MUTATION STUDY OF CELLOBIOSE DEHYDROGENASE FROM PHANEROCAETE CHRYSPORIUM IN A PROKARIOTIC SYSTEM FOR MULTIPLE BIOSENSOR APPLICATION

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ABSTRACT

The active site of cellobiose dehydrogenase from Phanerochaete chrysosporium is composed of two sub-sites, catalytic C and substrate-binding B subsite. The soluble flavin domain of the Phanerochaete chrysosporium Cellobiose dehydrogenase (CDH) has been mutated in residue F282 and successfully expressed in Escherichia coli. Substitution of Phe282 to Ala, Asp, and His shown to affect its enzymatic activity and altered the enzyme’s substrate specificity. While the wild-type cellobiose dehydrogenase efficiently oxidizes only cellobiose and lactose, the three mutated Phe282 shown significant activity for glucose and maltose. For all mutants type, cellobiose has retain its maximum activity but greatly decreased with lactose. The ability of CDH to recognize glucose provide great opportunities for the application in mult biosensor.

Keywords: cellobiose dehydrogenase, Glukosa, Laktosa, Maltosa, Phanerochaete chrysosporium

INTRODUCTION

Cellobiose dehydrogenase (CDH; EC1.1.99.18) has widely studied because it has many useful applications in biosensors (Stoica, Ludwig, Haltrich, & Gorton, 2006), bioremediation (M D Cameron & Aust, 1999), biopulping (Pricelius, Ludwig, Lant, Haltrich, & Guebitz, 2011), and biofuels (Harreither et al., 2012). CDH is a glycosylated extracellular protein produced by various fungi, including the white rot fungi (Michael D. Cameron & Aust, 2001). CDH is able to catalyze the oxidation process in cellobiose (GLC-b-1,4-GLC) and the type of disaccharide which has a β-1,4
bond or oligosaccharides at position C-1 (Hallberg, Henriksson, Pettersson, & Divne, 2002).

In molecular aspect, the heme domain in CDH allows enzyme to transfer electrons directly without the aid of artificial electron acceptors, making this protein is very promising to be applied to third-generation biosensor tool with the principle of direct electron transfer.

To achieve CDH in rapid and produce large numbers of amount, several number of studies have been conducted, by expressing CDH recombinant genes derived from several types of white rot fungi using a host that is commonly used in the process of gene expression (Yoshida et al., 2014). The isolation and characterization of the enzyme CDH Phanerochaeta chrysosporium (PcCDH) by using host P. Pastoris. (Desriani, Ferri, & Sode, 2010) successfully conducting CDH flavin domain gene expression in Phanerochaeta chrysosporium in E. coli. The success of the use of E. coli as a host for gene expression CDH became a gateway to speed up the production process of CDH in quantity, as well as other studies done to improve the quality and performance of CDH protein to meet the criteria of the ideal protein for biosensor applications in a shorter time.

Phanerochaeta chrysosporium belonging to the white rot fungi family (Henriksson, Pettersson, Johansson, Ruiz, & Uzcategui, 1991). CDH enzymes from this fungus has been known and well characterized, and its potential is also well known.

To improve the performance and quality of this enzyme, several numbers of mutations have been done in the catalytic region. So far, there have been no reports of PcCDH mutation studies using E. coli as a host. In order to improve the quality and performance of enzymes PcCDH, the study of flavin domain mutant expression of PcCDH, with mutation in one of the amino acids using E. coli as a host become interesting to explore.

MATERIAL AND METHODS

pET30c CDH

The sample used was the recombinant plasmid pET30c CDH obtained from the Laboratory of Protein Engineering Laboratory for Health Applications, Indonesian Institute of Sciences (LIPI)

Host

The bacterium used as hosts in the process of transformation is E. coli BL21 (DE3).

Vector

The vector used in this study is pET30c, pET30c_PcCDH_Domain Flavin F282A / D / H.

