified enzyme was then modified with DMA. The number of modified lysine residue was shown by the modification degree determined using trinitrobenzenesulfonic acid. The success in the chemical modification was done by comparing the thermal stability of the enzyme before and after modification. The results showed that the modified enzymes with DMA produced modified enzyme with modification degree of 69, 75 and 76%. The enzyme thermal stability of the modified enzyme with modification degree of 69, 75 and 76% at 60°C were shown with the following data: $t_{1/2}$ = 96.25 min, k_i = 0.0072 min^{-1}, ΔG_i 106.868 kJ $mole^{-1}$; $t_{1/2}$ = 119.48 min, k_i = 0.0058 min^{-1}, ΔG_i 107.466 kJ mol^{-1}; and $t_{1/2}$ = 210 min; k_i = 0.0033 min^{-1}; ΔG_i 109,028 kJ $mole^{-1}$, respectively, whereas the thermal stability of the purified enzyme has data of: $t_{1/2}$ = 52.1 min, k_i = 0.0133 min^{-1}, 105.168 kJ $mole^{-1}$. The chemical modification on the purified protease enzyme has been able to increase the thermal stability up to 2 – 4 times compare to that of the purified one based on the decrease of K_i value. [Nature and Science. 2009;7(2):68-75]. (ISSN: 1545-0740).

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

Introduction

Protease is enzyme that breaks the peptide bond into oligopeptide and amino acids. This enzyme is widely used in industrial sector such as food, skin, detergent and pharmaceutical industries (Rao *et al*., 1998). The use of this enzyme as biocatalyst in industry must fill some criteria, which are it must have high thermal stability and is able to work in wide pH range (Vieille and Zeikus, 1996). This condition is not own by most of the enzymes, as generally an enzyme works at physiological condition and cannot stand against extreme condition especially temperature and pH. In industrial processes which use the enzyme in enzymatic reaction, the use of en enzyme in an extreme condition from its optimum condition, can cause the enzyme lose its catalytic activity. Therefore in order to find an enzyme which can be used in industrial process, we must be able to get enzyme which is able to work in extreme conditions. This can be achieved in a few ways such as direct isolation from organism which lives in extreme condition (extremophilic) or by chemical modification to the enzyme from organism which does not live in extreme condition (mesophilic) (Wagen, 1984). The stabilization of enzyme obtained from mesophilic microbia is preferred way to get stable enzyme (Mozhaev and Martinek, 1984). According to Mozhaev *et al.* (1984), three ways to increase stability of an enzyme are amobilization, chemical modification, and directed mutagenesis. Chemical modification is a method to increase the stability of an enzyme which is soluble in water. The use of enzyme amobilization has a weakness, i.e. the decrease of binding capacity or enzyme reactivity due to the mass transfer inhibition by amobil matrix. In directed mutagenesis needs complete information about the primer structure and the picture of three dimension structure. According to Janecek (1993) in amobil process, the work mechanism of the enzyme in clinical sector, during interaction with receptor or other components of cellular membrane might be altered due to the presence of long matrix. While chemical modification, the interaction of enzyme with substrate is not hindered by the presence of insoluble

matrix, so the decrease of enzyme activity can be minimized. Based on this fact, the chemical modification is suggested way to increase the stability of enzyme.

In order to obtain the enzyme by chemical modification with stable covalent bond can be done by utilizing bifunctional reagent, modification with nonpolar reagent, addition of charge group or new polar group and hydrophilization the surface of protein (Mozhaev *et al*., 1990).

Modification with bifunctional reagents (cross-bond reagents) has been used many times to increase the enzyme stability. An example of bifunctional cross-bonded reagent is dimethyladimipidate. Kazan *et al.* (1996) reported that penicillin G acylase has increased its pH working range after reaction with DMA. Erarslan and Ertan (1995) have done thermostablization of penicillin G acylase from *E. coli* mutant with dimethyladipimidate (DMA), dimethylsuberimidate (DMS) and dimethyl-3,3'-dithiobispropionimidate (DTBP). The higher thermostabilization increased was observed after cross bond with DMA at temperature above 50^{o}C.

Based on the results of the above researches, the chemical modification using dimethyladipimidate to increase the thermal stability of protease enzyme from local bacteria isolate *Bacillus subtilis* ITBCCB148 is chosen.

