KINETIC AND THERMODYNAMIC CHARACTERIZATIONS OF THE PROTEASE FROM Bacillus licheniformis (ATCC 12759)

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Abstract: In this study, the kinetic of a thermo-stable extracellular protease produced by Bacillus licheniformis (ATCC 12759) cultured in skim latex serum fortified media was investigated. The enzyme was stable up to 65 °C after incubation for 60 min at pH 8. The Lineweaver-Burk exhibited $v_{\text{max}}$ (maximum rate) of 37.037 U/mg min⁻¹ and $K_M$ (Michaelis-Menten constant) of 8.519 mg/mL. The activation energy ($E_a$) of casein hydrolysis and temperature quotient ($Q_m$) were found to be 4.098 kJ/mol and 1.038 - 1.034, respectively, at a temperature ranging from 35 °C to 65 °C. The results of the residual activity test allowed estimating activation energy for irreversible inactivation of the protease (denaturation) which was approximately $E_{a(d)} = 62.097$ kJ/mol. The thermodynamic parameters for the enzyme irreversible denaturation were as follow enthalpy ($59.286 ≤ \Delta H^d ≥ 59.535$ kJ/mol), Gibbs free energy ($97.375 ≤ \Delta G^d ≥ 93.774$kJ/mol), and entropy ($-122.797 ≤ \Delta S^d ≥ -101.992$ kJ/mol). These thermodynamic parameters inferred that the thermo-stable proteases could be potentially important for industrial application, for example, in the detergent industries.

Keywords: Bacillus licheniformis; kinetic; protease; skim latex serum; thermostability

Abstrak: Pada penelitian ini, kinetika protease ekstraseluler termo-stabil yang diproduksi oleh Bacillus licheniformis (ATCC 12759), yang dikultur dalam media yang diperkaya serum lateks dengan konsentrasi 60 menit pada pH 8. Lineweaver-Burk menunjukkan $v_{\text{max}}$ (luaju maksimum) adalah 37.037 U/mg min⁻¹ dan $K_M$ (konstanta Michaelis-Menten) 8.519 mg/mL. Energi aktivasi ($E_a$) dari hidrolisis kasein dan suhu quotient ($Q_m$) ditemukan masing-masing sebesar 4.098 kJ/mol dan 1.038 - 1.034, pada suhu yang berkisar dari 35 °C hingga 65 °C. Hasil uji aktivitas residu memungkinkan estimasi energi aktivasi untuk inaktivasi irreversible dari protease (denaturasi) yang kira-kira $E_{a(d)} = 62.097$ kJ/mol. Parameter termodynamika untuk denaturasi enzim irreversible adalah sebagai berikut entalpi ($59.286 ≤ \Delta H^d ≥ 59.535$ kJ/mol), energi bebas Gibbs ($97.375 ≤ \Delta G^d ≥ 93.774$kJ/mol), dan entropi ($-122.797 ≤ \Delta S^d ≥ -101.992$ kJ/mol). Parameter termodynamika pada penelitian ini menyimpulkan bahwa protease termo-stabil dapat berpotensi penting untuk aplikasi industri seperti dalam industri deterjen.

Kata kunci: Bacillus licheniformis; protease; serum lateks skim; thermostabilitas
Introduction

Proteases or proteolytic enzymes are natural catalysts that act in the degradation of protein by hydrolyzing peptides bonds (Rao et al., 1998; Aguilar et al., 2019; Jisha et al., 2013). They usually require metals ions and or cofactors for the maximal efficiency. The catalytic activity of protease is affected by temperature, pH, the concentration of substrate, and the presences of similarly sized and shaped molecules called inhibitors (Mienda et al., 2014; Bhunia et al., 2012). As a catalyst, protease has a pivotal role in application areas such as food, textile, leather, environmental pollution abatement, pharmaceutical, and healthcare product (Bhunia et al., 2012). In order to perform their application effectively, the kinetic and thermodynamic studies of protease need to be investigated. The kinetic analysis of enzymes constitutes the information about activation and deactivation of enzymes and describes the capability of the enzyme to catalyze and enhance in a process. In biotechnological processes, studies of deactivation enzyme are essential because it provides the data to illustrate the enzyme stability and control over the activation processes. Moreover, the deactivation studies support investigates the correlation between structure and function of particular enzymes in which will provide valuable physical insight into the structure and function of enzymes (Prajapati et al., 2014; Bhuni et al., 2013).

