OPTIMIZATION OF EXPRESSION CONDITION, TWO DIMENSIONAL AND MELTING POINT-BASED CHARACTERIZATION OF RECOMBINANT HUMAN INTERFERON ALPHA-2A FUSION AND NON FUSION FORMS

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Abstract

Recombinant Human Interferon Alpha-2a (rhIFNα-2a) is a therapeutic protein that used in hepatitis and cancer treatments. In our previous research, we developed higher molecular weight of the protein through human serum albumin fusion. The fusion and non fusion form of rhIFNα-2a were produced in *Pichia pastoris*with 86 kDa and 19 kDa in size respectively. In previous research, protein yield was not reproducible due to unoptimized expression conditions. This reseach was aimed to optimize expression condition process and to characterize the fusion and non fusion forms of rhIFNα-2a. The parameters to observe in overproduction include nutrient (media and methanol concentration) and non nutrient (temperature andincubation period). Affinity and size exclusion cromatographicwere compared in protein purification. BCA assay was used to determine quantity of protein. Protein characterization was conducted using two-dimensional SDS PAGE and denaturation analyses. The optimal condition of expression was achieved using complex media with 1% of methanol for 3 day incubation period at 25°C. The protein yield was reproducible and higher comparing to previous research. Affinity chromatography resulted in higher purity of the proteins comparing to size exclusions. Characterization using two dimensional gel analysis revealed that isoelectric point of rhIFNα-2a is 6.5 for fusion form and 6.0 for non fusion form. The melting points of fusion protein were 56°C and 62°C whilst that of non fusion was 56°C.

Keywords : Recombinant Human Interferon • Alpha-2a • *Pichia pastoris* • 2D-SDS PAGE • melting point.

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Introduction

Interferon (IFN) is a cytokine that produced by eukaryotic cells that exposed to virus, bacteria, mitogen and antigen. The secreted interferon stimulates surrounding cells to produce other proteins, which regulate viral replication, immune response, cell growth and other cell functions.It is classified based on its receptor at cell surface, type 1 that consists of α, β, ω and τ and type 2 that consists of γ (Reddy *et al.,* 2002). HumanIFNα (hIFN-α)isreported to have wide biological activity including antiviral, antiproliferative and immunomodulatory as well. The IFNA2 gene is clustered with all other type I IFN genes. The genehas three allelic variants, namelyhIFN-α2a, hIFN-α2b and hIFN-α2c. hIFN-α2a is a glycoprotein which consists of 188 amino acids (23 residues are signal peptide and 166 residues are mature protein) and O- glycosylation at threonine position 106. The use of hIFN-α2a as therapeutic protein for hepatitis and cancer has been approved by the U.S. Food and Drug Administration since June 1986 (Samuel, 2001).

Approximately, 14.1 million cancer cases occur worldwide with 8.2 million of death and about 64% of the deatharises in developing countries (Jemal *et al.,* 2011; Ferlay *et al.,* 2015). Global population for hepatitic case is 257 millions for Hepatitis B and 71 millions for Hepatitis C with 1.3 millions of death (WHO, 2016). Therefore the needs of hIFNα2a as antihepatitis and cancer is indespensable. Despite of its wide advantage properties, the

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use of hIFN-α2a as therapeutic protein still remains the disadvantage. As a low molecular weight of protein, hIFN-α2a has short elimination half life due to rapid renal clearance. This property leads to higher therapy frequencyto maintain optimal drug concentration in the body. The unmodified hIFN-α2a has 5 hour elimination half life. For hepatitis, it needs three times of drug administration per week for 24-48 weekson the treatment. Therefore, to overcome the problem and to improve the protein elimination half life, several approaches have been reported. The molecular weight modification was applied using some technologies, such as pegylation (Reddy *et al.,* 2002), glycosylation (Ceaglio *et al.,* 2008) and albumin fusion (Ningrum *et al.,* 2017; Subramanian *et al.,* 2005).

