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The Chemical Modification of Cellulase Obtained from *Bacillus subtilis* ITBCCB148 With Dimethyladimipidate

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Cellulase obtained from *Bacillus subtilis* ITBCCB148 has successfully been isolated and purified. The native enzyme was modified with dimethyladipimidate (DMA) to increase the stability of the enzyme. The native and modified enzymes were characterized including determination of optimum temperature, optimum pH and thermal stability. The activity of the cellulase was determined based on Mendels method and the protein content was determined based on Lowry method. The results showed that the native enzyme has optimum temperature of 55°C and optimum pH of 5.5. The thermal stability for 60 minutes at temperature of 55°C indicated that the native enzyme has k_i = 0.034 min.t^{-1}, $t_{1/2}$ = 20.4 min., and ΔG_i = 100.9 kJ/mol. The modified enzyme with modification degrees of 64.5; 69.5 and 82% have optimum temperature of 55 °C and optimum pH of 6. The thermal stability for 60 minutes at temperature of 55°C of the modified enzyme with modification degrees of 64.5; 69.5 and 82% have k_i values of 0.024; 0.021 and 0.022 min.$^{-1}$, $t_{1/2}$ values of 28.9; 33.0 and 31.5 minutes, and ΔG_i values of 101.9; 102.3 and 102.2 kJ/mol, respectively. The modification with DMA has successfully increased the thermal stability of the modified enzymes between 1.4 – 1.6 times compared to that of the native enzyme. The decrease of ki values, increase of half-lives and ΔG_i indicated that the modified enzymes were more stable compared to the native enzyme.

Key words: Cellulase, *Bacillus subtilis* ITBCCB148, dimethyladimipidate.

The research on the enzyme stability is very interesting in the last decade due to the developing in industry, especially in detergent industry, liquid sugar syrup from amylum, synthetic organic compound industry, pulp and paper industry, feed stock industry and the use of the this enzyme in the treatment of industrial waste[1].

Enzyme in general is not stable at high temperature and extreme pH[2], the condition which generally required at many industrial processes. Therefore, the thermostable enzymes are needed, the enzymes which are able to work optimum at temperature range of 60°–125°C[1] and wide range of pH. To obtain the enzyme having high stability and activity at extreme condition, it can be achieved by direct isolation from natural organism living at that condition (extremophilic) or by chemical modification toward enzyme obtained from organism living at normal condition (mesophilic)[3].

According to Mozhaev and Martinek[4] the stabilization of enzyme obtained from mesophilic microbia is more preferable to obtain more stable enzyme since the direct isolation from thermophilic enzyme have some weaknesses such as requiring bioreactor design or new process method[5]. Mozhaev and Martinek[4] reported three ways for enzyme stabilization including immobilization, chemical modification and directed mutagenesis. Enzyme immobilization has some disadvantages as

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there will be decreased on binding capacity or enzyme reactivity due to mass transfer inhibition by immobile matrix. Directed mutagenesis requires complete information about the primier structure and complete image of the three dimentional structure. Thus, chemical modification is the more preferable method to obtain the more stable enzyme.

According to Mozhaev *et al.*[6] to obtain the modified enzyme with stable covalent bond can be achieved by (1) modification using bifunctional reagent to form cross-linking bond among the functional groups on the surface of protein; (2) modification using nonpolar reagent to increase hydrophobic interaction; (3) addition of charge groups or polar group to increase the ionic or hydrogen bonds; (4) hydrophilisation of protein surface to protect contact between hydrophoic groups and aqueous surrounding which is not preferred by the enzyme. Mozhaev and Martinek[4] stated that to increase the stability of the enzyme, chemical modification is the method chosen. The increase of enzyme stability can be achieved by the addition of a certain material. Chemical modification by cross linking or bifunctional reagent is a very useful method to increase stability of protein at thermal condition. The cross linking produces folding conformation of native enzyme and reduce thermal inactivation[7]. One the bifunctional reagent used is dimethyladipimidate (DMA)[7].

In our previous research, it has been performed chemical modification on á-amylase obtained from *B. subtilis* ITBCCB148 using dimethyladipimidate. The results showed that all modified enzymes increased their thermal stability compared to the native enzyme[8]. The cellulase obtained from *B. subtilis* ITBCCB148 has also been modified using low molecular weight modifier of glyoxylic acid[9]. The results showed that the modification was able to increase the thermal stability of the modified enzymes between 1.8-2.1 times compared to the native enzyme. In this paper we continue to perform chemical modification on native cellulase using dimethyladipimidate as modifier in order to increase its stability. The characterization to native and modified enzymes were also performed by varying temperature, pH and thermal stabiltiy.