However, finding the protease with novel and excel properties such as having stability and activity at high pH and temperature are desirable due to providing an economic advantage such as minimized enzyme consumption (Souza et al., 2015), enhancing the mass-transfer and reducing the substrate viscosity during the progress of hydrolysis substrate. Furthermore, thermostability of the enzyme at 60°C or above can avoid or reduce microbial contamination, particularly bioreactor containing immobilized enzyme (Nigam et al., 2013). Many researchers reported proteases that possess stability and activity at high pH and temperature using agri-industrial waste such as groundnut oil cake (Elumalai et al., 2020), soy fibre (Marin et al., 2018), and dried powder of wheat bran, rice bran, sugarcane bagasse, and sugarcane molasses (Rathod and Pathak, 2014). On the other hand, in the best of our knowledge, only a few studies about proteases production using skim latex serum as a basal medium for microbial growth (protease Bacillus licheniformis, ATCC 12759). Thus, in this study, we reported the heat-stable microbial protease from Bacillus licheniformis, ATCC 12759 that was the culture in much waste (skim latex serum) by different reporting method that is the deactivation kinetics of protease. As a consequent, the objective of this study was to examined kinetic parameters (Michaelis constant, $K_M$ and maximum
velocity, $v_{\text{max}}$) and its relation toward thermodynamic parameters (activation and deactivation energy, enthalpy, entropy and Gibbs free energy) of the protease from \textit{B. licheniformis} (ATCC 12759).

**Materials and Methods**

**Microorganism and Cultured Condition**

The \textit{Bacillus licheniformis} (ATCC 12759) used in this study was ordered from American Type Culture Collection (ATCC) USA. They were cultured in the nutrient broth media and routinely re-cultured every 12 days on Nutrient Agar (NA) medium at 37 °C, then stored at 4 °C (Mardina et al., 2018). As suggested in our previous optimization study (Mardina et al., 2015), the production of the protease was conducted using the prepared media containing the skim latex serum (70 %, v/v), galactose (1 %, w/v) and Luria Bertani (LB) (1 %, w/v). One hundred millilitres of the modified media was then inoculated with 4 % (v/v) of the inoculums (3x10$^9$ cells (CFU/mL) that were previously prepared in LB broth). The prepared started was cultured at 35 °C with a specific of pH 7, 75 rpm of rotation and 24 h for agitation and incubation period. All the liquid state fermentations were performed in triplicates. The extraction of protease from the production medium was carried out by centrifugation (10,000xg) for 10 min at 4 °C. The obtained cell-free supernatant (called crude enzyme) was stored for protease activity determination.

**Assay of Protease Activity**

The produced protease activity was confirmed using a casein substrate which was previously described by in our previous work (Mardina et al., 2015). An aliquot of 2.5 mL of the casein solution 1 % (w/v) was mixed to the protease and followed by 2.5 mL trichloroacetic acid (TCA, 0.11 M) and then incubated in the water bath for 10 min at 37 °C. Subsequently, the mixture was allowed at room temperature for 45 min. 1 mL supernatant was pick up from centrifugation process (10,000 xg for 10 min at 4 °C), then subjected to the mixture of 2.5 mL Na$_2$CO$_3$ (0.5 M) and 0.5 mL Folin reagent (0.1 N). The average of triplicate optical density at the wavelength of 660 nm was calculated for the determination of protease activity with the equation:

$$\text{Protease activity (U/mL)} = \frac{\mu\text{g of Tyrosine release} \times \text{reaction volume}}{\text{sample vol} \times \text{reaction time} \times \text{vol assayed}}$$

The quantity of enzyme that liberates 1 µg tyrosine per millilitre in 1 min under the assay condition was defined as one unit of protease activity.
Kinetic Parameters Determination