Albumin fusion technology is a reasonableprotein modification due to its modification efficiency.The modification uses human serum albumin (HSA) which is fused with targeted protein at its N or C terminal with or without linker in construction of DNA sequence. Therefore, it is more efficient comparing to pegylation. HSA is chosen based on its advantages properties, such as high molecular weight, longelimination half life (9 days), non immunogenic and soluble (Zhao *et al.,* 2008; Zhao *et al.,* 2012;Subramanian *et al.,* 2005). Several proteins have already reported using HSA as fusion tag, such as insulin (Duttaroy *et al.,* 2005),p53 (Joshi *et al.,* 2013), recombinant factor VIII (Schulte *et al.,* 2008), recombinant factor IX (Nolte *et al.,*, 2008), Somatostatin (Peng *et al.,* 2012), VEGF165b (Zhu *et al.,* 2012) and interferon alpha-2b (Subramanian *et al.,* 2005).

In previous study, we generated86 kDa HSA-hIFNα-2afusion protein that wasproduced in methilotropic yeast *Pichia pastoris* (Ningrum *et al.,* 2017). We also developed the non fusion form with 19 kDa in size. The fusion and non fusion forms have been characterized using anti hIFNα-2 monoclonal antibody. Thetwo proteins yield was not reproducible (ranging from 4 to 44 mg/L) due to non optimized expression conditions.This reserach was aimed to optimize expression condition and tofurther characterize the fusion and non fusion forms of rhIFNα-2a. The characterization was based on two dimensional electrophoresisand melting point profiles. The optimal condition is important in protein production steps to obtain highest and reproducible yield so further characterization can be performed.

Materials and Methods

Strains and Media

The recombinant *Pichia pastoris* GS115-hIFN-α and protease deficient mutant SMD1168-HSA-hIFNα-2a obtained from our previous study (Ningrum *et al.,* 2017) were used to produce fusion and fusion proteins. 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 100μg, 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 consisted 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 complete ultra tablet (Roche, Germany).

Optimization of expression condition

Single colony was cultivated overnight in 3 ml BMGY medium at 30˚C and 250 rpm until log phase (OD_{600}= 2-6). The cell suspensionwas centrifuged at 1500 x g for 5 min. The pellet was resuspended in 3 ml BMMY medium (OD_{600}= 1.0 containing 0.5% methanol as inducer). The induction was repeatedat 24 h cultivation time. Harvesting was carried out by centrifugation at 1500 x g for 5 minutes at room temperature. Supernatant containing rhIFNα-2a was collected. For media optimization, BMGY-BMMY and BMGH-BMMH were used in cultivation and expression stages. Various incubation time (24, 48, 72 h), temperature (27°C and 30°C) and methanol concentration (0.5 to 2.5% v/v) were observed. SDS PAGE and western blotting were used to monitor the expression. Highest expression level was determined based on Area Under Curve value using ImageJ. Optimal parameters were applied in three dependent experiment to monitor protein yield.

Purification

Supernatant containing fusion protein was collected and concentrated 10 times (v/v) using

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tangential filtration system with 10 kDa molecularweight cut off. Concentrated protein was purified using affinity chromatography method by blue sepharose 6 fast flow (Capto blue, GE healthcare, Germany) or filtration gel (Superdex, GE healthcare, Germany). For affinity chromatography, 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 protein was eluted from column by using 50 mM sodium phosphate and 1.5 M KCl pH 7. For gelfiltration, sample was loaded into superdexcolumn and eluted with 0.5 M *phosphate buffer*containing 0.15 M NaCl.

Characterization

SDS-PAGE was performed using 12% (w/v) polyacrylamide gel and coomassie blue staining solution (BioRad, USA) or silverstaining (Merck, Germany). 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 Blot Analysis. Protein quantification using BCA kit assay (Merck, Germany) was determined with standard range from 25 to 1000 ug of BSA. 200 uL of BCA solution was mixed with 25 uL of samples and incubated in 37°C for 30 minutes. The absorbance was read at 570 nm. Iso Electric Focusing was performed in glass tube with 2.3 mm inner diameter using 2% pH 3-10 Isodalt Servalyte for 9600 V for 1 hour. 1 µg of tropomyosin was used as a standard. The pH gradient attached to the gel tube and the gel was equilibrated for 10 minutes. SDS-slab gel electrophoresis was performed for 4 hours with a strong current of 15 mA. Melting point determined using temperature shift assay. 2.5 µL sample (200 µg/µL was mixed with 5 µL of a universal pH7 buffer and 12.5 µL aquadest. 5 µL SyproR Orange (dilution 1: 100) was added into the mixture. Sample was run at 20-95 °C gradient temperature range using Real Time PCR (BioRad, USA)with increment 0.5 ° C per 5 seconds.

