Purification of recombinant human granulocyte colony-stimulating factor from Pichia pastoris using two ninta chromatography methods

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Abstract. Human granulocyte colony-stimulating factor (hG-CSF) is a glycoprotein that stimulates the production of mature neutrophil and enhances its survival, proliferation, differentiation, and neutrophil precursor function. This study was carried out to determine the purity of recombinant protein employing two purification methods using NiNTA with imidazole and with pH gradient (without imidazole). The synthetic gene (gcsf-cmyc) was cloned into secretive expression vector pPICZαA and methanol utilizing alcohol oxidase (AOX1) promoters before being expressed in Pichia pastoris SMD1168H strain. The recombinant protein was purified using NiNTA chromatography with imidazole and pH gradient. All samples were analyzed using SDS PAGE, followed with detection using coomasie blue. The molecular mass of recombinant hG-CSF expressed in P. pastoris was ~23kD. The efficiency of hG-CSF purification using NiNTA with imidazole was ~63%, while with pH gradient was ~89%. Purification techniques use pH gradients gradients can be applied to avoid used of imidazole, so that it does not contaminate protein samples.

1. Introduction
Granulocyte colony-stimulating factor (G-CSF), also known as colony-stimulating factor 3 (CSF 3) and pluripoietin, is a glycoprotein, growth factor and cytokine produced by a number of different tissues to stimulate bone marrow to produce granulocytes and stem cells. In the body, G-CSF commonly used to treat neutropenia, sources from monocytes, mesothelial cells, fibroblasts, and endothelial cells; while the receptors for G-CSF are on precursor and mature neutrophilic granulocytes, monocytes, platelets, and endothelial cells [1-3].

Recombinant human granulocyte colony stimulating factor (hG-CSF) was expressed in methylotrophic yeast P. pastoris under the control of AOX1 promoter after integration of GCSF gene into P. pastoris genome [4,5]. Bahrami et al. (2008) [4] used P. pastoris GS115 transformed with pPIC9 vector in which the synthetic gene for hG-CSF was inserted into the XhoI and EcoRI sites. This study used pPICZαA vector as the expression vector for recombinant G-CSF, while the host cell was P. pastoris SMD1168H.

P. pastoris is methylotrophic yeast that can use methanol as its sole carbon and energy source in the absence of repressing carbon source. Derived from the alcohol oxidase 1 (AOX1) gene of methanol utilization pathway, AOX1 promoter is known to be one of the strongest and tightly regulated eukaryotic promoters [6]. Calik et al. (2015) [7] stated that P. pastoris has become one of successful and popular host systems for heterologous protein production. P. pastoris can produce structurally and functionally correct recombinant proteins, especially when it is derived from eukaryotic sources. The yeast has ability to perform post-translational modifications, has the chaperones for proper protein folding and disulfide bond formation, and has proteases for essential proteolytic process. Several strong
inducible and constitutive promoters have been identified in *P. pastoris*, making host ideal for recombinant protein expressions. Proteolysis may lead to contamination of protein products through degradation intermediates. One of the most effective methods to reduce degradation is the use of protease-deficient strains, such as SMD1165 (his4, pep4, and prb1 deletions), SMD1163 (his4 and prb1 deletions) and SMD1168 (his4 and pep4 deletions) [8]. Gong et al. (2013) [9] analyzed the O-linked glycosylation of recombinant human granulocyte colony-stimulating factor (rhG-CSF) derived from glycoengineered *P. pastoris*. Recombinant protein used in this study was *P. pastoris* SMD 1168H to reduce the degradation of recombinant protein as host cell.

The development of immobilized metal chelate affinity chromatography (IMAC) together with recombinant histidine-tag (His-tag/ NiNTA) protein these last few years has increased the need for the use of heavy metals for binding and large quantities of imidazole for elution [10]. IMAC has been used for purification of recombinant proteins. It was based on the known affinity of transition metal ions such as Zn$^{2+}$, Cu$^{2+}$, Ni$^{2+}$, and Co$^{2+}$ to histidine and cysteine in aqueous solutions. Purification of recombinant proteins with 6× His-tag/ NiNTA is the most commonly used method for the affinity purification of recombinant proteins and antigenic peptides. It is based on the high-affinity binding of six consecutive histidine residues (the 6× His tag) to immobilize nickel ions, giving a highly selective interaction that allows purification of tagged proteins or protein complexes. The tight association between the tag and the Ni-NTA resin allows contaminants to be washed away easily under stringent conditions, yet the bound proteins can be eluted gently by competition with imidazole, or a slight reduction in pH [11]. In this study, we compared techniques to elute recombinant protein, i.e. with imidazole and with a slight reduction in pH.