$K_M$ and $v_{max}$ of the enzyme were calculated through a double reciprocal (Lineweaver-Burk) plot of the protease activity versus casein concentrations (2 – 30 mg/mL). The calculation was carried out at 37°C and pH 8. The protease assay was performed using equation (1). In order to read the sample absorbance, 2.5 mL Na$_2$CO$_3$ and 0.5 mL Folin reagents were used to colour the substrate. After homogenically mixed, the substrate was tested in a specific absorbance reading of 660nm in every 1 minute and repeated for three times. The obtained data were then averaged to obtain the curve by plotting Δ660 nm versus time. The slope was calculated using the equation (2) and considered as the rate of reaction. The value of quality was converted using L-tyrosine standard curve and expressed in U/mg, and the concentration of casein [S] was reported in mg/mL.

$$m = \frac{(y_1-y_2)}{x_1-x_1}$$ .................................................(2)

Thermodynamic Parameters Determination

Determining the energy activation and temperature coefficient ($Q_{10}$)

The energy required for the activation was determined using methods developed by Souza et al. (2015), Castro and Sato (2014), and Aguilar et al. (2019). First, the protease was incubated on 1% of casein solution at various temperatures (35 to 65 °C) for 10 min. The correlation of the rate constants with temperature was expected to follow the Arrhenius law where $E_a$ was estimated from the slope of the plot of $1/T$ (in Kelvin) vs natural log ($ln$) of the enzyme activity, $E_a = -$slope x R, R is the gas constant with the value of 8.314 JK$^{-1}$ mol$^{-1}$, and T is the absolute temperature (K) (Castro and Sato, 2014; Pogaku et al., 2012). The effect of temperature on the rate of reaction was expressed as ($Q_{10}$). The temperature quotient was defined as a measure of the rate increases as a consequence of increasing the temperature by 10 °C and determined by the equation (Eq. 3) given by Castro and Sato (2014) and Aguilar et al. (2019).

$$Q_{10} = \text{antilog}_e \left( E_a \times \frac{10^{10}}{RT^2} \right)$$ ...................................................(3)

Kinetic parameters for thermal inactivation

The inactivation process of protease due to thermal effect (denaturation) was calculated by first-order kinetics as a function of the time (Eq. 4 and Eq. 5). The protease was incubated without substrate at various temperatures (range 35 °C – 75 °C) for 60 min. The residual activity was measured at 20, 40 and 60 min.

$$\frac{dA}{dt} = -K_d A$$ ............................................................(4)

so that,
Where $t$ was time, $A_0$ was the initial protease activity, $A$ was the protease activity at a particular time $t$, and $K_d$ was deactivation rate constant which is determined from the slopes gained by plotting $\ln [A/A_0]$ or $\ln$ [residual activity] versus $t$ (time) at an assay temperature (Castro and Sato, 2014; Pogaku et al. (2012)). From this, the deactivation energy ($E_{a(d)}$) could be determined based on the Arrhenius equation as given by Prajapati et al. (2014) and Bhunia et al. (2013).

$$K_d = K_o \exp \left( -\frac{E_{a(d)}}{RT} \right) \quad \ldots \quad (6)$$

The half-life ($t_{1/2}$) of the enzyme was the time when its residual activity reaches 50 % of the initial activity and calculated, as shown in Eq. 7 (Castro and Sato, 2014).

$$t_{(1/2)} = \frac{\ln(0.5)}{K_d} \quad \ldots \quad (7)$$

Furthermore, D-value of the enzyme that was related to $K_d$ can be defined as the time required for a 90 % reduction in the initial enzyme activity at a specific temperature and was calculated as shown in Eq. 8 (Prajapati et al., 2014).

$$D = \frac{2.303}{K_d} \quad \ldots \quad (8)$$

The remaining thermodynamic parameters of the enzyme were determined according to the equation given by Souza et al. (2015) and Aguilar et al. (2019).