Materials and Methods

Materials: Dimethyladipimidate (DMA) and other chemicals used were purchased from Sigma Aldrich and used without further purification. Local bacteria isolate *Bacillus subtilis* ITBCCB148 was obtained from Microbiolgy 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.

Production of protease enzyme: the production of enzyme is done using fermentation media containing peptone 0.5%, yeast extract 0.15%, glucose 0.036%, and NaCl 0.25% (Yandri *et al.* 2007).

Purification of protease enzyme: The purification of protease enzyme is done in few steps: the separation of enzyme liquid from the cell with cold centrifugation to get the raw enzyme extract, precipitation with ammonium sulphate, ion exchange column chromatography and molecule filtration column chromatography (Yandri *et al*., 2007, 2008).

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

The modification of purified enzyme with DMA: Into 10 mL (0.082 mg/mL) of purified protease enzyme in 20 mM of phosphate buffer pH 7 was added with solid DMA until the concentration of DMA were 0.5%, 1%, and 1,5% (^{W}/v) to reach inactivation of kinetic work at different pH and temperature and then leave them for 1 h at room temperature (Kazan *et al.*, 1996)..

Determination of modification degree: Determination of modification degree was done based on the method used by Synder and Sobocinski (1975) and as following 0.1 mL of modified enzyme is dissolved into 0.9 mL borate buffer (pH 9.0) and then added with 25 µl 0.3 M 2,4,6-trinitrobenzenesulfonic acid. The mixture is then shaken and left it at room temperature for 30 min. The standard solution is made with the same composition but using the purified enzyme, while the blank contains 1 mL borat buffer 0.1 M pH 9 and 25 µl 0.3 M 2,4,6-trinitrobenzenesulfonic acid. The absorbance is measured at the λ_{max} 420 nm.

Determination of optimum pH and temperature before and after the modification: To know the optimum pH of the enzyme before and after the modification, the phosphate buffer 0.1 M was used with pH variations of 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 50; 55; 60; 65 and 70°C, and then followed by the measurement of enzyme activity with Kunitz method.

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 0, 60, 120, 180, 240, and 300 min optimum pH and temperature based on the method applied by Yang *et al*. (1996)

Determination of half life (t½), ki and ΔGi: determination of ki value (rate constant of termal inactivation) of purified enzyme and the modified enzyme was done using the first order of inactivation kinetics equation (Equation 1) (Kazan *et al.* 1997

$$\ln(Ei / E0) = -ki\ t \quad (1)$$

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

$$\Delta Gi = -RT \ln (kih/kBT)$$

Results and Discussion

Determination of modification degree with DMA: Modification of the purified enzyme with DMA was done in three concentration variations of DMA which was mixed with 10 mL of purified enzyme in phosphate buffer pH 7.0. The concentration variations of DMA used were 0.5%, 1%, and 1.5%. The result of determination of modification degree with 2,4,6-trinitrobenzenesulfonic acid were 69, 75, and 76%. Determination of modification degree is based on the ratio of number of lysine residues after and before modification.

Effect of modification toward optimum pH: Based on the data obtained, the modified enzyme with modification degree of 75% get decrease its optimum pH , while the optimum pH of modified enzyme with modification degree of 69% and 76% are the same as the purified enzyme (Figure 1).

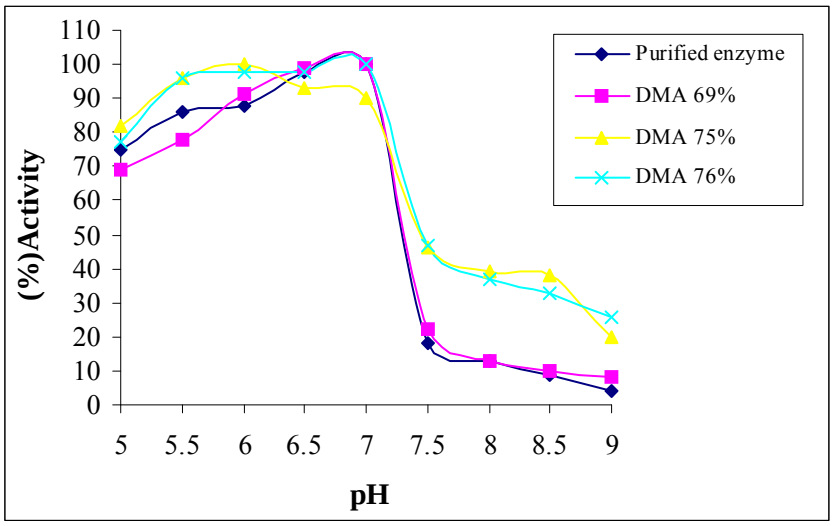

Figure 1. The Optimum pH of the Purified and Modified Enzyme (69%, 75%, 76%)