In this study, *P. pastoris* SMD 1168H was integrated with gcsf gene. The gene was inserted using the c-myc tag and six His-tag (6×His) at C-term. In our further study, the next version of recombinant protein hG-CSF no longer contained protein tag (His-tag). In addition, protocol purification with pH gradient will be further developed for several reasons, such as to facilitate the stages of purification processes for purified recombinant hG-CSF to be eluted in accordance with the composition of buffer solution formula used in the final commercial formulation. This study developed to compare the development of protein purification techniques for hG-CSF recombinant on which one is more efficient in providing the purified protein resulted in two methods of purification, i.e. using NiNTA with imidazole and with pH gradient (without imidazole) modified from protocol Apte-Deshpande et al 2009 [5].

2. Materials and Methods
2.1. Microorganism and vector system
Yeast *P. pastoris* SMD 1168H (Invitrogen) was used as the host for hG-CSF expression. This strain was transformed with a commonly available plasmid, pPICZa inducible expression system vector (Invitrogen), in which the hG-CSF-cmyc gene was inserted into XbaI site. Host cells were transformed with the plasmid following electroporation procedure (Biorad). Transformed cells were spread on several YPDS agar plates containing 100µg/ml zeocin (Invitrogen). All engineering DNA work were prepared by the method of Ausubel et al. (2003) [12].

2.2. Media and shake flask cultivation
To make starter culture, a single colony of recombinant *P. pastoris* clone was inoculated into 2 ml YPD (1% yeast extract, 2% peptone, 2% dextrose) containing 100µg/ml zeocin medium, grown for 48 h at 30°C. The starter culture was then inoculated into 50 ml BMGY (1% yeast extract [Caisson labs], 2% peptone [Caisson labs], 100 mM potassium phosphate buffer {132 ml 1 M K$_2$HPO$_4$ [Merck] and 868 ml 1 M KH$_2$PO$_4$ [Merck]} pH 6.0; 1.34% YNB [Bio Basic Inc]; 4×10$^{-5}$% biotin [Sigma-Aldrich]; 1% glycerol [Bio Basic Inc]) medium, grown for 72 h at 30°C and harvested using centrifugation. The harvested culture was then re-suspended in 10ml BMGY (1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer pH 6.0, 1.34% YNB, 4×10$^{-5}$% biotin, 0.5% methanol [Merck]) induction
medium (OD<sub>600</sub> 5.0) before being inoculated into 150 ml BMMY medium. Secretion of rhGCSF was obtained by adding 0.5% (v/v) methanol in a fed-batch mode every 12 h for 72 h a total of 150 ml culture was harvested using centrifugation (10.000 rpm, 15 min, 4°C). Sampling for analyse the protein and the optical density (OD) of cell suspension was measured at 600 nm was conducted every 24 h. Cell biomass was stored at -20°C and the cell-free culture medium (supernatant) was stored at 4°C for further protein analysis.

2.3. Purification of Recombinant hG-CSF

For purification of rhGCSF, the culture cell-free supernatant used samples of 48 hour fractions were subjected to salting out using ammonium sulphate precipitation. Ammonium sulfate was added slowly to 115 ml recombinant hG-CSF protein with constant stirring at 4°C to make saturation degrees 80%. The precipitates were collected using centrifugation at 6500 ×g for 10 min, and dialyzed in 50 mM Tris-Cl (pH 7.4) buffer. The protein was subsequently purified.

2.3.1 Purification of proteins using imidazole. Ni-NTA agarose (His Pur<sup>TM</sup>Ni-NTA Resin, Thermo Scientific) was re-suspended in its bottle by inverting and gently tapping the bottle repeatedly. A total of 500 µl resin was pipetted into 0.8 ml Pierce Centrifuge Purification Column. The resin was allowed to settle completely by gravity (5 minutes) and 6 ml sterile, distilled water was added before the resin was resuspended by alternately inverting and gently tapping the column. The supernatant was gently aspirated using low-speed centrifugation for 20 seconds at 5000 rpm. A total of 250 µl dialyzed recombinant hG-CSF samples was added into a prepared purification column and bound for ±4 h at 4°C using gentle agitation to keep the resin suspended in the sample solution. The resin was settled using low-speed centrifugation for 20 seconds at 5000 rpm. Flow through (FT) supernatant was kept at 4°C for SDS-PAGE analysis (Biorad®) [13]. The resin was then washed with 3 × cv (250 µl) washing buffer (phosphate buffer + 30mM imidazole, pH 8.0) incubation for 5 minutes. The washed supernatant was then aspirated using low-speed centrifugation for 20 seconds at 5000 rpm. Washed (W) supernatant was kept at 4°C for SDS-PAGE analysis purpose. The column was clamped in vertical position and the cap was snapped off at the lower end. The protein was eluted with 1× cv (250 µl) Elution Buffer (phosphate buffer + 500 mM imidazole, pH 8.0) and incubated for 5 minutes. The eluted supernatant was aspirated using low-speed centrifugation for 20 seconds at 5000 rpm. Eluted (E) supernatant was kept at 4°C for SDS-PAGE analysis (Biorad®) purpose. Samples were eluted in 6× replications.

