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Comparison of Immobilized Metal Affinity Chromatography Ni-NTA and Co-TALON for the Purification of Recombinant Human Erythropoietin

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Abstract

The purification of recombinant proteins is an important stage in biopharmaceutical research. A commonly used technique is immobilized metal affinity chromatography (IMAC). One of the main advantages of this type of chromatography is that the column can easily be regenerated for subsequent purification work. The mechanism of IMAC is based on bonding between metal ions immobilized on a matrix with a specific amino acid. Because of the strong interactions of the electron donor group on the imidazole ring, histidine is often used in the IMAC purification system. Two types of commercial IMAC resin use a nitrilotriacetic acid (NTA) matrix: a nickel-based (Ni-NTA) and cobalt-based (Co-NTA), better known as TALON. This study was aim to investigate the effect of the metal ions Ni^{2+} and Co^{2+} to purify recombinant human erythropoietin (rhEPO) expressed in yeast system *Pichia pastoris*. The results indicated that both Ni-NTA and Co-TALON gave almost the same level of protein purity; however, Ni-NTA has a higher binding affinity than Co-TALON might be due to the higher stability complex of Ni^+. The average amount of protein bound by Ni-NTA and Co-TALON was 183.5 and 38.7 µg/mL, respectively.

Abstrak

Perbandingan Immobilized Metal Affinity Chromatography Ni-NTA dan Co-TALON untuk Pemurnian Rekombinan Erythropoietin Manusia. Teknik pemurnian rekombinan protein merupakan tahap yang sangat penting dalam riset biofarmasetik. Teknik yang umum digunakan adalah kromatografi afinitas logam terimobilisasi (IMAC). Salah satu kelebihan dari kromatografi jenis ini adalah kolom dapat dengan mudah diregenerasi untuk digunakan pada proses purifikasi berikutnya. Mekanisme purifikasi IMAC berdasarkan pada ikatan yang sangat spesifik antara metal ion yang terimobilisasi pada matriks dengan asam amino tertentu. Karena interaksinya yang kuat sebagai kelompok donor elektron pada cincin imidazol, maka asam amino histidin sering digunakan pada sistem purifikasi IMAC. Terdapat 2 jenis resin IMAC komersial yang menggunakan matriks asam nitriloasetat (NTA), yaitu resin IMAC berbasis-nikel (Ni-NTA) dan berbasis-kobalt (Co-NTA) atau lebih dikenal dengan TALON. Penelitian ini bertujuan untuk mengetahui pengaruh penggunaan ion logam Ni^{2+} dan Co^{2+} dalam memurnikan protein rekombinan erithropoietin manusia (rhEPO) yang diekspesikan dalam sistem ragi *Pichia pastoris*. Hasil penelitian menunjukkan bahwa baik Ni-NTA maupun Co-TALON mempunyai tingkat kemurnian yang hampir sama, namun Ni-NTA mempunyai afinitas pengikatan yang lebih tinggi dibandingkan dengan Co-TALON kemungkinan disebabkan oleh kestabilan komplek logam Ni yang lebih tinggi. Jumlah rata-rata protein yang diikat oleh Ni-NTA dan Co-TALON masing-masing sebesar 183.5 µg/mL dan 38.7 µg/mL.

Keywords: Co-TALON, IMAC, Ni-NTA, purification, rhEPO

Introduction

Research on human erythropoietin (hEPO) has increased as researchers attempt to obtain therapeutic agents. Cloning and gene expression techniques have developed hEPO into recombinant human erythropoietin (rhEPO) as a drug. Since 1980, rhEPO has been a major commodity in the biotechnology industry; sales have increased from year to year until they reached billions of dollars [1-2]. rhEPO has been used for clinical treatment of patients

with anemia caused by cancer, human immunodeficiency virus infection (HIV) infection, kidney failure, and bone marrow transplantation [3].