Results

Optimization of Expression Condition

Inoculation and expression media were observed using Western Blotting analysis. BMGY-BMMY and BMGH-BMMH media were compared with and without the use of antiprotease (Figure 1). The result showed that both proteins have higher expression level using BMGY-BMMY media. BMGY (Buffered Glyserol-complex Medium) and BMMY (Bufered Methanol-complex Medium) are rich media supplemented yeast extract and peptone that allow better growth and biomass accumulation. The use of antiprotease improved expression level of fusion and non fusion proteins. However, the use of antiprotease was not highly improved non fusion yield.

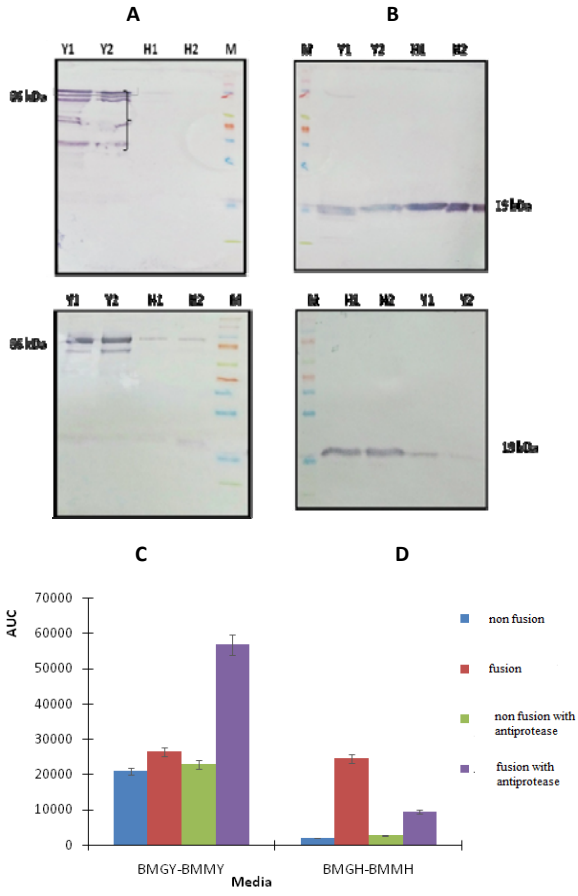

Figure 1. Optimization of media, Y= BMGY-BMMY; H= BMGH-BMMH. (A)= fusion protein without antiprotease; (B)= non fusion protein without antiprotease; (C) = fusion protein with antiprotease; (D)= non fusion protein without antiprotease ; (E) = semiquantitative analysis by Image

Optimization of incubation time revealed that highest expression was achieved on 72 hour of incubation (Figure 2, gel was not shown). The difference of expression level between 27°C and 30°C was not considerable (Figure 2). Optimization of methanol concentration showed that the best concentration was 1.5% (Figure 2).