The decrease of pH optimum might be due to the change in enzyme conformation which causes the change on the active site of the enzyme, so the reaction ability of enzyme is also changed in its environment. Although there is decrease in optimum pH, but the (%) activity of the modified enzyme (DMA 75% and 76%) is higher at pH 7.5-9.0 when they are compared to modified enzyme with DMA 69% and the purified one. The high activity value (%) of the modified enzyme illustrates that the modified enzyme attain stability increase toward pH when compared to that of the purified one. The purified enzyme can retain its stability at pH 6.0-7.0, but there is a drastic decrease at pH 7.5-9.0. Whereas the modified enzymes with DMA 75% and 76% have wider working pH range, i.e. 5.0-7.0, although there is activity decrease at pH 7.5-9.0, but the decrease is not too high and the (%) activity is higher in that pH range compared to modified enzyme with DMA 69% and the purified enzyme. The activities (%) of purified and modified DMA 69% at pH 9.0 were 4% and 9 %, respectively, while those for modified DMA 75% and 76% were 20% and 26%, respectively. These results showed that the modified enzymes are more stable toward pH especially at basic condition than the purified enzyme. This phenomenon can be explained that at basic condition, the higher concentration of OH^- ion will bind to H^+ ion of carboxyl group on enzyme molecule to form H_2O. By modifying the enzyme, besides the binding of NH_2 groups of enzyme molecule by carbon group from DMA molecule, the hydrogen bond between H atom from carboxyl group with N of amine group in DMA. Due to the binding of H^+ of carboxyl group of enzyme by OH^- to form H_2O is not occurred. Therefore, the modified enzyme will be more stable at basic pH. The activity increase of modified enzyme was also occurred at acidic condition, but not too high and less significant. Based on this discussion it has been shown that DMA is able to increase enzyme stability toward pH.

The effect of modification toward optimum pH: The modified enzymes with DMA 69%, 75%, and 76% have optimum pH the same as the enzyme before it is modified (Figure 2), however their activity is higher than that of purified one.

Soemitro (2005) stated that the activity increase occur due to temperature increase did not change the accuracy of the tertier structure of enzyme before and after modification which bring close the side chain of some amino acids of stereospecific substrate side bond maker or amino acids of catalytic side support, so the optimum temperature of the enzyme did not change. This result agreed to those reported by Yandri *et al.,* (2008), Kazan *et al*. (1997) and Francis *et al*. (1992) that the chemical modification must not always cause the change of optimum temperature of the modified enzyme. Although the optimum temperature of the modified enzyme did not change, but the stability of the enzyme has increased at temperature range of 55 – 65^{o}C. At 55^{o}C the purified enzyme has activity of 75%, while at 65^{o}C was 24%. The modified enzymes with DMA 69%, 75%, and 76% at 55^{o}C have activity of 87%, 82%, and 96%, respectively, while at 65^{o}C has activity of 42%, 60% and 40%, respectively. This result illustrated that the modification caused the enzyme was more rigid, so the enzyme was more endure toward temperature.

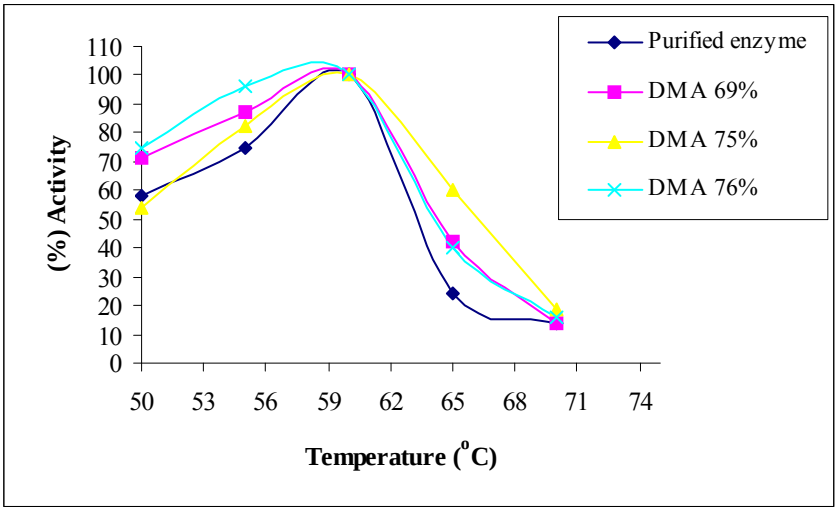

Figure 2. Optimum Temperature of the Purified and Modified Enzyme (DMA 69%, 75%, 76%)

