

PURIFICATION AND CARBOHYDRATE ANALYSIS OF RECOMBINANT HUMAN ERYTHROPOIETIN EXPRESSED IN YEAST SYSTEM *Pichia pastoris*

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Abstract

For clinical purposes, pure protein and identification of carbohydrate structure from recombinant erythropoietin are needed. Purification was done by Immobilized Metal Affinity Chromatography (IMAC) column charged with Ni²⁺ (His-Trap affinity chromatography) and continued with gel filtration chromatography column to get purer protein. The carbohydrate group which is oligosaccharide from the resulting pure protein then can be recognized by using N- and O-glycosidase. Pure oligosaccharide was hydrolyzed to produce various monosaccharide through incubation with 4 N HCl in 100 °C temperature for 6 hours and the result was applied on High Performance Liquid Chromatography (HPLC) column to learn the composition of its monosaccharide.

Keywords: EPO, gel filtration chromatography, His-Trap affinity chromatography, monosaccharide

1. Introduction

Erythropoietin (EPO) is a glycoprotein that can stimulate the production of red blood cells. Recombinant EPO is used in the medication of anemia caused by kidney failure on dialysis patient in order to increase the production of autologous blood in a normal subjects, and to reduce anemia duration of a chemotherapy patient. Therapy with recombinant EPO can decrease the need for blood transfusion, thus reducing the risk of coming down with illness such as viral hepatitis or HIV AIDS [1].

Recombinant EPO for clinical purposes is largely available from the result of mammalian cell expression, i.e. CHO (Chinese hamster ovary) cells [2]. However, the impediment in using mammalian cell culture to produce recombinant EPO is the inefficiency and high cost production [3], therefore, an alternative system that is more efficient and cheaper is needed.

Pichia pastoris is a methylotrophic yeast system that offers an expression system beneficial in producing recombinant protein. Compare to other eukaryotic system, *P. pastoris* has more profit, which is an expression system with high efficiency because it uses the methanol inducible alcohol oxidase gene (AOX1) promoter and vector that is integrated on the *Pichia* genome, has high secretion level on free protein medium, easy fermentation process, limited or no over-

glycosylation, and no highly antigenic terminal, that is α -1.3-mannose linkages, otherwise present in *Saccharomyces cerevisiae* [4].

Research Center for Biotechnology LIPI has succeeded in expressing recombinant EPO in yeast system *P. pastoris*. The aims of this research are to get the pure protein with the appropriate purification technique and to characterize the carbohydrate content of recombinant EPO for clinical purposes.

2. Methods

Recombinant human Erythropoietin (rhEPO). rhEPO is produced from supernatant result of transformant culture of *Pichia pastoris* strain X-33 which has been cultivated for 48 hours on 30 °C temperature with 0.5% methanol induction every 24 hours. The presence of EPO protein has been proven with Western blot analysis.

His Trap Affinity Chromatography. Isolation of rhEPO from culture was performed by affinity chromatography method using nickel chelating agent as stationary phase (hisrap column) (GE Healthcare). In this process, the matrix in the column was equilibrated in the binding buffer (phosphate buffer pH 7.4) which contain 20 mM sodium phosphate, 500 mM NaCl, and 20 mM Imidazole. A 20 mL supernatant of *Pichia pastoris* transformant containing EPO was applied to

the equilibrated column. Then, the column was washed using 15 column volume (CV) binding buffer. Elution was conducted using 5 CV elution buffer containing 20 mM sodium phosphate, 500 mM NaCl and 500 mM imidazole. Elution result was fractionated individually. These fractions being analyzed with Western blot analysis method to find the highest recombinant EPO-containing fraction.

Gel Filtration Chromatography using Sephadex LH-20. The fractions obtained from hisrap column that containing rhEPO were applied to 40 mL Sephadex LH-20 column to omit imidazole and to get a purer protein. The packed Sephadex LH-20 column was equilibrated with 80 mL phosphate buffer containing 50 mM sodium phosphate pH 6.7 with 125 mM NaCl. Then 4 mL eluate from His-Trap result that contained EPO protein was applied in the column then eluted by 60 mL phosphate buffer with a flow rate of 1mL/minutes. Elution result was fractionated 1 mL each, continued by the analysis using UV spectrophotometer on 280 nm wavelength to know the fraction containing targeted protein.