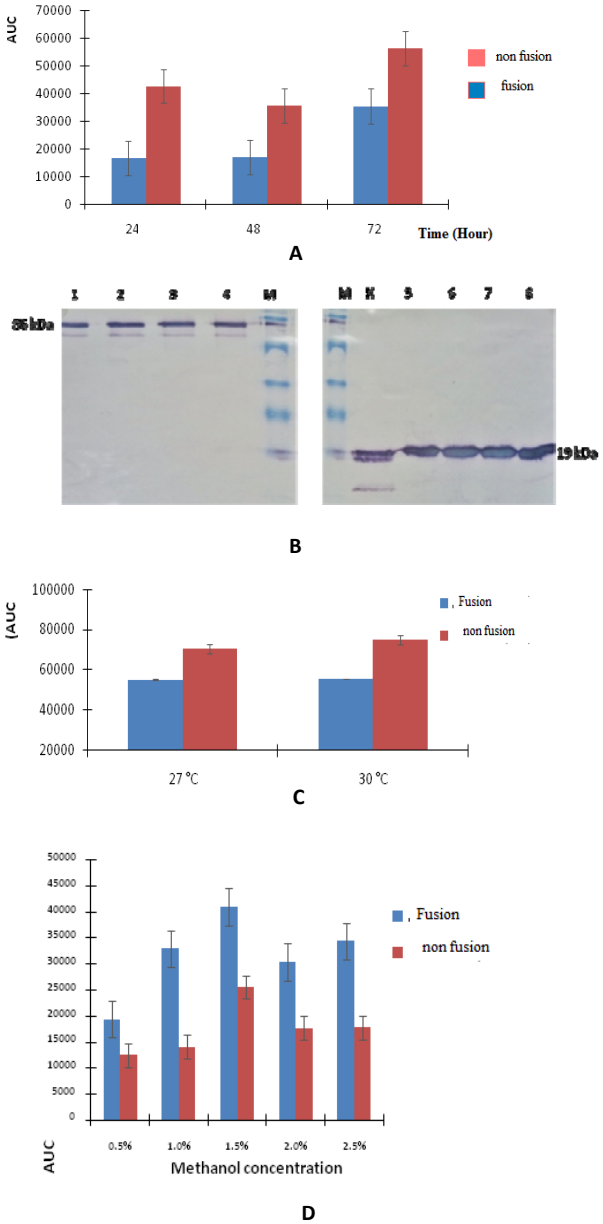

Figure 2. Optimization of (A).incubation time;(B). temperature and methanol of overproduction, 1-2 = fusion protein at 27ºC, 3-4 = fusion protein at 30ºC, M = marker, K= control, 5-6 = non fusion protein at 27ºC, 7-8 = non fusion protein at 30ºC; (C). methanol concentration

Purification

The affinity chromatography result showed that fusion and non fusion proteins can be captured by affinity resin with single band profile (Figure 3). To monitor the purity, single band purified proteins were also stained using silverstain (Figure 3). No additional band detected in SDS PAGE profile indicated that the proteins had high purity.

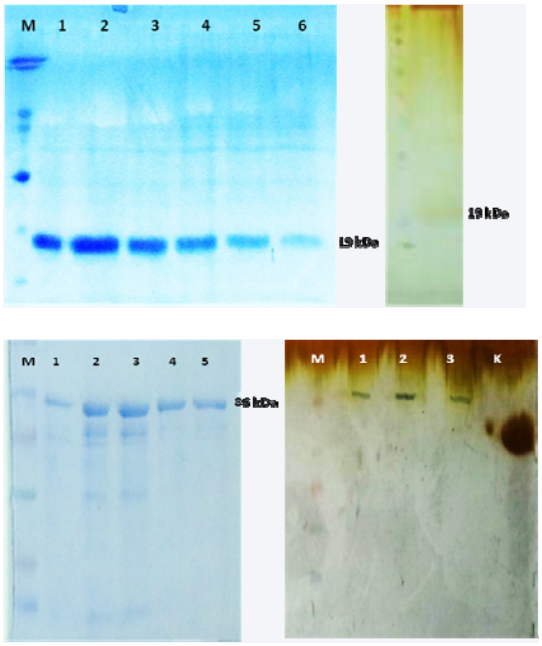

Figure 3. Purification using affinity chromatography; Non fusion and fusion proteins with coomassie blue and silver staining , M = marker, K = control, 1 to 6= eluat number

To compare the purity, size exclusion chromatography was used. The SDS PAGE profile informed that purified non fusion proteins still contained many impurities using comassie blue and silver staining as well (Figure 4). The matrix can exclude non fusion protein but based of the SDS band profile and imagej analysis (data not shown), the recovery still lower comparing to affinity chromatography. So Affinity chromatography was furtherly used to purify the proteins and BCA assay usedto determine protein yield. The yield of purified proteins were shown in Table 1.

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Table 1. Yield of purified non fusion and fusion proteins

No	Sample	Average Yield (mg/L)	
		Previous	Current
1	Non fusion	Not reproducible, 10-44 mg	48±1 mg
2	Fusion	Not reproducible, 4-16 mg	16±2 mg