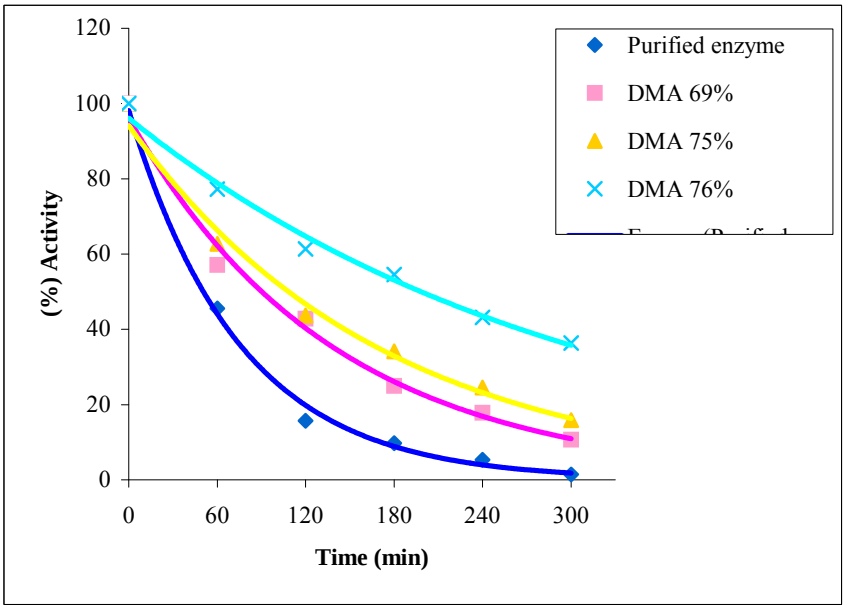

Figure 3. Thermal Stability Expressed as Graphic between Residual (%) activityPurified Enzyme and Modified Enzyme (DMA 69%, DMA 75%, DMA 76%) at 60°C *vs* Time

The effect of modification toward thermal stability: The test of thermal stability effect to the modified enzyme was done by calculating the percentage of residual activity of purified and modified enzymes. This was done by incubating each enzyme at 60°C for 300 minutes. The activity was measured every 60 minutes, and the result is presented in Figure 3.

Figure 3 illustrates the residual (%) activity of each enzyme. The purified enzyme has residual activity of 1.47% and then after modification with DMA 69%, DMA 75% and DMA 76% the residual activities were 10.71%, 15.87% and 36.36%, respectively. Based on graph in Figure 3, it is clear that the modified enzymes have much higher thermal stability than that of purified one.

In line with modification degree, the higher modification degree, the higher the thermal stability of the enzyme, and the use of DMA as the modifying agent has a good effect on the structure stability. The mechanism of enzyme structure stability by DMA is occurred through cross-bond of inter and intramolecular of DMA to form small circles on enzyme, to protect the unfolding of tertier structure of enzyme and protein oligomer dissociation into subunits. The cross bond to lysine residue in the surface will protect the hydrophobic site of the enzyme to contact with solvents. Besides that, there is addition of hydrogen bond between the nitrogen atom of amine group in DMA molecule and hydrogen atom of carboxyl groups of the enzyme. Since DMA is bifunctional reagent with two functional groups which can bind to two enzyme molecules, consequently, the hydrogen bond form will more. Kazan *et al*. (1996) reported that the change of ionization degree of organic groups on enzyme due to the formation of hydrogen bond causing the rigidity increase of penicillin G acylase structure modified with DMA. The more hydrogen bonding formed, the higher the modification degree used, so the enzyme rigidity is also increased. This result supported previous result obtained that the higher the modification degree, the more stable the enzyme will be (Kazan *et al*., 1996).