The Research Center for Biotechnology, Indonesian Institute of Sciences (LIPI) expressed recombinant α-hEPO in the yeast expression system (*Pichia pastoris*) and showed with the western blotting technique that the supernatant contained rhEPO. For the next stage, appropriate purification techniques are required to obtain pure protein. A purification technique that utilizes specific interactions between the protein and a ligand (metal ions) has been developed. This technique is known as immobilized metal affinity chromatography (IMAC) [4-5]. Depending on the specific interactions, IMAC can be used to purify protein at up to 95% purity [6].

IMAC utilizes specific interactions between the side chains of amino acids with borderline Lewis metal ions (such as Cu^{2+}, Co^{2+}, Ni^{2+}, and Zn^{2+}) [7-11]. Metal ions immobilized through a chelating agent attach to the stationary support. The chelating agents most widely used for this application are iminodiacetic acid (IDA) and nitrilotriacetic acid (NTA) [12]. The target protein is "tagged" with hexahistidine (6xHis); thus, there is specific binding between the recombinant protein with a ligand of the IMAC. Given the specific binding, the target protein to purify can be separated from other proteins [4,8]. Two types of commercial IMAC resin use NTA matrix as a chelating agent, nickel-based (Ni-NTA) and cobalt-based (Co-NTA) IMAC resin. The latter is well known as TALON. In this study, the affinity of metal ions Ni^{2+} and Co^{2+} was compared to find a suitable IMAC technique for purifying rhEPO protein.

Materials and Methods

Selection of yeast transformant. *Escherichia coli* DH5 alpha (Invitrogen, San Diego, CA) was used as the host for the recombinant plasmid cloning [7] experiment and grown in LB medium (1% tryptone, 0.5% NaCl and 0.5% yeast extract, plus 2% agar in plates). Zeocin was added at standard concentrations to screen the bacteria and the selection of the yeast transformant, *P. pastoris* strain X-33 (Invitrogen). The yeast was cultured in the yeast peptone dextrose (YPD) medium (1% yeast extract, 2% peptone and 2% dextrose, plus 2% agar in plates). The composition of the media for the expression study was buffered glycerol complex medium (BMGY) (1% yeast extract, 2% peptone, 1.34% YNB, 4×10^{-5}% biotin, 1% glycerol and 0.1 M potassium phosphate, pH = 6.0) and buffered methanol complex medium (BMMY) (BMGY medium containing 0.5% methanol instead of glycerol). The yeast cultures were grown at 30 °C. The cell-producing strain was selected based on the expression level. Several clones were screened for the level of expression of the EPO protein with western blotting using polyclonal anti-EPO antibodies. The highest expressing clone was selected for subsequent study.

Recombinant human erythropoietin (rhEPO) production. The *P. pastoris* transformant containing the hEPO gene was grown on the YPD medium (1% Bacto yeast, 2% Bacto peptone, 2% glucose 20% and 2% Bacto agar) at 30 °C for 2 days until the clones appeared. The clones were screened with western blot analysis using human polyclonal anti-EPO antibodies. The highest-expressing clone was selected for the study. A single colony was grown in the BMGY medium (1% yeast, 2% peptone, potassium phosphate 100 mM, 1.34% yeast nitrogen base, 0.00004% biotin, and 1% glycerol) at 30 °C for 1 day (24 h). Growth of the cells was then resumed at a concentration of optical density (OD) 1 in the BMMY medium (the BMGY medium with 1% glycerol was replaced with 0.5% methanol) at 30 °C for 2 days. After 2 days, the cell culture was centrifuged for 10 min at $2,300 \times$ g. The supernatant was then analyzed with the western blot technique [14].

Ni^{2+}-NTA/Co^{2+}-TALON IMAC purification. A 200 mL supernatant was produced from the same culture. Then 10 mL of the supernatant was mixed with the IMAC resin (Ni-NTA/Co-TALON), which had been equilibrated with phosphate buffer pH 7.4 (20 mM sodium phosphate and 500 mM NaCl) and then shaken with a shaker (150 rpm) for 60 min. The mixture was inserted into the empty column, and then the filtrate samples (flow-through) were accommodated. The column was washed with 10 mL washing buffer (20 mM sodium phosphate and 500 mM NaCl) 1 time. Then the column was eluted with the elution buffer containing 20 mM sodium phosphate, 500 mM NaCl and 500 mM imidazole. The elution results were fractionated (each 1 mL) and stored in 1.5 mL polypropylene tubes [15]. This process was repeated four times. Subsequently, the fraction solutions were analyzed with western blotting and measured with a spectrophotometer.