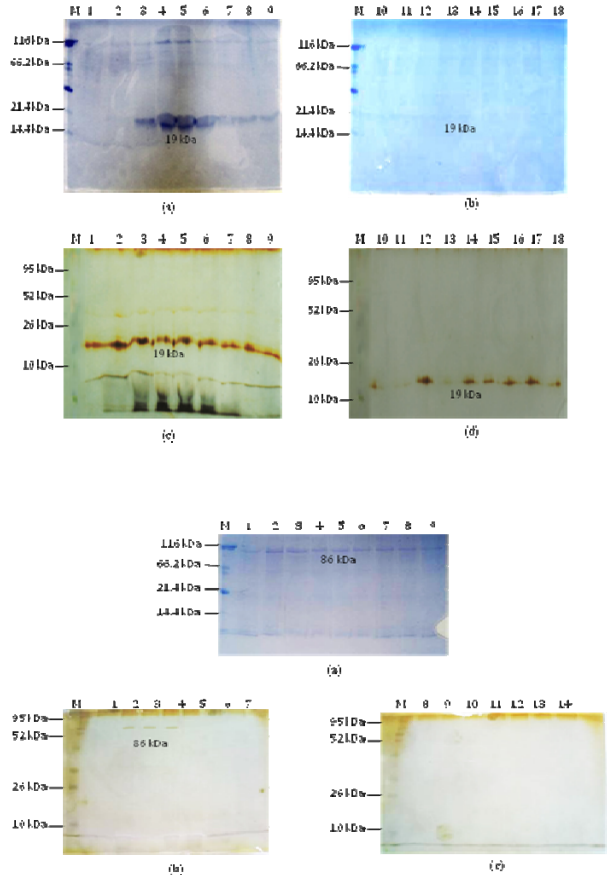

Figure 4. Purification using size exclusion chromatography. Fusion and non fusion proteins with coomassie blue staining and silver staining, M = marker, 1 to 18= eluat number.

Characterization

Two dimensional electrophoresis was used to characterize molecular weight and isoelectric point of the protein. The method also can be used to purify individual protein from complex samples and to monitor the purity of the protein as well. The first dimension is isoelectric focusing and the second is SDS PAGE. In this research, tropomyosin with 5.2 isoelectric point and 30 kDa in size was used as a standard. The electrophoregram profiles of non fusion protein showed some impurity spots ranging to 60-94 kDa. This indicated that the silver stained single band of SDS PAGE may still contain some undetected impurities. Theoretically non fusion protein as interferon alpha-2a has 5,99 of pI and 19 kDa in size (Drugbank, 2018). The profile showed a very slight spot of non fusion protein at 6.0 of pI (Figure 5). The electrophoregram profiles of fusion protein showed 4 spots of impurities with obvious spot of target protein. The fusion protein had 6.6 of pI with 86 kDa in size. Human serum albumin has 4.7 of pI and 65 kDa in size (Drugbank, 2018). The melting point curve showed that the non fusion form had 56°C of melting temperature. Fusion form showed two different melting points, 56°C and 62°C. (Figure 6).

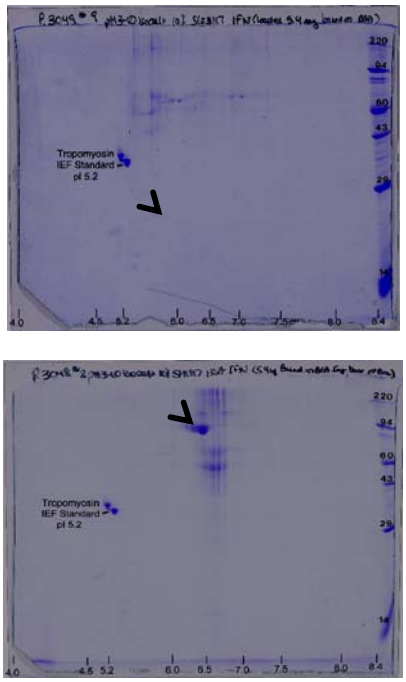

Figure 5. Analysis of two dimensional electrophoresis, A = non fusion protein; B=fusion protein. Tropomyosin was used as a standard. Non fusion protein had6.0 of pI with 19 kDa in size and fusion protein had 6.6 of pI with 86 kDa in size.

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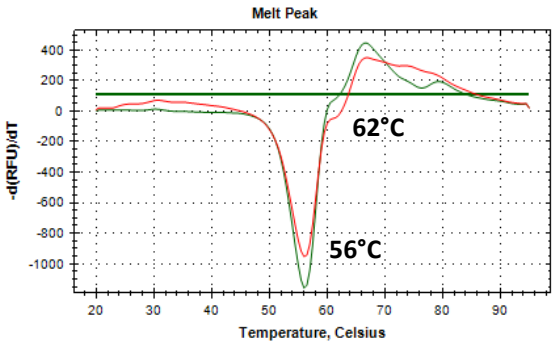

Figure 6. Analysis of melting point, greeen = non fusion protein; red=fusion protein. The non fusion form had 56°C of melting temperature and fusion form showed two different melting temperature, 56°C and 62°C.