Table 1. The Values of Rate of Thermal Inactivation (k_i), Half-life ($t_{1/2}$) and Denaturation Energy Change (ΔGi) of Purified and Modified Enzymes (DMA 69%, DMA 75%, DMA 76%)

Enzyme	k_i (min^{-1})	$t_{1/2}$ (min)	ΔGi (kJ $mole^{-1}$)
Purified enzyme	0.0133	52.1	105.168
DMA 69%	0.0072	96.25	106.868
DMA 75%	0.0058	119.48	107.466
DMA 76%	0.0033	210	109.028

Half-life (t½) and rate of thermal inactivation (ki): The half lifes of the modified enzymes have increased 2-4 times compared to the purified enzyme. Stahl (1999) stated that half-life will determine the stability of the enzyme. The results obtained in this research, the half-lives of modified enzyme with DMA 69%, 75% and 76% have all increased from 52.1 minutes to 96.25, 119.48 and 210 minutes, respectively. These data showed us that the higher the modification degree used, the longer the half-life will be. The decrease of k_i value (Table 1) showed that the decrease of enzyme denaturation has occurred on the modified enzymes. The increases of enzyme stability based on the decrease of ki value are 2-4 times compared to that of purified enzyme. The lower of value k_i illustrates that the enzymes is less flexible in water due to the bond formation between DMA and NH_2 group of side chain lysine residue on the surface. This causes the decrease in protein unfolding so the structure of enzyme will be more rigid and more stable. The same result was also obtained by Kazan *et al*. (1996) where the rate of thermal inactivation of DMA cross-bonded with penicillin G acylase (PGA) was always lower than the pure PGA.

The energy change due to denaturation (ΔGi): Table 1 shows the ΔGi increase of modified enzyme compared to that of the purified enzyme. The higher ΔGi value of the modified enzyme demonstrates that the enzyme is more rigid and less flexible in water. The tertier structure of the denaturated enzyme has changed and the enzyme structure that is more rigid has stronger bond, so the enzyme conformation is not easily opened that the tertier enzyme structured can be maintained. To denature this sort of enzyme will require more energy, so the ΔGi value obtained will also be higher. Therefore the higher ΔGi value indicates that the enzyme is more rigid, less flexible and is hard to denature and *vice versa.* Kazan *et al.* (1996) reported that the higher ΔGi of

penicillin G Acylase which crossed-bond to DMA indicated the stabilization of PGA by cross bond at extreme pH. In this research, the ΔGi value of purified enzyme obtained was 105.168 kJ mole^{-1} while for the modified enzyme with DMA 69%, 75%, 76% were 106.868; 107.466; 109.028 kJ mole^{-1}, respectively. The increase of enzyme stability is higher with the increase of modification degree, although the ΔGi value is not too high, but the thermal stability is increased 2-4 times.

Conclusion

Based on the results obtained and the discussion above, it can be concluded that: the optimum temperature of the modified enzyme is the same as the purified enzyme, but the stability is increased at 55 – 65^{o}C. The thermal stability test at storage temperature of 60^{o}C for 300 min shows the modified enzyme with DMA (69%) has residual activity of 10.71%, $t_{1/2}$ = 96.25 min; k_i = 0.0072 min^{-1}; ΔGi = 106.868 kJ mole^{-1}.

DMA (75%) has residual activity of 15.87%; $t_{1/2}$ = 119.48 min; k_i = 0.0058 min^{-1}; ΔGi = 107.466 kJ mole^{-1}. DMA (76%) has residual activity of 36.36%; $t_{1/2}$ = 210 min; k_i = 0.0033 min^{-1}; ΔGi = 109.028 kJ mole^{-1}. While the purified enzymes has residual activity of 1.47%; $t_{1/2}$ = 52.1 int, k_i = 0.0133 min^{-1}, 105.168 kJ mole^{-1}. The modification of enzyme with DMA has been able to increase the thermal stability of the enzyme up to 2-4 time based on the decrease of k_i value.

Future works and suggestion

Based on the results obtained, we suggest to do further research to find out the three dimension structure of protease enzyme *B. subtilis* ITBCCB148 to explain the stability of the enzyme. The modification of this enzyme with other bifunctional reagent is interesting to be done to compare the stability might be obtained.

Acknowledgements

The authors would like to thank to The Directorate of Research and Community Services, Directorate General of Higher Education, The Ministry of National Education of Republic of Indonesia that provide fund for this project to be undertaken through Hibah Bersaing XV/2 Research Grant Scheme 2008 with contract number of 010/SP2H/PP/DP2M/III/2008, 6 March 2008

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12/21/2008