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Purification of Thermophilic Lipase *E.coli* BL21(DE3)-pET30(a)-lipITB1.*2* Using Immobilized Metals Affinity Chromatography Ni-NTA

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Abstract: Lipase as biocatalyst displays three major types of selectivity (chemo-, regio- and diastereo-, and enantio-selectivity) and useful in many industrial applications. In order to increase the enzyme performance, purification of lipase is the essential thing and need to be investigated. In this research, Immobilized Metals Affinity Chromatography (IMAC) Ni-NTA was applied to purify a local thermophilic lipase lipITB 1.2**.** The purification of lipase was performed at the optimum condition of reaction (Ni-NTA - enzyme supernatant ratio of 1:1 and continuous stirring at 100 rpm for 1 hour). Elution was carried out at a flow rate of 0.02 m/s. 50 mM phosphate buffer pH 7.5, 300 mM NaCl, and 5mM imidazole solutions were used as eluents. Based on the results, purification of thermophilic lipase lipITB1.2 by IMAC Ni-NTA was successfully performed by increasing 17-folds the specific activity of heated supernatant of crude enzyme.
Keywords: thermophilic lipase; enzyme; immobilized metals affinity chromatograph (IMAC); Ni-NTA; specific activity.

1-Introduction

Lipase (acylglycerol hydrolases, 3.1.1.3) is highly chemo-, stereo, -regio-, and enantio- selective enzyme which has ability to catalyze the hydrolysis of fats and oils to produce glycerol and free fatty acids [1,2]. Lipase also shows a very high versatility in catalyzing various reactions such as aminolysis, alcoholysis, esterification, and transesterification[3]. Therefore, lipase has a wide range of applications in various industries, such as food (dairy, food, bakery, fats, and oil), biodiesel, cosmetic, pharmaceutical, leather, textile, detergent, agro-chemicals, and paper[1,3,4].

Due to its potential applications, the interest of researchers in the synthesis of lipase from different sources (microorganisms, animals, and plants) is getting increase in the last decade[5]. A recombinant microorganism bearing lipase gene from local thermophiles lipase has been developed in Institut Teknologi Bandung (ITB) [6]. Previously, thermostable Lipase ITB1.1 activities was investigated and showed a highest activity at 85^{o}C and pH 9.5[7]. In this research, lipase ITB2.1 has an optimum condition at 70^{o}C and pH 9.0[6]. However, the method to purify that lipase is not established yet and need to be developed.

Among many current protocols, metal-chelate affinity chromatography is the most frequently used for separating recombinant proteins. It employs a nitrilotriacetic acid (NTA)-attached resin to immobilize nickel

ions (Ni^{2+}) in order to separate the recombinant proteins then six consecutive histidine residues (6x His) were obtained. Many examples showed that the proteins fused with 6 or 10 consecutive histidine residues can be effectively purified via immobilized metal affinity chromatography (IMAC)[8,9]. Therefore in this research, immobilized metals affinity chromatography of Ni-NTA was used to purify the enzyme from crude extract.

Materials and Methods

Materials

NaCl, tripton, yeast extract, kanamycin, IPTG, lysozyme, Na_2HPO_4, NaH_2PO_4, DEAE-Cellulose, Ni-NTA-Sepharose resin, Triton-X 100, imidazole, NaOH were supplied from Merck, glycine, p-nitrophenyl palmitate (pNPP), p-nitrophenol (pNP), acetonitrile, ethanol, bovine serum albumin, Bradford reagent, molecular weight marker kit, Coomassie Brilliant Blue R-250 were supplied from Thermoscientific Inc.

Methods

Organism

A local isolate of thermophilic bacteria papandayan-2 (PPD2) expressed at *E. Coli* BL21 (DE3), called *E. Coli* BL21(DE3)-pET30(a)-lipITB1.2, was used as microorganism to produce lipase. This bacteria was kindly donated from Biochemistry Laboratory. The bacteria were stored at -20^oC in glycerol (glycerol stock).

Production of *E. coli* BL21(DE3)-pET30(a)-lipITB1

10 uL glycerol stock of *E. coli* BL21(DE3)-pET30(a)-lipITB1.2 was cultivated in 10 mL sterile medium LB (1% (w/v) NaCl, 1% (w/v) Tripton and 0.5% (w/v) yeast extract) added by 10 uL kanamycin 30% (w/v). The media was incubated at temperature 37ºC with agitation 150 rpm for 16 hours. 1 mL of the culture was pipetted and grown in 100 mL sterile media LB (1% (w/v) NaCl, 1% (w/v) Tripton and 0.5% (w/v) yeast extract) added by 100 uL kanamycin 30% (w/v). The media was incubated at 37ºC with agitation 150 rpm for 2.5 hours. After that, the optical density (OD) of culture medium was measured by UV-Vis 600 nm (OD_{600} must be at 0.5-0.8). The media was added by 100 uL IPTG 1 M and incubated at 37ºC with agitation 200 rpm for 4 hours. The fermentation culture was harvested and centrifuged at 6,000 rpm for 30 minutes and stored at -20^oC until used.