Discussion

Several studies reported that the choice of culture conditions for *P. pastoris* expression system is an important factor to improve the level of targeted recombinant protein (Ayed *et al.,* 2008; Ghosalkar *et al.*,2008 ; Hong *et al.,* 2001). Various optimal conditions for expression are different based on the strain that was used and the property of protein that was expressed. Incubation time, temperature of induction and methanol concentration are the most crucial non-nutritional factors that influence protein production in *P.pastoris*. Other involving factors are pH, cell density and medium composition or additives (Files *et al.,* 2010; Sinha *et al.,* 2003). On this research, culture conditions were not only optimized for expression increment but also proteolysis control. Protease contaminates the product with degradation fragments that have similar physicochemical and affinity characteristic, reduces protein yield and deprives biological activity as well. There are several proteases that mostly produced in *P. pastoris*, namely proteinase A, proteinase B, carboxylpeptidase and aminopeptidase (Sinha *et al.,* 2003).

Controlling proteolysis on protein production in *P. Pastoris*can be performed by several strategies, such as cultivation-level (non nutritional and nutritional factors of culture condition), cell-level (protease deficient stain) and protein-level (linker engineering or aminoacid substitution). Intheprevious research, cell-level strategy was used using protease deficient mutant SMD1168 (Ningrum *et al.,* 2017), whilst in this research, cultivation level strategy that cover nutritional (media and antiprotease) and non nutritional factors (induction time, methanol concentration and temperature) were applied.

In previous research, it was revealed that the use of SMD1168 was still not completely deprived the proteolysis problem in fusion protein (Ningrum *et al.,* 2017). The knock out of *pep4* that encodes proteinase A did not completely eliminated the proteolysis. *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 B (pRb1). A study reported the role of yapsin 1 protease as glycosylphosphatidylinositol (GPI)-linked aspartyl proteases family that has ability to cleave protein at the C-terminal side of basic amino acids (Bourbonnais *et al.,* 2000). Therefore in this study, antiprotease was used in the protein production.

Some studies have reported that lowering temperature could improve recombinant protein production, decrease cell lysis, reduce the release of intracellular protease to culture medium and decrease the proteolytic activity (Hong *et al.,* 2001; Jahic *et al.,* 2001). In this study, 27ºC was used to monitor the lowering effect. Methanol is an inducer that regulates promoter *AOX1* to supply energy and carbon source on fermentation process. Optimization of methanol concentration showed that the best concentration was 1.5% . This research was in line with previous publication that reported *P.pastoris* still tolerate methanol concentration from 0.3 to 3% (Khatri *et al.,* 2005).

Protein purification was performed using two different chromatography methods, i.e. affinity and size exclusions. Capto blue resin was applied in affinity chromatography. It contains highly rigid agarose base matrix with cibacron blue ligand which able to capture human serum albumin, blood coagulation factors, enzymes and recombinant protein such as interferon (GE healthcare, 2018). To compare the methods, size exclusion chromatography was applied. Superdex 75 resin has 3-70 kDa protein pore size and able to exclude high molecular weight of protein, such as the fusion protein with 86 kDa in size. Superdex75 is composed by matrix of crosslinked dextran and highly cross linked agarose.

To characterize the protein, two dimensional electrophoresis and thermal shift assat were used. Thermal shift assay was

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applied to determine the protein melting point property. The assay quantifies the change in thermal denaturation temperature of a protein under varying conditions, such as pH, salt concentration, mutation, additives and so on. This research used thermofluor based method with SYPRO orange that able to bind nonspecifically to hydrophobic surfaces. When the protein unfolds by temperature increment, the exposed hydrophobic surfaces bind the dye, resulting in an increase in fluorescence by excluding water. The stability curve and its midpoint are obtained by gradually increasing the temperature to unfold the protein and measuring the fluorescence at each poin. So far there is no experimental melting point property of interferon alfa-2a that reported. The most similar protein is interferon alfa-2b with 61°C of experimental melting temperature. Fusion form showed two different melting points, 56°C and 62°C. This result was in line with previous report that stated h*uman serum albumin* have two *melting point* temperatures, 56°C and 62°C (Das, 2014).

As the conclusions, the optimal condition of overproduction and purification were obtained and result in more reproducible protein yield comparing to previous study. Protein characterization using two dimensional gel analysis and thermal shift assayconfirmed the hIFNα-2a properties.

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