2.3.2 Purification of proteins using pH gradient. Purification using pH gradient also used NiNTA agarose (His Pur<sup>TM</sup>Ni-NTA Resin, Thermo Scientific) but with different buffer. In early stages of pH gradient purification, the preparation of resin was the same with that of using imidazole. For purification, 5x column volume equilibrate buffer (phosphate buffer + 500mM NaCl, pH 8) was added prior to incubation for 5 minutes. The supernatant was then gently aspirated using low-speed centrifugation for 20 seconds at 5000 rpm. A total of 250 µl dialyzed recombinant hG-CSF samples was added into a prepared purification column and bound for ±4 h in 4°C with gentle agitation to keep the resin suspended in the sample solution. The resin was settled using low-speed centrifugation for 20 seconds at 5000 rpm. Flow through (FT) supernatant was kept at 4°C for SDS-PAGE analysis purpose. The resin was then washed with 3 × cv (250 µl) washing buffer (20 mM Na-acetate, 5% sorbitol pH 6.3) prior to incubation for 5 minutes. Washed supernatant was aspirated using low-speed centrifugation for 20 seconds at 5000 rpm and the washed (W) supernatant was kept at 4°C for SDS-PAGE analysis purpose. The column was clamped in vertical position and the cap was snapped off at the lower end. The protein was eluted with 1× cv (250 µl) Elution Buffer (10 mM Na-acetate, 5% sorbitol, 0.004% tween 80, 500mM NaCl pH 3.0) prior to incubation for 5 minutes. The eluted supernatant was aspirated using low-speed
centrifugation for 20 seconds at 5000 rpm. Eluted (E) supernatant was kept at 4°C for SDS-PAGE analysis purpose. Samples were eluted in 6× replications.

3. Results and Discussion
3.1. Protein recombinant Production
A recombinant *P. pastoris* strain which can express protein G-CSF-cmyc was constructed in our previous study [14]. Recombinant hG-CSF-cmyc-21-09 appeared on SDS PAGE as a protein of ~23 kDa (Figure 1, which corresponds with a theoretical molecular weight of 23 kDa). Production hG-CSF in *P. pastoris* in shake flask to get a time course investigation was done in BMMY medium. As shown in Fig. 1 production recombinant protein in 48 h was higher than other sampling time, so that the culture cell-free supernatant used samples of 48 hour fractions were subjected to salting out using ammonium sulphate precipitation and purification. Meanwhile, the growth curve of the recombinant *P. pastoris* showed an optical density (OD) after 72 h still rise (Figure 2). The measurement done with two replication.

![Figure 1. Production recombinant protein hG-CSF by the recombinant *P. pastoris*.](image)

![Figure 2. A time course of growth hG-CSF by the recombinant *P. pastoris*.](image)

3.2. Purification of recombinant protein hG-CSF
Recombinant protein hG-CSF-cmyc clone 23 was purified using NiNTA resin with two different protocols. The first protocol is a common one, i.e. using imidazole solution as counter ion at elution stage, while the second protocol used pH gradient at stage of purification and elution of target proteins. Both protocols were developed to compare the development of protein purification techniques for hG-CSF recombinant which one is more efficient in providing the purified protein yield. Figure 3.A showed protein purification using imidazole at elution stage (Protocol 1); while Figure 3.B showed protein purification using pH gradient at elution stage (Protocol-2). IMAC is used to purify recombinant hG-CSF with histidine-tag (His-tag) in various studies and it uses imidazole for elution. Do et al. (2014) [3] separated hG-CSF from the PDb’a’-hG-CSF fusion protein using two rounds of IMAC. The fusion protein was expressed in *E. coli* and eluted in IMAC buffer containing 500 mM imidazole. According to Do et al. (2014) [3], IMAC was possible because all tags used in the study contained an additional His6 or His8 tag at their N-terminal end with purity of approximately 80%. Figure 3A showed SDS PAGE results of purification using imidazole in elution buffer. The recombinant protein G-CSF-cmyc occurred at 6 His-tag as marker and can be purified using IMAC containing 500 mM imidazole. In this study, the efficiency of purification using first protocol (with imidazole) was 63.50% (Table 1).
A. Electrophoregram purification of recombinant hG-CSF using imidazole solution. M: protein Marker; 1: The initial sample (C); 2: flow-through (FT) fraction; 3: washing fraction (W); 4-7: elution fractions (E1-E4). B. Electrophoregram purification of recombinant hG-CSF using pH gradient. M: protein Marker; 1: The initial sample (PH buffer 8.0); 2: flow-through (FT) fraction; 3-4: washing fraction (W1-W2) (pH buffer 6.3); 5-9: elution fractions (E1-E5) (pH buffer 3.0).