$$\Delta H_{a}^* = E_{a}^* - RT \quad \ldots \quad (9)$$

$$\Delta G_{a}^* = -RT \ln \left[ \frac{k_B h}{K_B T} \right] \quad \ldots \quad (10)$$

$$\Delta S_{a}^* = \frac{\Delta H_{a}^* - \Delta G_{a}^*}{T} \quad \ldots \quad (11)$$

where $\Delta H_{a}^*$, $\Delta G_{a}^*$, $\Delta S_{a}^*$ are the changes of enthalpy, Gibbs free energy and entropy between the denatured and the active form of the enzyme respectively; $K_B$ and $h$ are the Boltzmann and Planck constants, respectively. The Boltzmann’s constant $(R/N)$ is 1.38 x $10^{-23}$ J K$^{-1}$, $T$ is the absolute temperature (K), $h$ is Planck’s constant with the value of 6.626 x $10^{-34}$ J s, $N$ is Avogadro’s number $(6.02 \times 10^{-23}$ mol$^{-1}$) and $R$ is gas constant at 8.314 J K$^{-1}$.
Results and Discussion

Kinetic Studies

The kinetic parameters $K_M$ and $v_{max}$ were estimated at pH 8 and 37 °C varying $S_0$ from 2 – 30 mg/mL. The result of Lineweaver-Burk of the enzyme showed Michaelis-menten-type kinetics with $v_{max} = 37.037$ U/mg min$^{-1}$ and $K_M = 8.519$ mg/mL, with good correlation ($R^2 = 0.993$). According to Souza et al. (2015), the activity of an enzyme depends on the type of substrate used and the purity of the enzyme. Thus it isn't easy to compare. However, the $K_M$ value of this result that is quite closed by other reports for protease from Bacillus licheniformis (5 mg/mL) (Nasdeem et al., 2013) and alkaline protease from Lactobacillus brevis (3.33 mg/mL) (Ola et al., 2012) demonstrated the good affinity for the substrate casein.

Thermodynamic Studies

Proteases are biocatalysts that minimize the activation energy ($E_a$), which was required to convert the substrates into the product. In this study, the activation energy barrier was carried out at pH 8.0, $S_0 = 100$ mg/mL and varying temperature from 35 °C to 65 °C. In order to reduce the effect of temperature on the loss of protease activity due to denaturation, only the initial of protease activity ($v_0$) was occupied into consideration as suggested by Souza et al. (2015). The apparent activation energy for the hydrolysis of casein value was calculated from the slope of the Arrhenius plot (Fig. 2) and found to be 4.098 kJ/mol with satisfactory correlation ($R^2 = 0.852$). The lower value of $E_a$ thermodynamically demonstrated the feasibility of casein hydrolysis reaction by protease at a lower temperature. This result was lower than those of similarly reported proteases; for
instance, $E_a$ value of 19.03 kJ/mol, 15.53 kcal/mol (64.98 kJ/mol), 10.59 kcal/mol, and 44.30 kJ/mol for protease from Aspergillus foetidus, Yersinia ruckeri, and Bacillus clausis and Bacillus licheniformis LBA 46 respectively. If the low $E_a$ value in this study, it might indicate that only small amount energy was required to create the activated complex of casein hydrolysis and the reaction is also highly reactive (Souza et al., 2015; Kezia et al., 2011; Aguilar et al. (2019).

![Figure 2. Arrhenius plots for determining the activation energy ($E_a$) of B. licheniformis protease in the temperature range from 35 – 65 °C.](image)

The $Q_{10}$ values were determined to infer the influence of reaction that being examined was controlled by temperature and or other factors. Samanta et al. (2014) and Aguilar et al. (2019) stated that enzymatic reaction generally had $Q_{10}$ value between 1 and 2 and any deviations from these value indicate the involvement of some factors other than the temperature in regulating the reaction rate. A $Q_{10}$ value of 2 recommended doubling of the rate of reaction with every 10 °C increase in temperature. The $Q_{10}$ value of the Bacillus licheniformis protease in this study showed in 1.038 – 1.034 at a temperature ranging from 35 °C to 65 °C. This finding was supported by Castro and Sato (2014), who reported protease from A. oryzae had a $Q_{10}$ value of 1.64 – 1.53 at the temperature range of 30 to 55 °C. Other study reported the $Q_{10}$ in the range of 1.59–1.87 for protease from Bacillus licheniformis LBA 46 (Aguilar et al. (2019).