SDS PAGE and Western Blot Analysis. In order to affirm protein purification result and the completion of glycosidase reaction, the protein were being analysed by SDS PAGE with a separating gel containing 12% acrylamide. After electrophoresis, protein bands were detected by staining with Coomassie brilliant blue (Biorad) and Silverstain reaction (GE healthcare). Western blot analysis was performed to detected each fraction after purification on gel filtration chromatography. After SDS PAGE process, protein inside the gel was transferred into Hybond nitrocellulose membrane (GE Healthcare) by using electroblotting. Immunodetection was achieved by using anti-hEPO antibody (Calbiochem) as primary antibody and anti-rabbit IgG peroxidase conjugate (Biorad) as secondary antibody. The band was visualized by NBT-BCIP staining reaction (Promega).

Carbohydrate Analysis Using HPLC. As much as 0.5 mL pure rhEPO was incubated with 25 units of enzyme N- and O-glycosidase (Roche) on a temperature of 37 °C for 24 hours in 50 µl of sodium phosphate buffer 200 mM pH7.2. A volume of 80% methanol preserved in -20 °C temperature was added to precipitate oligosaccharide (and protein). Oligosaccharide in the precipitate was resolubilized with a small volume of 60% aqueous methanol in a cold condition without dissolving the protein [5]. After being centrifuged, the supernatant was eliminated by drying it to obtain pure oligosaccharide. In order to obtain monosaccharide, pure oligosaccharide was hydrolyzed through incubation in 4N HCl on 100 °C for 6 hours at nitrogen atmosphere (oxygen was eliminated by flowing in nitrogen and then sealing the tube tightly). Following the neutralization process, monosaccharides were

separated using HPLC autosampler Hitachi L-2000 with Purospher® STAR NH₂ (Merck) diluted with 3:1 acetonitrile/water with a flow rate of 0.6 mL/minute. The separated monosaccharide was detected with RI detector Hitachi L-2490 at 40 °C.

3. Results and Discussion

Purification of rhEPO. The result of purification of supernatant *P. pastoris* strain X-33 which contained EPO by the His-Trap affinity chromatography method using Ni²⁺ column [6] showed two fractions containing recombinant EPO with a high concentration. Western blot analysis showed protein bands only existed in fraction 1 and 2 (as shown on Figure 1).

Gel filtration chromatography was done as a smoothing step in the purification process [7]. When the eluate from His-Trap was applied into the Sephadex LH-20 column, more than one peak was detected with spectrophotometer on the 280 nm wavelength [8]. Data was shown in Figure 2.

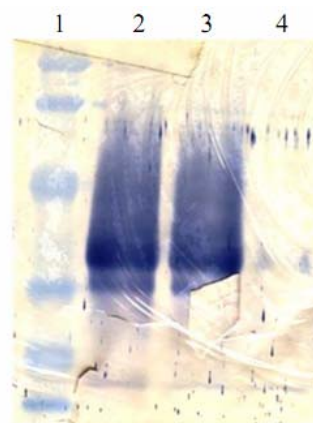


Figure 1. Western Blot Analysis for His-Trap Fraction. Lane 1: Protein Marker; Lane 2: Fraction 1; Lane 3: Fraction 2; Lane 4: Fraction 3

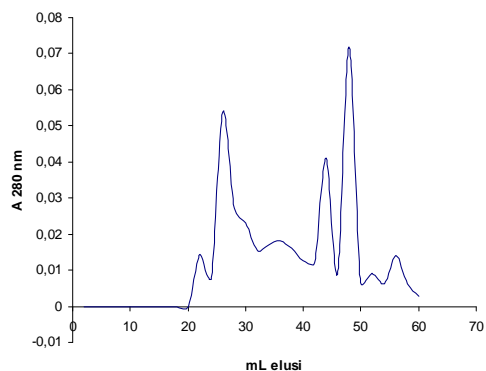


Figure 2. The Result of Gel Filtration Chromatography Fractions Measured with Spectrophotometer on 280 nm Wavelength



Figure 3. Western Blot Analysis of Gel Filtration Chromatography. -: there was no EPO Protein; +: there was EPO Protein



Figure 4. SDS PAGE with the Silver Colouring After the Purification Process. Lane 1: Purification Combination Using His-Trap Column and Sephadex LH-20 Column. Lane 2: Protein Marker. Lane 3: Purification Using His-Trap Column

In order to recognize the eluate which contained rhEPO, every fraction with the highest absorbance was detected using Western blot analysis (shown by Figure 3).