SDS-PAGE and western blot analysis. To confirm the protein purification result, the protein was analyzed with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with a separating gel containing 12% acrylamide. Western blot analysis was performed to detect each protein fraction after purification on IMAC. Following SDS-PAGE, the protein inside the gel was transferred into Hybond nitrocellulose membrane (GE Healthcare) by using electroblotting. Immunodetection was achieved by using an anti-hEPO antibody (Calbiochem) as the primary antibody and anti-rabbit IgG peroxidase conjugate (Bio-Rad) as the secondary antibody. The band was visualized with the nitro-blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt (BCIP) staining reaction, then the density of the rhEPO bands was calculated based on area under the curve (AUC) determined with ImageJ software (http://imagej.en.softonic.com).

Analysis of total protein by bicinchoninic acid (BCA) protein assay kit. A series of standard solutions of bovine serum albumin (BSA) with concentrations of 25, 125, 250, 500, 750, 1000, 1500, and 2000 µg/mL was prepared. The working solution was prepared by mixing 50 mL of solution A (sodium carbonate, sodium bicarbonate and sodium tartrate bicinchoninic acid in 0.1 M sodium hydroxide) with solution B containing 4% Cu-sulfate. A total of 0.1 mL of standard, blank samples was pipetted into a clean test tube, and then 2 mL of the working solution was added to each tube and shaken gently until well mixed. The mixture was incubated in a water bath with a temperature of 37 °C for 30 min. After the solution had cooled to room temperature, the solution was immediately measured using a UV-visible spectro-photometer at a wavelength of 562 nm [16].

Results and Discussion

Identification of the highest-expressing clone. The expression and subsequent purification of recombinant proteins are widely performed in molecular and biochemical studies. A powerful purification method involving the use of peptide affinity tags, which are fused to the protein of interest and used to expedite protein purification via affinity chromatography, was extensively applied. The commonly used commercial Ni-based IMAC resin (Ni-NTA) and cobalt-based IMAC resin (Co-NTA) was compared and analyzed. In the current experiment, purification of rhEPO, which had been fused with six histidine amino acid residues at the carboxy terminal, was studied.

The expression level of the rhEPO protein is shown in Figure 1. In each lane, a band the same size as the rhEPO standard, which is about 37 kDa, can be observed. These results indicate that the rhEPO protein was contained in the supernatant. The result in lane 3 indicates a greater number of rhEPO in clone 18. The highest-expressing clone (clone number 18) was selected for the study.

Quantification of rhEPO protein. Clone 18 was then expressed for further analysis. For a better idea of how effective the use of Ni-NTA and Co-TALON resin was for purifying the protein, the expression study using clone number 18 was repeated four times from the same supernatant. The expressed and purified protein was then analyzed with western blotting. The result is shown in Figure 2. The protein eluates using Ni-NTA are shown in lanes 2, 3, 4, and 5. The protein eluates using Co-TALON resin are in lanes 6, 7, 8, and 9. The density of the rhEPO bands was analyzed based on the AUC determined with ImageJ; the results are shown in Table 1 and Figure 3. This analysis clearly shows that the density of the rhEPO bands purified using Ni-NTA was higher than that of Co-TALON, indicating that more rhEPO protein can be captured with Ni-NTA than with Co-TALON.

