Purification and Stability Expression of Open Reading Frames encoding Fusion and Non Fusion Human Interferon Alpha-2a produced in Methilotropic Yeast Pichia pastoris

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Abstract. Recombinant human interferon alpha-2a (hIFNα-2a) is therapeutic protein that widely used in hepatitis B/C and several cancer treatments. We developed higher molecular weight of hIFNα-2a to improve protein pharmacokinetic profile. The protein was designed as a fusion protein with human serum albumin as protein tag. The protein was produced in Pichia pastoris with 85 kDa in size. This research was aimed to purify, characterize and determine the stability expression of open reading frame (ORF) encoding Fusion and Non fusion forms of hIFNα-2a. The proteins were purified using affinity chromatography and characterized using SDS PAGE and Western Blotting methods. Protein recovery yield was determined by ELISA. Stability expression was applied in generation time until 90th generation. The results showed that the Fusion and Non fusion proteins were successfully purified with 74-79% of protein recovery. The proteins can be recognized by specific monoclonal antibody and verified as hIFNα-2a Fusion and Non fusion with 85 kDa and 19 kDa in size respectively. The expression stability showed that the proteins were still produced in Pichia pastoris until 90th generation time with no significant difference of expression level. To conclude, the expression level of ORFs encoding Fusion and Non fusion hIFNα-2a was stable until 90th generations.

1. Introduction
Cancer and hepatitis infection are major public health problem worldwide. World Health Organization (WHO) stated that currently 350 million people worldwide are living with chronic hepatitis B and 150 million people are living with Hepatitis C. The mortality rate in the world due to hepatitis is about 1.5 million people per year [1,2]. According to Indonesian Ministry of Health, Indonesia is the second country in South East Asian Region (SEAR) with the highest hepatitis B case. A study in 2013 predicted that 10 from 100 persons were infected by hepatitis B or C. Currently, there are 28 millions of people that are hepatitis B or C positive, with 14 million are potential to be chronic status and 1.4 million potentially develop to liver cancer. The comparison of hepatitis incidence showed that there was a twofold increase of patients in 2013 comparing to 2007[1,3]. Hepatitis is a liver inflammation disease that mainly caused by hepatitis viruses. Currently, there are five different types of hepatitis based on the infecting virus; A, B, C, D and E. Hepatitis B and C are chronic diseases that potentially develop into hepatocarcinoma and cirrhosis on unappropriate treatments [4]. It was reported that approximately 12.7 million cancer cases occur worldwide with 7.6 million of mortality rate. About 64% of death arise in developing countries. Breast cancer in women and lung cancer in men are the biggest cause of death, followed by stomach, liver, cervix, and prostate cancers [2]. The incidence of cancer in Indonesia is 136 cases per 100,000 population [5].

The human interferon alpha2a (hIFNα2a) is a therapeutic protein that widely used as therapeutic protein for hepatitis B and C and several types of cancer. The use of hIFNα2a as protein therapeutic has been approved by the United States Food and Drug Administration (US FDA) since 1986 [6,7]. It is used in a single therapy or combination therapy with nucleos(t)ide analog, such as lamivudine, telbivudine, entecavir, adefovir or tenofovir for hepatitis B andribavirin, sofosbuvir, simeprevir, daclatasvir, sofosbuvir/ledipasvir, paritaprevir/ombitasvir/ritonavir or dasabuvir for hepatitis C [4,8].
anticancer hIFNα2a is used as therapeutic in combination with cytarabin, vinblastine, 5-fluorouracil, tamoxifen, or interleukin-2[9].

Despite its wide activity as antihepatitis and anticancer, the use of hIFNα2a protein still has major limitation. As low molecular weight protein, hIFNα2a has short elimination half-life due to rapid renal clearance [10,11]. In our previous research, we developed native and higher molecular weight of recombinant hIFNα2a in Pichia pastoris. Higher molecular weight protein was modified by human serum albumin fusion as fusion tag [12,13]. This research was aimed to purify the fusion and nonfusion forms of hIFNα2aand determine the stability of open reading frame (ORF) encoding hIFNα2a in 90th generations.

2. Materials and Methods

2.1. Strains and Media

Recombinant Pichia pastoris that harboring fusion and nonfusion forms of hIFNα2a were obtained from previous research [12,13]. The host strain for nonfusion was GS115 and for fusion one was SMD1168. Cultivation media was YPD (1% yeast extract, 2% peptone, 2% dextrose), screening media was YPDS (1% yeast extract, 2% peptone, 2% dextrose, 1M sorbitol) containing zeocin from 500 to 2000 μg, and expression media were BMGY (1% yeast extract, 2% peptone, 100mM potassium phosphate pH 6, 1.34% YNB, 1% glycerol, 0.2% Biotin), and BMMY that consist of 1% yeast extract, 2% peptone, 1.34% YNB, 0.2%Biotin and 0.5% methanol, respectively. The protease inhibitor that added during the expression was a compleuletra-tablet (Roche, Germany).