An analysis using SDS PAGE with silver staining was performed in order to compare the purity of the protein as a result of purification using His-Trap and a combination of His-Trap with the gel filtration chromatography [9]. The clearer band was produced on the purification combination using the His-Trap column with Sephadex LH-20 (as shown on Figure 4).

Monosaccharide from rhEPO carbohydrate group.

The carbohydrate analysis was performed to confirm that the rhEPO is glycoprotein. The N- and O-glycosidase enzymes were used to digest glycoprotein and the results were shown in Figure 5 [10].

The result of SDS PAGE clearly showed the decreasing of the molecular weight of the rhEPO protein when treated with glycosidase enzymes. Lane 1 which was rhEPO without any treatments has ± 37 kDa molecular weight, while on lane 3 which was rhEPO with the glycosidase enzyme treatment has ± 22 kDa molecular weight. The result has confirmed that approximately 40% of the molecular mass of the mature hEPO is made up by 4 carbohydrate chains (3 N-linked at Asn24, Asn38, and ASn83, and 1 O-linked at Ser126) [11]. Proper glycosylation of EPO is not required for its receptor binding but it is however quite essential for elongation of its biological half-life. Even though glycosylation pattern of CHO and *P. pastoris* may differ to some extent, but still glycosylation pattern of secreted proteins by *P. pastoris* is the most similar pattern to mammalian cells [12]. Some glyco-engineering system strategies had been reported for further development of human-type N- and -O glycosilation pathway engineered in *P. pastoris* system [13-15].

An analysis using HPLC with the normal phase system was done to confirm the content of monosaccharides in the carbohydrate group [16]. HCl 4N was used to hydrolyzed the carbohydrate which is an oligosaccharide to be monosaccharides. The data from the HPLC analysis will be shown in Table 1.

The type of monosaccharides contained in the rhEPO carbohydrate group were still could not be confirmed based on the data produced. As a comparison, the data from Sjöblom's dissertation regarding protein expression and the recombinant vaccine as a result of

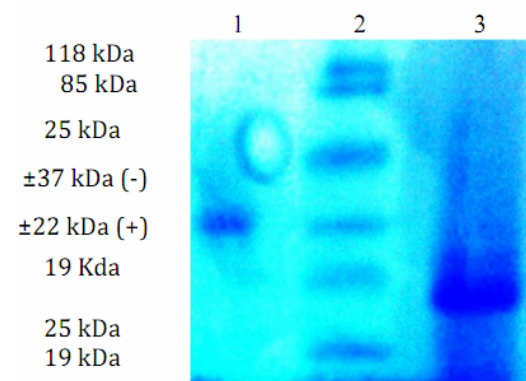


Figure 5. Glycoprotein Analysis. Lane 1: rhEPO without Treatment (-); Lane 2: Protein Marker; Lane 3: rhEPO with Glycosidase Enzyme Treatment (+)

Table 1. Analysis of Monosaccharide using HPLC

Sample	Retention Time(minute)	Peak Area
Standard mannose 259 ppm	8.273	225.805
Sampel dilution 30×	10.187	2.966.770

the expression on *P. pastoris* and Hamilton *et al.* stated that the monosaccharides that would be consisted in the glycan structure from the glycoprotein as the product of expression on *P. pastoris* for the wild-type strain consisted of mannose and N-acetyl glucosamine [17-19]. Kakehi and Honda stated that only amino sugar like glucosamine and galactosamine will be hydrolyzed with 4N HCl in the condition of nitrogen atmosphere, while neutral sugars such as galactose, fucose, and mannose are easily degraded using 4N HCl [20]. Based on the outcomes of that research, then the highest peak possibility of the result from detection using HPLC was amino sugar, which was N-acetyl glucosamine. Meanwhile, mannose was not detected, possibly because it was destroyed during the hydrolysis with 4N HCl, as seen in the data above, the retention time from the standard mannose was different from the sample.

4. Conclusion

In the purification step, the combination of His-Trap affinity chromatography and gel filtration chromatography was very important to obtain a purer protein target. The use of 4N HCl acid solution for the hydrolysis reaction on 100 °C for 6 hours was not effective in determining the monosaccharide content. For the next study, a correct reaction condition will be needed, consisted of the type of acid and temperature used in the carbohydrate hydrolysis process to determine the type of monosaccharide it contains.

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