Lysis of *E. coli* BL21(DE3)-pET30(a)-lipITB1.2 Pellet

1 gram pellet was suspended with 5 mL lysozyme 100% (w/v) and incubated at 25^oC for 4 hours. The pellet was sonicated for 10 times (30 second ON and 30 second OFF) and centrifuged at 12,000 rpm 4^oC for 30 minutes. The supernatant was heated at 60^oC for 30 minutes and collected by centrifuge at 12,000 rpm, 4^oC for 30 minutes and stored at 4^oC before used.

Lipase purification

DEAE-Celulose Purification

DEAE-cellulose Resin was washed three times by sterile distilled water and equilibrated by 0.05 M Na-Phosphate buffer pH 7.5. The DEAE-Cellulose was added with enzyme supernatant (1:1). The mixture was shaken 100 rpm for 1.5 hours and centrifuged at 5,000 rpm, 4^oC for 3 minutes. The supernatant was collected and stored at 4^oC before used.

Ni-NTA Purification

The purification of enzyme produced from DEAE-Cellulose was carried out by ultra-filtration using vivaspin 6 cut off 30 kDa at 5,000 rpm, 4^oC for 10 minutes. The residue was added by Triton-X 100 and NaCl to get final concentration of 1% (v/v) and 100 mM, respectively. After that, the residue was poured to Ni-NTA (1:1) which has been equilibrated by 50 mM Na-Phosphate buffer pH 7.5, 100 mM NaCl, and 1% (v/v) Triton-X 100. The mixture was shaken at 100 rpm for 1 hour. After homogeny mixture was formed, it was moved to column then eluted by 50 mM buffer Na-fosfat pH 7.5, 300 mM NaCl, and 0-20 mM imidazole.

Enzyme assay

Enzyme purity assay

The purity of lipase was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method reported by Laemmli[10]. The SDS-PAGE electrophoresis used a separating zone of 10% polyacrylamide gels and a concentration zone of 5% polyacrylamide. The gels were stained by Comassine Blue. Page Ruler Unstained Protein Ladder from Thermo scientific was used as a marker.

Assay of lipase activity and protein concentration

Lipase activity was measured with a modified spectrophotometric method with p-nitrophenyl palmitate (p-NPP) as substrate. 2 mg p-NPP with a final concentration of 0.04 mg/mL was dissolved in 0.5 mL acetonitrile and mixed with 2 mL ethanol and 47.5 mL of 50 mM sodium glycine buffer pH 9.0. The reaction mixture was composed of 0.9 mL substrate solution and 0.3 mL of appropriately diluted enzyme solution, and incubated at 70^{o}C for 15 min. The p-nitrophenol (p-NP) produced in the reaction was quantified by UV/visible spectrophotometry at 410 nm. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μmol p-NP per min under standard assay conditions.

The protein concentrations in this study were determined by Bradford method using bovine serum albumin as a standard [11].

Results and Discussion

E. coli BL21(DE3)-pET30(a)-lipITB1.2 is a recombinant bacteria which made by inserting a codding gen thermophilic lipase from Papandayan bacteria (PPD2) to *E. coli* bacteria (Coli BL21 (DE3)). These recombinant bacteria are able to produce thermally stable lipase at room temperature. Table 1 shows the average weight of *E. coli* BL21(DE3)-pET30(a)-lipITB1.2 produced in one liter of Laura Bertani (LB) media. Based on that Table 1, one liter of LB media produced 2.73 g of bacteria pellet.

Table 1. Mass of *E. coli* BL21(DE3)-pET30(a)-lipITB1.2 pellets produced in one liter of LB media.

No	Volume (L)	Mass (gram)	Average (gram.L^{-1})
1	1.375	3.76	2.73
2	1.375	3.92	2.85
3	1.375	3.28	2.39
4	2.100	6.00	2.86
5	1.950	5.49	2.82
Average (gram.L^{-1})			2.73

Lipase ITB2.1 was extracted from cell pellets using lysozyme then followed by sonication. The procedure was conducted at room temperature based on the optimum condition of lysozyme activity. Lysozyme has an ability to degrade the cell wall of bacteria and make the bacteria membrane was easy to break by sonication at 4^{o}C [12].