Preparation of Competent Cells

BL21_DE3 E. coli grown into a new LB medium, incubated for 24 h at 37 °C. E. coli colonies proceed to contamination assay, then transferred to 5 mL LB medium, incubated at 37 °C for 24 hours at a speed of 200 rpm. E. coli overnight cultures that have proceed contamination assay transferred to liquid LB 50 mL, in shaking at 200 rpm at 37 °C to obtain 0.6 OD (approximately 1.5 hours incubation). After obtained OD 0.6, centrifuge has done to obtain E. coli BL21 DE3 pellet. On pellets (effendorf 2 mL) was added 2 mL MgCl2, homogenized (pipetting) and incubated in ice for 20 minutes and centrifuged at a speed of 10,000 rpm for 10 minutes at a temperature of 10 °C, then pellet and supernatant can be obtained. At the pellets are added 2 mL CaCl2 homogenised (pipetting), incubated in ice for 20 minutes and centrifuged at a speed of 10,000 rpm for 10 minutes at a temperature of 10 °C, then pellet and supernatant can be obtained. In the pellet was added 150 mL CaCl2 homogenized (pipetting) and added 18.75 mL of 80% glycerol were resumed with homogenized (pipetting), obtained homogeneous mixture which is E. coli BL21 DE3 competent cells.

Electrophoresis

PCR results can be viewed by using electrophoresis. Used agarose 1% as a gel, and as standar by mixing 1 kb ladder
marker (Thermo Scientific) by 2 mL to 4 mL of loading dye (Thermo Scientific). 6 mL standard pipette into the gel and 5 mL for each sample. Do running electrophoresis. To see the results of running the electrophoresis, the gel was soaked in ethidium bromide for 15 and the gel was photographed with transilluminators.

**Transformation CDH recombinant**

Competent cells as much as 168.75 mL plasmid added with 5 mL pET30c. The plasmid used is pET30c without inserted plasmid, pET30c PCDH_Domain Flavin F282A / D / H. Competent cells and plasmid mixture was incubated in ice for 30 minutes, followed by heating in a water bath for 45 seconds at a temperature of 42 °C, and then incubated again in ice for 5 minutes. After incubation in ice, add 1 mL of liquid LB, incubation for 45 minutes at a speed of 200 rpm at 37 °C incubation. Incubation result was centrifuged for 10 minutes at a speed of 13,000 rpm at a temperature of 10 °C. Supernatant was discarded and the centrifugation results spared about 100 mL, homogenized (pipetting) and the results of this transformation were grown in LB medium without antibiotics, with the addition of LB medium ampicillin and LB medium with the addition of kanamycin.

**Expression and production of recombinant CDH**

CDH expression of recombinant done with auto induction method, after the cells were grown on a PA-5052 growth medium containing 50 mM Na2HPO4, 50 mM KH2PO4, 25 mM (NH4) 2SO4, 2 mM MgSO4, trace metals, 0.5% glycerol, 0.05% by Glucose, 0.2% α-Lactose, and 200 mg / L, all the amino acids except cysteine and tyrosine.

**Extraction of CDH**

Cells were collected by centrifugation and resuspended in 50 mM ammonium acetate buffer, pH 5. The cells are broken down by ultrasonication and centrifuged to obtain a crude extract enzyme CDH.

**Past This**

Supernatant (crude extract CDH) and the pellet diluted 5x using phosphate buffer pH 7. Dilution results were 30 mL pipette and put into tube effendorf. On effendorf added 30 mL tube loading dye. E.coli control BL21_DE3 without inserts did the same with the enzyme preparation. After preparation was complete, samples and controls immersed in a water bath for 10 minutes at a temperature of 95 °C. After incubation the samples along with control done running electrophoresis for 1.5 hours. After running process was complete, do the staining and destaining the gel.

**Characterization of CDH**

Enzyme activity Recombinant determined at room temperature use 0.12.6 mM dichloroindophenol (DCIP) / in 50 mM Ammonium acetate buffer pH 5 at various substrates concentrations. DCIP absorption was detected at 525 nm with molar absorption coefficient 8.25M⁻¹cm⁻¹. Substrates (Cellobiose, Lactose, Glucose, dan Maltose) were used to determine enzyme activity.