Table 1. The protein concentration and purification efficiency (yield) using imidazole and gradient pH

| Protein sample | Protein concentration (µg/ml)¹ | Total Protein before purification (µg)² | Total protein after purification (µg)³ | Purification efficiency (%)⁴ |
|----------------|-------------------------------|--------------------------------------|-------------------------------------|-------------------------------|
| Initial sample | 1830                          | 457.5                                | -                                   | 63.50                         |
| Elution sample (combined) | 1162                         | -                                     | 290.5                               |

Purification using pH gradient

| Initial sample | 984                            | 346                                  | -                                   | 89.84                         |
| Elution sample (combined) | 84                             | -                                     | 221                                 |

1: protein concentration in sample, calculated using the Quant-It colorimetric kit (Invitrogen).
2: total protein in initial sample = concentration × volume; (volume sample 250µl).
3: total protein elution is the sum of purified recombinant protein (volume sample 250µl).
4: purification efficiency indicates the percentage (yield) protein before and after purification.

The recombinant hG-CSF with c-myc and histidine-tag (His-tag) was expressed in P. pastoris. Figure 3B (and Table 1) showed SDS PAGE result of purification technique with second protocol (pH gradient), indicating purification efficiency of 89.84%. It appears that the purification technique using pH gradient indicated higher efficiency than that of using imidazole. The pH gradient technique, used wash buffer with pH lower than that of the binding buffer (pH 6.3), can be employed to remove non-specifically bound protein [15]. This indicates that technique with pH gradient is more efficient than using imidazole. This is to be taken into account in further purification activities in our laboratorium. Purification protocol with pH gradient will be further developed for several reasons, such as the next version of protein hG-CSF no longer contained protein tag (His-tag) and to facilitate the stages of purification processes for hG-CSF recombinant purified to be eluted in accordance with the composition.
of buffer solution formulation used in the final commercial formulation. Expression of hG-CSF as secretory protein in P. pastoris was also carried out by Saeedinia et al. (2008) [16] using GS115 strain. IMAC purification was carried out in phosphate buffer pH 8.0 with ionic strength of equivalent to 300–500 mM NaCl. HEPES buffer (and, to a lesser extent, Tris buffer) pH 7.5-8.0 may also be used. It has been consistently observed that conditions with high ionic strength (for example, 500 mMNaCl) maintain the solubility and stability of various proteins. A modest amount of imidazole should be included in the cell extraction buffer to reduce binding of less histidine-rich proteins to the IMAC column [17].

Recombinant proteins was expressed in fusion with an epitope containing six or more histidine residues, the His tag. The most common form of His tag consists of six consecutive histidine residues (H6). Due to the relatively high affinity and specificity of the His tag, a single IMAC purification step, in most cases, leads to purity of the target protein preparation that is sufficient for many applications. The structure of the tag, that is its position, sequence, and length, can influence production of a protein on several levels: expression rate, accessibility for binding to the IMAC ligand, protein three dimensional structure, protein crystal formation, and—although to a minor extent—solubility and activity [18]. In this study, one of the aims of tag removal was to develop hG-CSF biosimilar product which is identical to the originator. “Biosimilars” or “follow-on-protein products” by the European (European Medicines Agency) and the American regulatory agencies (Food and Drug Administration), respectively, are extremely similar one to another and to their reference molecule, but are not identical, no matter how close their similarities are biosimilar G-CSF (Biograstim/ Filgrastimtriptopharm/ Ratiograstim/ Tevagrastim (XM02); Zarzio and Nivestim) are manufactured in facilities with state-of-the-art technology [19]. Biosimilar may bring benefits such as improved cost-effectiveness associated with similar safety and efficacy profile compared to reference product [20].

4. Conclusions
Product recombinant protein used to purified is production in 48 h. Purification with imidazole showed lower purification efficiency (63.50%) than that of with pH gradient (89.84%). Purification method with pH gradient is expected to facilitate purification of recombinant protein without tag. This is to be taken into account in further purification activities.

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6. References
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