The supernatant of crude enzyme was heated at 60^{o}C for 30 minutes and centrifuged at 12000 rpm, 4^{o}C for 30 minutes. This step was performed to heat the precipitation of cell free extract where the protein with thermally unstable characteristic will be precipitated and the stable one will stayed at supernatant phase [13]. Therefore, the heating will reduced the complexity of enzyme supernatant and made the Ni-NTA purification was easier.

The separated enzyme from thermally unstable proteins then mixed with the DEAE-Cellulose. The DEAE-Cellulose is a basic anion exchange which absorbed the proteins with wide network structure and hydrophilic properties [14,15]. As seen from Figure 1 (the SDS-PAGE electropherogram number B), the proteins with larger structure was lost after mixing with DEAE-Cellulose (no band of weight protein). However, bands of small structure proteins were seen under target protein and need to be purified.

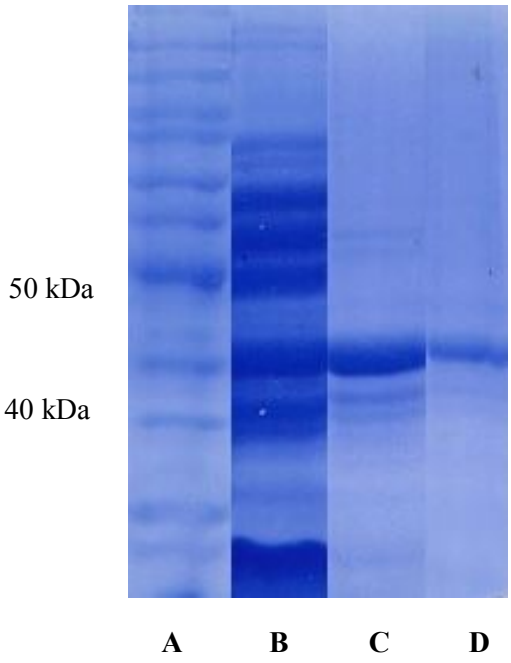

Fig. 1. SDS-PAGE of Lipase; A: Marker, B: Crude enzyme (heated at 60^{o}C, 30 minutes), C: DEAE-Cellulose, D: Ni-NTA, Eluent (50 mM Phosphate buffer pH 7,5; NaCl 100 mM; 5 mM).

Product of DEAE-Cellulose was filtrated by vivaspin6 30 kDa. This step was carried out to concentrate the supernatant of enzyme [16]. Protein as residue during the filtration process has a molecular weight of 30 kDA (46 kDa). This residue was used at IMAC of Ni-NTA.

In IMAC, adsorption of proteins was performed based on the interaction between an immobilized metal ion and electron donor groups located on the surface of proteins. Protein can specifically attached the metal ion coordination sites through certain amino acid residues, such as cysteine, hystidine, and tryptophan [17].

Lipase produced from *E. coli* BL21(DE3)-pET30(a)-lipITB1.2 has histidine as an active site which would interact with Ni-NTA. Chemically bonds between lipase and Ni-NTA will be obtained by mixing the vivaspin6 residue with lipase. The residue of enzyme was conditioned by 50 mM Na-phosphate buffer pH 7.5, 100 mM NaCl and 1% (v/v) Triton-X 100.

The Ni-NTA column was washed by 50mM Na-phosphate buffer pH 7.5, 100 mM NaCl and 1% (v/v) Triton-X 100 with flow rate of 0.002 mL .s^{-1}. The elution will remove the unattached protein from Ni-NTA column. On the contrary, lipase will be longer stay at the column. For the next step, the lipase will be eluted by the same eluent and replacing 1% (v/v) Triton-X 100 with imidazole 5 mM. In this case, imidazole acted as a ligand competitor to protein and substituted the bond formed between histidine and Ni-NTA [18]. Figure 1 (number D) shows that the band at the target protein (46 kDa) has a single band which is mean the purification was successfully performed.

Table 2. Concentration and activity of protein at each fraction.

No	Sample Code	Protein Concentration (mg.mL^{-1})	Activity (unit)	Total Activity (unit. mL^{-1})	Specific Activity (unit. mg^{-1})	Specific activity (-fold)
1	B	2.24	0.0045	4.52	2.09	1
2	C	0.61	0.0045	4.49	7.33	3
3	D	0.12	0.0043	4.31	34.94	17

Purification by using IMAC of Ni-NTA shows the increasing of enzyme activity 17-folds to heat the crude enzyme (Tabel 2).

Conclusions

Purification of thermophiles lipase lipITB1.2 by IMAC Ni-NTA was done by increasing 17-folds the specific activity of heated supernatant of crude enzyme. The optimum condition during the purification was using 50 mM Na-phosphate buffer pH 7.5, 300 mM NaCl, and 15mM of imidazole as eluent with an elution flow rate of 0.02 $mL.s^{-1}$.

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