**RESULTS AND DISCUSSION**

**Transformation CDH recombinant**

Plasmid pET30c that already carry genes CDH with three variations of mutant F282A, F282D, and F282H respectively transformed into E. coli BL21 (DE3) using a heat shock method. Before performing the transformation process, checking was done for each plasmid by performing migration analysis method using 1% agarose electrophoresis. (Figure 1).
The results of the transformation by using the heat shock method was grown on solid media LB + kanamycin selection, recombinant grow further confirmed by colony PCR method. PCR results showed that each plasmid carries the gene CDH with each mutant F282A, F282D, and F282H. (Figure 2)

In Figure 2 to confirm the target recombinant, a pair of primer were used which recognize the inserted flavin domain PcCDH gene with targeted product size is 1000 bp. Result shows transformation was done successfully, obtained the size of 1000 bp of the three different recombinant mutant.

**SDS-PAGE CDH Recombinant**

Expression of CDH flavin domain in E. coli was performed using auto induction method at a temperature of 25°C (Desriani et al., 2010b). auto induction method was chosen to obtain the target soluble and functional protein in prokaryotic systems. Crude protein extracted from E. coli subsequently checked using SDS-PAGE. E. coli is used as a negative control pET30c. SDS-PAGE is a method for the separation and estimate the size of a protein (Saini & Sarin, 2012).
Table I. PcCDH_Flavin Domain F282 A/D/H activity at 100 mM substrat (U/mg).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>F282A</th>
<th>F282D</th>
<th>F282H</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellobiose</td>
<td>0.1422</td>
<td>0.0513</td>
<td>0.0573</td>
</tr>
<tr>
<td>Lactose</td>
<td>0.0940</td>
<td>0.0309</td>
<td>0.0470</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.0025</td>
<td>0.0017</td>
<td>0.0013</td>
</tr>
<tr>
<td>Maltose</td>
<td>0.0030</td>
<td>0.0015</td>
<td>0.0013</td>
</tr>
</tbody>
</table>

Table I shows all the mutants have highest activity against Cellobiose substrate, followed by Lactose and Maltose and Glucose. According to (Desriani et al., 2010), PcCDH F282 flavin domain has activity against Cellobiose and Lactose were 0.28 U / mg; 0.33 U / mg, whereas activity for Glucose and Maltose could not be detected.

In this study, CDH flavin domain of P.chrysosporium have been mutated in amino acid position number 282 fenilialanin, capable of degrading Glucose and Maltose. The highest unit activity obtained for mutant F282A PcCDH of 0.0025 U / mg to glucose and 0.0030 U / mg against Maltose. In contrast to the results obtained from previous studies, the same method using flavin domain PcCDH F282 showed no activity against Glucose and Maltose. CDH works by cutting the β-1,4-glycoside bond in its substrate to produce compounds glukonolakton. Cellobiose, Lactose, cellotriose, cellotetrose known to have -1,4 glycoside bonding, while Glucose and Maltose have lack of those bonding, making them as poor substrate for CHD. Although the structure of flavin domain CDH has similarities with Glucose oxidase from Aspergillus, which has been used for glucose biosensors, but CDH have Km values 1600 mM and 2400 mM per each for Glucose and Maltose (Zamocky et al., 2006).

CONCLUSIONS

The enzyme domain of PcCDH flavin has been successfully well expressed, indicated by the appearance of the size of the 54 kDa protein corresponding to the targeted, and the size of the protein bands were not found on the negative controls. Most proteins have been successfully expressed in soluble form, only in a low percentage expressed to insoluble form (Figure 3 (b)). Insoluble proteins may due to the formation of inclusion body. Inclusion body is insoluble protein aggregates that are usually found in recombinant bacteria that forced to produce the heterologous protein (García et al., 2011).

REFERENCES