MATERIALS AND METHODS

Materials

All chemicals used were of high grade (pro analysis) materials. Bacteria isolate *B. Subtilis* ITBCCB148 was obtained from Microbiology and Bioprocess Technology Laboratory, Chemical Engineering Department, Bandung Institute of Technology, Bandung, Indonesia.

Research procedure

The following research phases were performed: the production, isolation, purification and characterization of the native enzyme were based on our previous report[10].

Activity test of cellulase and determination of protein content

Activity of cellulase was determined using dinitrosalycylic acid[11]. The protein content was determined based on the method by Lowry *et al.*[12].

Chemical modification of the native enzyme using dimethyladipimidate (DMA)

To 10 mL of native cellulase in 0.1 M buffer borate pH 8 was added with solid DMA with concentration variation of 5, 10 and 15 mg. The mixture was magnetically stirred at room temperature for 1h[13].

Determination of Modification Degree

Modification degree was determined using method developed by Synder and Sobocinski[14].

Characterization of enzyme before and after modification

Determination of optimum temperature

The determination of optimum temperature was performed by varying the temperature at 55, 60, 65, 70, 75 and 80°C.

Determination of optimum pH

Determination of optimum pH was performed by varying pH at 5; 5.5; 6; 6.5; 7; 7.5; 8; 8.5; 9.

The thermal stability test of enzyme

The thermal stability of native and modified enzymes was performed by measuring the residual activity of the enzyme after being incubated for 0, 10, 20, 30, 40, 50, and 60 minutes at temperature of 55°C[15].

Determination of half life ($t_{½}$), k_i and ΔGi

Determination of k_i value (thermal inactivation rate constant) of the enzyme was

performed using the first order of inactivation kinetics equation (Eq. 1)[7]:

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

The change of energy due to denaturation was performed using Equation 2[7].

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

RESULTS AND DISCUSSIONS

Determination of modification degree

The modification of the native enzyme with DMA was performed with 3 concentration variation i.e. 5, 10 and 15 mg as shown in Table 1. The determination of modification degree was based on the comparison of lysine residue before and after the modification. The data in Table 1 indicated that the modification with DMA with concentration of 5, 10 and 15 mg produced modification degree of 64.5; 69.5 and 82%, respectively. The calculation of the modification degree was based on the comparison of the number of lysine residues which were not modified at the modified enzymes and native enzyme. The ammine group on the lysine residues which were not modified will react with TNBS reagent to form yellow complex. The more the ammine group on the lysine residue were modified, the less the ammine group will react with TNBS, as a result the complex will be pale yellow. Based on Table 1, the higher concentration of DMA, the higher the modification degree.

Determination of optimum temperature

Fig. 1 showed that the optimum temperature of the native and modified enzymes was the same, i.e. at 55 ºC. All modified enzymes were observed to be more stable compared to the native enzyme at temperature range 50-65ºC. The data in Fig. 1 indicated that the modified enzymes with modification degree of 64.5; 69.5 and 82% at 55ºC have % activity of 93, 94 and 95%, respectively, while the native enzyme was 76%. At 65ºC the similar result was also observed where the modified enzymes had % activity of 72, 78 and 90%, while the native enzyme had 58%. At 70ºC, the modified enzymes were shown to be much better with %activity of 57, 68 and 89% while the native enzyme was only 40%. The results obtained indicated that there was an increased on the enzyme stability to the modified enzymes at higher temperature compared to the native enzyme, although the increase of optimum temperature was not observed. The results obtained similar to those reported by others[7,16]. that the chemical modification was not always cause the optimum temperature increase, rather the increase of the enzyme stability.

Table 1. Determination of Modification Degree with TNBS

Sample	ΔA_{420} nm	Modification (%)
Native Enzyme	0.5858	0
Modified with DMA 5 mg	0.5714	64.5
Modified with DMA 10 mg	0.5703	69.5
Modified with DMA 15 mg	0.5675	82

Determination of Optimum pH

Fig. 2 showed that the native and modified enzymes have working pH range of 5 – 7.5. The optimum pH of the native enzyme was 5.5 and the modified enzymes were 6, thus there was a slight increased in the optimum temperature. The data in Fig. 2 also indicated the increase of enzyme stability for the modified enzyme compared to the native enzyme especially the modified enzyme with modification degree of 69.5 and 82%. At pH 6.5, these two modified enzyme have % activity of 96 and 97%, while the native enzyme was 82%. At pH 9, the % activities of these two modified enzymes were 73 and 75%, while the native enzyme was only 59%. Based on these data, it can be concluded that the cross linking formation using DMA was able to increase the stability of the enzyme against pH.

