Expression of No Affinity Tagged Recombinant Human Interferon Alpha-2a in Methilotropic Yeast *Pichia pastoris*

Neng Herawati, Andri Wardiana, and Ratih Asmana Ningrum*

Research Center for Biotechnology, Indonesian Institute of Sciences (LIPI), Indonesia

Abstract

Recombinant human interferon alpha-2a (rhIFNα-2a) has been widely used for clinical therapy as antiviral, anticancer as well as immunomodulator. In this study, the open reading frame (ORF) encoding synthetic hIFNα-2a was constructed to be in framed with N-terminal alpha factor secretion system in methylotropic yeast *Pichia pastoris.* This research aimed to construct, express and analyse the non-affinity tagged recombinant human interferon alpha-2a in the methilotropic yeast *P. pastoris*. We used pPICZαB plasmid for cloning and expression vector. The confirmed recombinant plasmid containing the correct DNA sequence of hIFNα-2a was linearized by *SacI* restriction enzyme, then transformed into *P. pastoris* genome using electroporation. We screened two multi-copy recombinants in YPDS plates containing Zeocin™. Buffered complex medium containing 0.5 % methanol (BMMY) was used for protein expression for 48 hours in the culture condition. The recombinant protein was purified by blue sepharose affinity chromatography. Analyses of hIFNα-2a protein by SDS-PAGE and Western blot confirmed that protein band in which was observed around 19.2 kDa, was recombinant hIFNα-2a. The quantification of purified rhIFNα-2a using colorimetric binichoninic assay (BCA) informed that the yield was 44 mg/L culture (OD_{600}= 2-3).

Keywords: recombinant hIFNα-2a, synthetic gene, protein expression, *Pichia pastoris*

---

*Corresponding author:
Cibinong Science Center, Jl. Raya Bogor Km. 46, Cibinong 16911, Indonesia
Tel. +62-21-8754587, Fax. +62-21-8754588
E-mail. ratih.asmana@gmail.com, rati004@lipi.go.id

Introduction

Interferon α (IFN-α) family is the most dominant class of interferon produced by stimulated blood leukocytes. Human interferon alpha (hIFN-α) consists of 188 amino acids (23 residues are signal peptide and 165 residues are mature protein) that has been widely applied as therapeutic proteins because it has antiviral, antiproliferative and immunomodulator activities. Two recombinants of hIFN-α (2a and 2b) have been recognized by the U.S food drug administration (FDA) on June 1986 as therapeutic proteins for hepatitis and cancer treatments. Human interferon alpha 2a (hIFN-α2a) is widely used in clinical treatment to overcome several types of tumor and cancers such as melanoma, AIDS-related Kaposi's Sarcoma and Chronic Myelogenous Leukemia (CML) (Ferenci, 1993; Bekisz *et al*., 2010).

Several different expression systems have been used for interferon production. The first approved products on the market were Roferon A (IFN-α2a; Hoffmann-La Roche) and Intron A (IFN-α2b; Schering-Plough), both of them were produced in *Escherichia coli* (Meager, 2006). However, the overexpression of human alpha interferon proteins in *E. coli* often forms inactive protein or inclusion bodies (Babu *et al*., 2000). The inclusion bodies are usually misfolded as well (Villaverde & Carrio, 2003). The refolding process of inclusion bodies to obtain correct folding of protein can be laborious and also time consuming (Ningrum *et al*., 2012), consequently, overexpression in certain expression host to obtain soluble form is required. The hIFN-α2a products that are produced in methylotrophic yeast *P. pastoris* neither widely used, nor commercially available. Fast growth rate, high protein yield, low production cost, correct protein folding and most importantly, the formation of disulfide bonds and glycosylation are some advantages of producing proteins in the

methilotrophic yeast *P. pastoris* expression system (El-Baky & Redwan, 2015). The purification process of targeted protein becomes easier because *P. pastoris* slightly secretes its protein out of the secretory pathway.

Extra sites for tagging (*e. g*. histidine tag) on gene expression vector construction, will encode proteins biosimilar, aims to facilitate the purification stage of the protein expressed by the host cell selected. The existence of this site makes the protein production has no resemblance to the originator. It is reported that affinity tags in recombinant protein may have immunogenic implication and alter characteristics or functions (Terpe, 2003). Sometimes, peptide tags are less likely to interfere when fused to the protein. However, in some cases, they may stimulate negative effects on the tertiary structure or biological activity of the fused protein (Bucher *et al*., 2002; Klose *et al*., 2004; Khan *et al*., 2012). Therefore, in this study we expressed the interferon alpha- 2a protein with no affinity tag in yeast *P. pastoris,* which is expected to have close resemblance with its originator product.