2.2. Protein Characterization

The clones were inoculated in YPD medium containing 100 μg/ml of zeocin at 30°C for 24-48 h. Single colony was cultivated in 3 ml BMGY at 30°C and 250 rpm for 24 h. The pellet was collected, resuspended in 3 ml BMMY (OD=1) and cultivated at the same condition. Methanol (0.5%) was added at 24 h of cultivation time. The supernatant was collected by centrifugation at 1500 x g for 5 min. The protein was characterized by SDS-PAGE and Western Blotting. SDS-PAGE was performed by using 10% (w/v) polyacrylamide gel and coomassie blue staining solution (BioRad). 1: 1000 dilution of mouse anti IFNα-2 (Merck 407290-500 UGCN, Germany) or 1:1000 dilution of mouse anti HSA (Sigma) and 1: 7500 dilution of anti mouse IgG alkaline phosphatase conjugate (Promega, USA) with NBT/BCIP detection (Merck, Germany) were applied in Western Blotting method.

2.3. Protein Purification and Quantification

Overproduction was applied by using shake flask method with 50 mL of BMMY media. A single colony was grown overnight in 25 ml BMGY medium at 30°C and 250 rpm until log phase (OD600=2-6). The culture was centrifuged at 1500 x g for 5 min. The pellet was resuspended in 50 ml BMGY medium (OD600 = 1.0 containing 0.5% methanol as inducer). Harvesting was performed after 48 h inoculation time by centrifugation at 1500 x g for 5 minutes. Supernatant containing fusion protein was collected and concentrated 10X (v/v) by using tangential filtration system with 10 kDa molecular weight cut off. The concentrated protein was purified by using affinity chromatography method by blue sepharose 6 fast flow (Capto blue, GE healthcare, Germany). Sample pH was adjusted to neutral pH, filtered through 0.22 μm filter and loaded into the column. The column was washed with starting buffer (50 mM sodium phosphate buffer pH7) to remove weakly bound impurities. The fusion protein was eluted from the column by using 50 mM sodium phosphate and 1.5 M KCl pH 7. Purified protein was characterized based on its molecular weight and identity by SDS PAGE and Western Blotting Methods. Protein was quantified using enzyme linked immunosorbent assay (ELISA) for human interferon alpha-2 (Verikine, USA).
2.4. Stability Assay
The generation and doubling time were determined using growth curve. 1 mL of representative samples was taken every 30th generation from 50 mL of BMMY media. The optical density was measured by spectrophotometer at 600 nm. Protein stability test was carried out in three dependent experiments. The protein expression was monitored using Western Blotting. The generation time and doubling time was determined using the formula:

**Generation time:**

\[ G = \frac{\log\left(\frac{N_t}{N_0}\right)}{0.3} \]

**Doubling Time:**

\[ dt = \frac{t}{G} \]

N0 = # of cells or OD600 at start, Nt = # of cells or OD600 at the end, t = time cultured, G = number of generations and dt=doubling time.

3. Results and Discussions
The *Pichia pastoris* SMD1168 was used as a host for fusion protein. The SMD 1168 is a protease deficient strain that able to decrease proteolysis. Proteolysis is major problem of protein production in *Pichia pastoris* because it reduces protein yield, deprives biological activity and contaminates the product with degradation fragments that have similar physicochemical and affinity characteristic [14].

In our previous research, the use of SMD1168 to produce fusion protein was still not completely deprived the proteolysis problem which indicated that the knockout of pep4 encodes proteinase A did not completely eliminated the proteolysis [13]. pep4 encodes a major vacuolar aspartyl protease which is able to activate itself as well as other proteases such as carboxypeptidase Y (PRC1) and proteinase

![Figure 1](image-url)

**Figure 1.** Characterization of hIFNα-2a nonfusion protein by (A) Western Blot, 1=positive control, 2 and 3= samples (B). SDS PAGE, 1 and 2 = samples
B (pRb1). Several studies reported the role of yapsin 1 protease in degradation of recombinant protein produced in host. Yapsin is a glycosylphosphatidylinositol (GPI)-linked aspartyl proteases family that has ability to cleave protein at the C-terminal side of basic amino acids [15]. To overcome the proteolysis problem, we used protease inhibitor to reduce the protease activity in fusion protein production.