To measure how much rhEPO protein was purified using these two types of resin, the amount of protein eluates was estimated using BCA protein assay. The assay showed the average amount of protein purified using Ni-NTA was 183.5 µg/mL, while that of Co-TALON was 38.7 µg/mL (Table 2). Some small variations were observed within the four replications; however, overall, these data clearly correlate with those shown in Figure 2
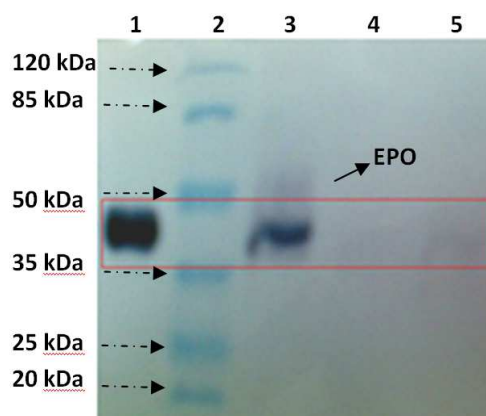

Figure 1. Western Blot Analysis of rhEPO. Lane 1: Standard rhEPO, Row 2: Protein Marker, Rows 3, 4, and 5: Supernatant rhEPO Clones 18, 11, and 9
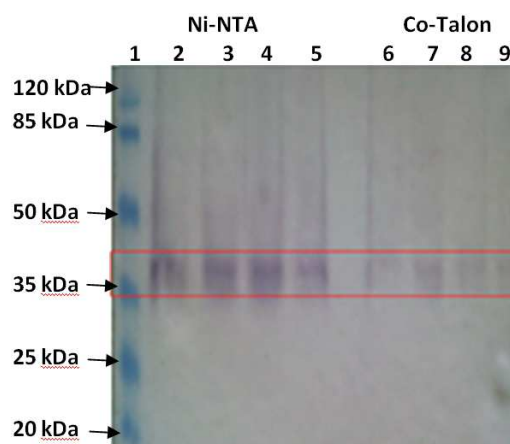

Figure 2. Western Blot Analysis of Purified rhEPO. Lane 1: Protein Marker, Lanes 2–5: Ni-NTA Eluate Fractions, Lanes 6–9: Co-TALON Eluate Fractions

Table 1. Area under Curve (ImageJ) of the rhEPO Bands Ni-NTA and Co-TALON

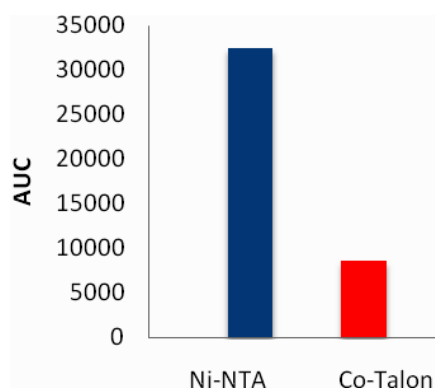| Repetition	Area Under Curve (AUC)	
	Ni-NTA	Co-TALON
1	36533.40	6299.229
2	36717.61	10637.96
3	35299.56	6916.995
4	21125.56	10348.61
Average	32419.03	8550.69

Figure 3. The average AUC of rhEPO Bands for Ni-NTA and Co-TALON

Table 2. Total Protein of the Eluate Purified with Ni-NTA and Co-TALON

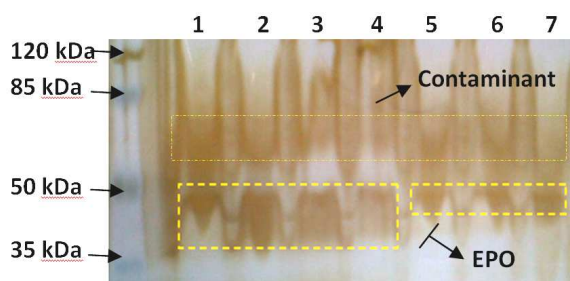| Repetition	Concentration (µg/mL)	
	Ni-NTA	Co-TALON
1	275.6	63.7
2	145.2	41.4
3	206.7	25.4
4	106.4	24.4
Average	183.5	38.7

Figure 4. Silver Staining Analysis of purified rhEPO. Lanes 1–4: eluate Ni-NTA, lane 5-7: Eluate Co-TALON

that demonstrated the Ni-NTA resin bound the rhEPO protein higher than Co-TALON. The differences of ligand field stabilization energy (LSFE) influenced the geometry stability of metal complex [17]. The relative value of LFSE Ni$^+$ is higher than LFSE Co$^+$, causing the complex of Ni is more stable. Therefore, the Ni-NTA resin can capture more proteins.