Materials and Methods

Construction and Cloning of rhIFN-α2a ORF in *E. coli*. We used a codon optimized synthetic gene in order to obtain higher expression level. DNA encoding hIFNα-2a (from gene bank), which was constructed in the PUCIDT-AMP plasmid, was digested by *Xho*I and *Xba*I restriction enzymes then ligated into pPICZαB expression vector. The ligation mixture was transformed into XL1 blue *E. coli* competent cells by heat shock at 42 ^{o}C for 2 minutes then incubated at 37 ^{o}C with shaking in LB liquid medium for 1 hour. After incubation, the transformed cells were plated into LB agar medium containing ampicillin. The clone selection was performed after overnight incubation at 37 ^{o}C. Recombinant plasmids from the clones were characterized and their sequences were confirmed by sequencing analysis. Recombinant plasmid that had correct size and sequence was transformed into GS115 yeast strain for protein expression.

Transformation, Screening, and Expression of rhIFN-α2a in *P. pastoris*. For yeast transformation, recombinant plasmid pPICZαB-IFNα2a was linearized by *Sac*I restriction enzyme. Preparation of competent *P. pastoris* cells strain GS115 was performed by resuspending the cells pelet in 1 mL of ice-cold 1 M sorbitol. As much as 10 µg of linearized plasmid were transformed into 80 µL of yeast competent cells using electroporation method as given in the protocol (Invitrogen). The transformation product was spreaded into YPDS plates (1 % yeast extract, 2 % peptone, 2 % dextrose, 1 M sorbitol, and 2 % agar) containing 100 µg/mL of Zeocin™ then incubated at 30 ^{o}C for several days until colonies were formed. All single colonies were screened on YPDS plate containing 500, 1000 and 2000 µg/ml Zeocin™ and incubated at 30 ^{o}C for 2-7 days. Survived transformants were used for expression study. Single colony of each transformant was inoculated into 2 mL BMGY liquid medium (1 % yeast extract, 2 % peptone, 100 mM potassium phosphate pH 6, 1.34 % yeast nitrogen base with ammonium sulfate, 4×10^{-5} % biotin, 1 % glycerol) then incubated at 30 ^{o}C with shaking at 250 rpm until the culture reaches an OD_{600} = 2-6. The cells were harvested and resuspended in 2 mL of BMMY liquid medium (1 % yeast extract, 2 % peptone, 100 mM potassium phosphate pH 6, 1.34 % yeast nitrogen base with ammonium sulfate, 4×10^{-5} % biotin, 0.5 % methanol absolute) (final OD_{600} = 1) at 30 ^{o}C with shaking (250 rpm) for 48 hours. Methanol 0.5 % was added every 24 hours for maintaining the induction. The culture was harvested with centrifugation at 1,500 g for 5 minutes. Supernatant was collected and stored at certain temperature for further analyses.

Overproduction, Purification and Quantification of rhIFN-α2a. The GS115 yeast cells carrying pPICZαB-hIFNα-2a were grown in 25 mL BMGY medium. The culture was incubated overnight at 28-$^{30\ oC}$ with saking at 250 rpm and harvested at an OD_{600} = 2-6 by centrifugation at 1,500 g for 5 minutes. The cells pellet was resuspended in 50 mL BMMY medium (final OD_{600} = 1). The addition of 0.5 % methanol was done for every 24 hours during 48 hours of induction. Harvesting process was performed by centrifugation at maximum speed for 5 minutes at room

temperature to collect the supernatant (Invitrogen, 2010).

The supernatant was concentrated into 5 mL using 10 kDa of Minimate™ TFF system (PALL, USA). The concentrated fraction was applied into prewashed Blue sepharose resin (GE Healthcare) using wash buffer containing 1×PBS pH 7.0. After 3 times of sample loading, the resin was washed 5 times using wash buffer, then eluted with 5 mL elution buffer containing 1×PBS pH 7.0 and 1.5 M NaCl. The purified protein was filtered by Amicon® (Millipore) with 3 kDa cut off, then characterized by SDS-PAGE and Western blot. Quantification of purified proteins were determined by bicinchoninic acid assay protein kit (Pierce, USA) with various concentration of bovine serum albumin (25 to 2,000 µg/mL) as a standard. The absorption of the purified proteins was measured at 562 nm (Ningrum *et al*., 2015).