Thermal Stability of the enzyme

The data on Fig. 3 shown that the native enzyme has residual activity (%) 14.1% after being incubated at 55ºC for 60 minutes, while the modified

Table 2. The change of Inactivation rate constant (k_i), half life ($t_{1/2}$), and energy due to denaturation (ΔG_i) of the enzymes

Enzyme	ki (min.$^{-1}$)	$t_{1/2}$ (minute)	ΔGi (kJ/mol)
Native	0.034	20.4	100.9
DMA 64.5%	0.024	28.9	101.9
DMA 69.5%	0.021	33.0	102.3
DMA 82%	0.022	31.5	102.2

enzymes with modification degree of (64.5; 69.5 and 82%) have 25.7; 26.1 and 26.7, respectively. All modified enzymes have increased their thermal stability against temperature compared to the native enzyme.

Half life ($t_{1/2}$), thermal inactivation constant (k_i), and the change of energy due to denaturation (ΔG_i)

Table 2 tabulated the data of half life ($t_{1/2}$), thermal inactivation constant (k_i), and the change of energy due to denaturation (ΔG_i)

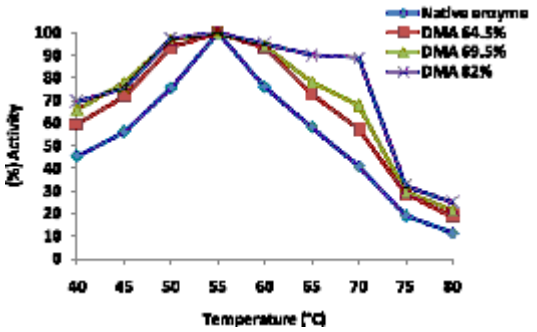

Fig. 1. Optimum temperature of purified and modified enzyme (64.5; 69.5 and 82%)

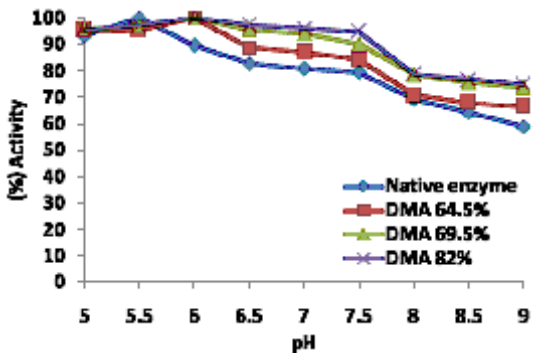

Fig. 2. Optimum pH of native and modified enzymes (64.5; 69.5 and 82%)

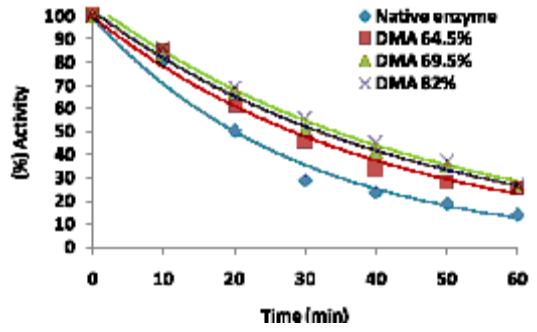

Fig. 3. Thermal stability of the native enzyme and modified enzyme (64.5; 69.5 and 82%). At temperature 55ºC *vs* time

Based on the data on Table 2, it can be seen that the half life ($t_{1/2}$) of modified enzymes were increased 1.4 – 1.6 than the native enzyme. According to Sthal[17], the increase of half life will determine the stability of the enzyme. The result obtained indeed indicated that the stability of the modified enzymes were higher than the native enzyme.

In conclusion, the chemical modification using DMA on cellulase obtained from *B. subtilis* has successfully increased the thermal stability of the native enzyme. The thermal stabilities of the modified enzymes were increased 1.4 – 1.6 times compared to the native enzyme. The decrease of k_i value, the increase of half-life and $\ddot{A}G_i$ values showed that the modified enzymes were more stable than the native enzyme.

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