![Figure 2](image_url)

**Figure 2.** Characterization of hIFNα-2a fusion protein by (A) Western Blot using anti hIFNα-2, (B) Western Blot using anti HSA, (C) SDS PAGE

Protein was characterized using SDS PAGE and Western Blot methods. The results showed that the non fusion protein was about 19 kDa in size as its theoretical size. The protein also identified as hIFNα-2a by Western Blot analysis using specific monoclonal antibody anti hIFNα-2 (Figure 1). The HSA-hIFNα-2a fusion protein had 85 kDa in size with positive recognition of anti HSA monoclonal antibody and anti hIFNα-2 (Figure 2). The result of SDS PAGE analysis confirmed that both proteins were identity verified. There was molecular weight improvement from 19 kDa of native to 85 kDa of fusion protein. Human serum albumin (HSA) as a protein tag was chosen because it has a high molecular weight with 9 day half-life, widely distributed in the body and not immunogenic. Another advantage is that HSA can increase protein solubility and stability [16].

![Figure 3](image_url)

**Figure 3.** Purified protein, (A) Fusion; (B) Non fusion

Protein purification by affinity chromatography column showed that the nonfusion and fusion proteins had been purified as shown in Fig. 3. The highly cross linked agarose matrix containing cibacron blue ligand was used in purification. The ligand has affinity to human serum albumin as well as hIFNα-2a. The protein recovery from purification process was 74-79% (Table 1). The protein loss may cause by physical treatment using tangential flow filtration and protein binding process in
chromatography. In this research one step purification approach was used as purification strategy. HSA was chosen because it has binding interaction with cibacron ligand as well. Comparing to multistep purification that consists of capture, intermediate and polishing processes, the one step purification is more efficient [17].

Table 1. Protein Quantification using ELISA

| Samples                | Crude  | Yield (Purified Protein) | Recovery |
|------------------------|--------|-------------------------|----------|
| Fusion (HSA-hIFNα-2a)  | 19 mg/L| 14 mg/L                 | 74%      |
| Nonfusion (hIFNα-2a)   | 55 mg/L| 44 mg/L                 | 79%      |

The choice of culture conditions used for P. pastoris expression system is an important factor to improve the productivity of the recombinant protein production. Optimal conditions for expression is vary according to the kind of strain used and the foreign protein expressed. Temperature of induction, incubation time and methanol concentration are the most common crucial non-nutritional factors that can influence protein production in P. pastoris expression system. There are many other different factors that can influence protein expression in P. pastoris, such as pH, cell density and medium composition [14]. In our previous research, the optimal overproduction condition was obtained. In this research we observed the cell density and protein expression in optimal production process to determine the generation and doubling time of host cell. The information can be used in large scale protein production. BMGY media was used to cultivate the host cells and the BMMY media used to produce the fusion and non fusion protein.

Figure 4 showed that the log phase was 24 hours and stationary phase was after 48 hours. Using generation and doubling time formula, it was obtained that the SMD1168 harboring fusion protein had 40 minute generation time (GT) with 7 hour doubling time (DT). The GS115 harboring non fusion protein had 43 minute GT with 8 hour DT (Table 2).

The doubling time of log phase was in line with another study in methanol media which stated that the doubling time was 7 hour [18]. The expression stability of Pichia pastoris was determined by DNA integration process. Pichia pastoris is capable of integrating multiple copies of transforming DNA via recombination into the genome at sites of sequence homology. Although the exact
Table 2. Generation and Doubling time of host

| Samples                        | Generation time (GT) | Doubling time (DT) |
|--------------------------------|----------------------|-------------------|
| SMD1168 harboring fusion protein (HSA-hIFNα2a) | 1.5 generation/h     | 7 h               |
| GS115 harboring non fusion protein (hIFNα-2a)   | 1.4 generation/h     | 8 h               |

mechanism of multiple integration events is not fully understood, such events are reasonably common among the transformants. Successful expression of the gene of interest to useful levels may depend upon the generation of a recombinant strain that contains multiple copies integrated at the AOX1 locus [18]. In this study, stability assay was performed by expression analysis for every 30th generation. The result showed that there was no significant difference of protein profiles among the generations (Figure 5). AUC determination using Image J software confirmed that no decreasing and the expression were stable until 90th generations. Further analysis may still needed to state the expression stability such as multicopy integration analysis by southern blot.

**Figure 5.** Stability assay of 30 generations of *Pichia pastoris* SMD1168 and GS115, (A). Fusion protein, (B). Nonfusion protein, (C) Image J analysis
4. Conclusions
So to conclude the HSA-hIFNα2a fusion protein and hIFNα2a protein had been overproduced, characterized and purified. The stability assays informed that the proteins had stable expression level until 90th generations.

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6. References
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