Characterization of rhIFN-α2a. Polyacrylamide gel (Bio-Rad, USA) containing separating gel (15 %) and stacking gel (4 %) was used in SDS-PAGE analysis. After electrophoresis, the protein bands were visualized according to standard procedures by staining with coomassie brilliant blue G250 (Bio-Rad, USA). For western blotting, the gels were transferred into nitrocellulose membrane by electroblotting. Immuno-detection was achieved using anti-human IFNα monoclonal antibody (Merck 407290-500 UGCN, Germany) as primary antibody followed by the anti-mouse IgG alkaline phosphatase conjugate (Promega, USA) as secondary antibody. The membrane was stained with NBT/BCIP solution (Invitrogen, USA).

Results

Construction and Cloning of rhIFN-α2a ORF in *E. coli*.

In this work, the gene was constructed without any fusion of protein tags attached to protein of interest. The cDNA fragment encoding human IFNα-2a gene was ligated between the *Xho*1 and *Xba*1 sites of shuttle vector pPICZαB as shown in Figure 1.

Transformation, Screening and Expression of rhIFN-α2a in *P. pastoris*.

Positive clones were determined by antibiotic selection. The transformation resulted in 11 positive clones with 3 clones were furtherly characterized. Restriction enzyme analysis (double digestion with *Xho*I & *Xba*I) confirmed that the recombinant plasmids contained two fragments which were about 534 bp (*hifnα-2a*) and 3,600 bp (pPICZαB) (Figure 2 lane 4, 6 and 8). The DNA sequencing was conducted to confirm the correct sequence of ORF in recombinant plasmids (data not shown). The results show that the *hifnα-2a* gene was inserted correctly into the *P. pastoris* expression vector pPICZαB.

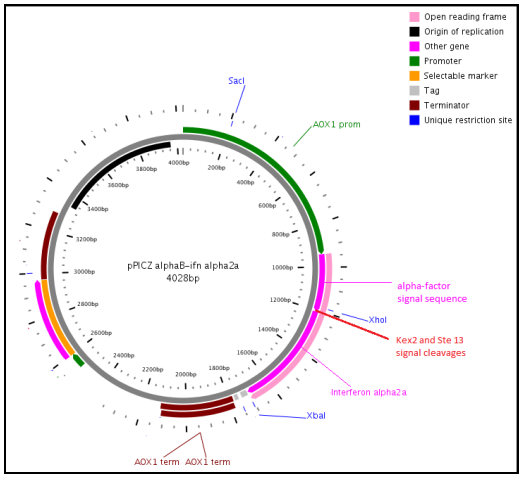

Figure 1. Schematic Mapping of the recombinant expression vector pPICZαB/hifnα-2a. The *hifnα-2a* gene was subcloned into pPICZαB using *Xho*1 and *Xba*1 sites.

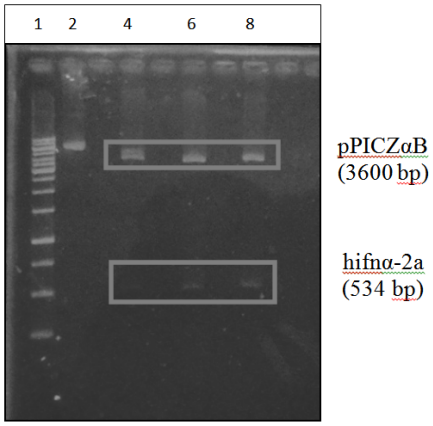

Figure 2. The recombinant plasmid pPICZαB containing hifnα-2a gene, lane 1: 1 kb DNA Ladder; lane 2: undigested recombinant pPICZαB; lane 4, 6, 8: digested recombinant pPICZαB by *Xho*1 and *Xba*1.

We used GS115 (*his4*) expression host to be grown with the presence of methanol for the wild-type rate (methanol utilization plus /Mut$^+$) phenotype. Therefore, previous recombinant plasmid was linearized by *Sac*1 restriction enzyme before transformation into GS115 cells (Figure 3).

Characterization and Purification of hIFNα-2a.