Purity of the expressed protein rhEPO. To evaluate the rhEPO protein after it was purified using Ni-NTA and Co-TALON, the eluates were analyzed using SDS-PAGE and stained using Silver staining (Fermentas). The result (Figure 4) showed that the rhEPO protein was clearly observed at the size of, approximately, 37 kDa. The amount of rhEPO purified when Ni-NTA was

used was much higher than when Co-TALON was used. These data correlate well with the data shown in Figures 2 and 3 and Tables 1 and 2, where the Ni-NTA resin was more efficient that Co-TALON, indicating that the Ni-NTA resin has more binding capacity than Co-TALON.

With the advent of genetic engineering, it has become easy to design a protein that has a structure for a simplified purification procedure. One of the most popular means to do is by adding 6–10 histidines on the N-terminus of a protein [18-20]. The protein is then purified by its ability to bind tightly to a column that contains chelated Ni^{2+} or Co^{2+} in which it can be washed and then eluted with free imidazole or by lowering the pH to 5.9, where histidine becomes fully protonated and no longer binds to the chelated metal.

However, although IMAC technology is very promising and superior to affinity chromatography, the purity level of the protein obtained (Figure 4) is not absolute. Protein impurities can still be recognized in this Silver stained gel. These impurities might come from the media or the host cell proteins containing histidine residues. Subsequently, these proteins may bind to the IMAC resin.

Although histidine occurs infrequently (2% of all protein residues are histidine), some cellular proteins may contain two or more adjacent histidine residues. These proteins have an affinity for the IMAC matrix and may coelute with the protein of interest, resulting in significant contamination of the final product. The problem of this type of impurity is more common in eukaryotic cells than in prokaryotic cells. This is because mammalian cells have a higher natural abundance of proteins containing consecutive histidine residues. In addition to the presence of natural consecutive histidine residues, the formation of a disulfide bond between the protein of interest and other proteins can also lead to contamination [8, 21]. Thus, although IMAC is a versatile method that can result in 100-fold enrichments in a single purification step [22] and can achieve at up to 95% purity, the possibility of impurities was still present, especially if the protein of interest is eukaryotic in origin.

Morganti et al. (2002) reported that aromatic nitrogen-containing ligands (e.g., histidine and tryptophan) are considered borderline Lewis bases. Metal ions at the boundary of hard-soft acids such as Co, Zn, Cu, and Ni can coordinate with the aromatic nitrogen atom (the base border) and with the sulfur atom (the soft base) [9]. Therefore, Ni and Co can bind in coordination with other proteins from the media or host cells that also have histidine or tryptophan residues. However, Ni affinity is greater than Co. The Ni addition to the rhEPO binding protein can also bind to other proteins more.

This makes the total protein yield purification with Ni-NTA larger than that with Co-TALON.

Protein purification is very challenging, particularly when one considers the mixture of macromolecules present in a cell extract. In this study, the advantage of affinity chromatography, in particular the use of the His tag system, was explained. However, the presence of impurities clearly shows that a combination of several other chromatography techniques are needed for purification to be effective and for acceptable results.

Conclusions

Both IMAC resins can purify proteins sufficiently, although not completely. Therefore, additional purification studies are required. The Ni-NTA resin can capture more rhEPO protein than the Co-TALON resin. In this study, the average of the total protein was 183.5 μg/mL with Ni-NTA and 38.7 μg/mL with Co-TALON. In our experience, the protein resulted from this purification procedure is sufficient enough for in vitro analysis. With the combination of size exclusion chromatography we found that the purified protein was even suitable for biological analysis experiment.

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