In term of the economic parameter in industries, $t_{1/2}$ and $D$ values were significant, due to if higher their value, it means higher enzyme thermostability. The thermodynamic results of these assessments were summarized in Table 1 that exhibited the $K_d$ value of 0.0015 min$^{-1}$, $t_{1/2}$ of 7.77 h and a $D$ value of 25.59 h at 55 °C. These values were according to the trend of the enzymes reported in the literature, such as protease from Aspergillus foetidus ($K_d = 0.018$ h$^{-1}$); $t_{1/2} = 37.6$ h; $T = 55$ °C) (Souza et al., 2015) and Aspergillus oryzae (($K_d = 0.071$ min$^{-1}$); $t_{1/2} = 97.63$ h; $T = 57.2$ °C) (Castro RJS and Sato, 2014), thus this produced enzyme acted to be thermostable.
Table 1. Thermodynamic parameters for inactivation of the produced protease

| T (°K) | K_d (min^-1) | t_1/2 (h) | D (h) | R^2 | ΔH_d (kJ/mol) | ΔG_d (kJ/mol) | ΔS_d (J/mol K) |
|--------|---------------|-----------|-------|-----|---------------|---------------|---------------|
| 308.15 | 0.0002        | 57.75     | 191.9 | 0.8322 | 59.535        | 97.375        | -122.797      |
| 318.15 | 0.0013        | 8.88      | 29.5  | 0.9616 | 59.452        | 95.663        | -113.817      |
| 328.15 | 0.0015        | 7.77      | 25.59 | 0.9597 | 59.369        | 95.366        | -109.696      |
| 338.15 | 0.0023        | 5.02      | 16.69 | 0.9968 | 59.286        | 93.774        | -101.992      |

Semi-log plot of ln k_d versus 1/T recommended for calculating the activation energy of protease denaturation or energy required for thermal inactivation (E^*_d) that was found to be 62.097 kJ/mol (R^2 = 0.7938). This value is similar to those reported for alkaline protease from Bacillus licheniformis (32.79 – 41.69 kJ/mol) (Bhunia et al., 2013) and Aspergillus fumigatus (69 kJ/mol) (Martinez et al. 2011) and Bacillus licheniformis LBA 46 (144.50 kJ/mol) Aguilar et al. (2019). E^*_d means enzymes require a similar amount of energy to be inactivated.

Besides, there was the relation between E^*_d in Eq. (7) and the activation enthalpy of denaturation (ΔH^*_d), which was another essential thermodynamic parameter expressing the total amount of energy necessary to denature the protease; thus, large and positive values of E^*_d and ΔH^*_d should be associated with high enzyme thermostability. The protease in this study had a ΔH^*_d at 65 °C (59.29 kJ/mol) (Table 1) which was much higher than alkaline protease from B. licheniformis (30.06 – 39.97 kJ/mol) (Bhunia et al., 2013).

The other reliable predicting parameters for evaluating the stability of enzyme due to thermal denaturation include the Gibb free energy (ΔG^*_d) and the activation entropy (ΔS^*_d). The ΔG^*_d and ΔS^*_d of irreversible thermal inactivation (a 55 °C) of B. licheniformis protease was equal to 93.774 kJ/mol and -101.992 J/mol K respectively (Table 1). An increase in temperature would decrease in free energy while there was a slight increase in entropy. However, a slight decrease in ΔG^*_d in this study exhibited resistance of the enzyme to denaturation (thermostable). The lower value of ΔG^*_d of protease from B. licheniformis indicates its higher affinity to catalyse soluble casein hydrolysis (Souza et al., 2015). Furthermore, the low entropy values in this study also showed compaction of the enzyme molecule and such changes could arise from the formation of charged particles around the enzyme molecule and the ordering of solvent molecule (Prajapati et al., 2014).
Conclusions
The protease obtained from *Bacillus licheniformis* (ATCC 12759) cultured in skim latex serum basal media potentially used in several industries such as detergent industry because it has high stability up to 65 °C after incubation for 60 min at pH 8.

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