Characterization of protein was performed based on its molecular weight and identity. Molecular weight analysis shows a correct protein size as expected (19 kDa) and was recognized by anti-interferon antibodies in immunodetection analysis (Figure 4). This result strongly indicates that the ORF encoding interferon protein had been successfully expressed in *P. pastoris* strain GS115. Because clone number 1 had higher expression level than clone number 2, thus it was chosen for overproduction and purification of hIFNα-2a protein

Protein purification was carried out based on affinity chromatography using blue sepharose. The results showed that the purified rhIFNα-2a produced in the *P. pastoris* yeast was represented as single band on SDS PAGE and gave positive result on immunodetection analysis (Figure 5). The protein quantification informed that the yield of our purified rhIFNα-2a was 44 mg/L (at OD_{600} = 2.4). This result is higher than bacteria expression system (16 mg/L) conducted by Bis *et al*. (2014), however lower than protein expression study done by Kim *et al*. (2002).

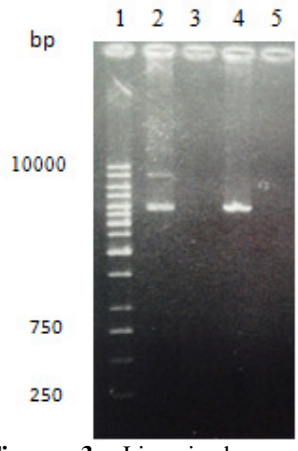

Figure 3. Linearized recombinant pPICZαB-hIFNα2a (lane 4); Lane 2: undigested; lane 1: DNA Ladder 1 kb

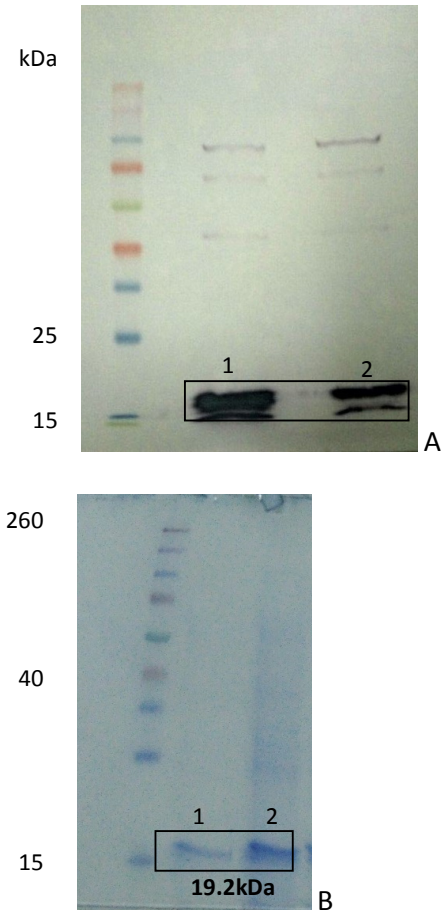

Figure 4. Characterization of rhIFNα2a, 1 = clone number 1 and 2 = clone number 2, A. Protein identification analysis by immunodetection on Western Blot, B. Molecular weight characterization by SDS PAGE

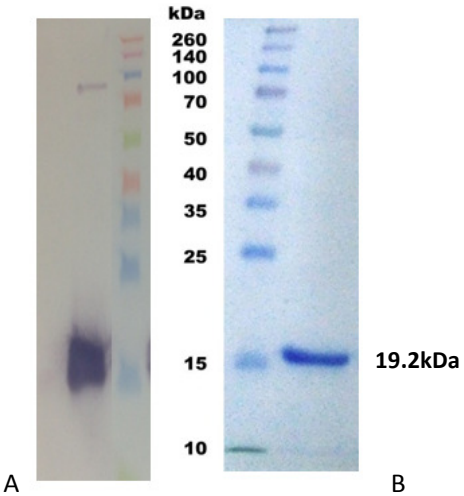

Figure 5. Purified rhIFNα-2a protein characterization using (A) Western blot and (B) SDS PAGE

Discussion

Construction and Cloning of rhIFN-α2a ORF in *E. coli*.

The expression vector contains tigthly regulated *AOX1* promoter that strongly repressed by glucose, glyserol or ethanol. It also contains α-factor matting signal sequence that involve in protein secretion. The processing of α-factor matting signal sequence in pre fusion protein is performed by *KEX2* and *STE13* gene products. *KEX2* cleavage occurs between arginine and glutamine in the sequence Glu-Lys-Arg-Glu-Ala-Glu-Ala. The GluAla repeats are furtherly cleavaged by STE13. We fused the ORF with KEX2 cleavage site to obtain N native terminus hIFNα2a.

Transformation, Screening and Ex-pression of rhIFN-α2a in *P. pastoris*.

Linear vector DNAs are able to generate stable transformants of *P. pastoris* due to homologous recombination between homologous sequences in the vector and host genome. The homologous recombination as single cross-over events is more frequently happened in *P. pastoris*. The insertion event at *AOX1* of GS115 loci arises from single crossover between the loci and *AOXI* region in pPICZαB vector (Cregg *et al*., 1985).

Working stage of protein production using Mut^+ strain GS115 occurs in two phases. Firstly, the use of glycerol to obtain a certain amount of biomass with high cell density and secondly, is the reduction of glycerol and methanol used for recombinant protein biosynthesis (Brierley *et al*., 1990 in Celik *et al*., 2009). The *AOX1* gene is responsible for most of the alcohol oxidase activities in the cells. As a result, GS115 is very responsive at medium containing methanol. The regulation of the *AOX1*gene is similar to the mechanism of the *GAL1*gene of *S. cerevisiae.* GAL1 covers two mechanisms, a repression/ derepression and induction. However, unlike *GAL1* regulation, derepressing conditions of AOX1 gene do not result in substantial transcription. The presence of methanol is very essential to induce high levels of transcription (Tschopp *et al*., 1987). Previous publication reported that *P. pastoris* was managed to tolerate methanol concentration from 0.3 to 3 % (Khatri & Hoffman, 2005). In this study, we used methanol 0.5 % to induce expression for 48 hours incubation time (Herawati *et al*., 2014).

The pPICZαB vector was constructed with gene resistance to zeocin™ (Invitrogen). Linear recombinant plasmids which had been electroporated, directly selected on YPDS plates supplemented with 100 µg/mL zeocin™. In this study, two single colonies were grown on this selection media. Zeocin can be used for transformants selection and screening of high-copy strains by rising its concentration. The two colonies were grown on YPDS containing 500, 1000 and 2000 µg/mL zeocin, and after 3 days of incubation time, the colonies grew appropriately (data not shown).

Characterization and Purification of hIFNα-2a.

Affinity chromatography was done using blue sepharose to purify the protein, this method resulted in a single band that strongly indicated that rhIFN-α2a with no affinity tag was successfully purified. Protein amino acid sequence identification will be conducted to confirm our purified protein of interest.

Conclusion

In conclusion, we have achieved high level expression of human IFNα-2a in the recombinant yeast *P. pastoris.* The characterization of protein had also been confirmed by SDS-PAGE and Western blot analyses. The absence of fusion tag of rhIFNα-2a was prepared as compliance regulation for biosimilar products, since the originator product of this protein has no affinity tag.

Acknowledgements

The authors would like to thank to Adi Santoso, Ph. D as Head of the Laboratory of Therapeutic Proteins and Vaccines, as well as to Syaipul Bahri for their assistance in this research. This research was funded by 'Kegiatan Riset Unggulan LIPI Ketahanan Pangan dan Obat' Programme in 2015.

References

Babu, K. R., Swaminathan, S., Marten, S., Khanna, N., & Rinas, U. (2000). Production of interferon-α in high cell density cultures of recombinant *Escherichia coli* and its single step purification from refolded inclusion body proteins. *Applied Microbiology and Biotechnology, 53*(6), 655-660.

Bekisz, J., Baron, S., Balinsky, C., Morrow, A., & Zoon, K. C. (2010). Antiproliferative properties of type I and type II interferon. *Pharmaceuticals, 3*(4), 994-1015.

Bis, R. L., Stauffer, T. M., Singh, S. M., Lavoie, T. B., & Mallela, K. M. (2014). High yield soluble bacterial expression and streamlined purification of recombinant human interferon α-2a. *Protein Expression and Purification, 99*, 138-146.

Brierley, R. A., Bussineau, C., Kosson, R., Melton, A., & Siegel, R. S. (1990). Fermentation development of recombinant *Pichia pastoris* expressing the heterologous gene: bovine lysozyme. *Annals of the New York Academy of Sciences, 589*(1), 350-362.

Bucher, M. H., Evdokimov, A. G., & Waugh, D. S. (2002). Differential effects of short affinity tags on the crystallization of *Pyrococcus furiosus* maltodextrin-binding protein. *Acta Crystallographica Section D: Biological Crystallography, 58*(3), 392-397.

Cregg, J. M., Barringer, K. J., Hessler, A. Y., & Madden, K. R. (1985). *Pichia pastoris* as a host system for transformations. *Molecular and Cellular Biology, 5*(12), 3376-3385.

El-Baky, N. A., & Redwan, E. M. (2015). Therapeutic alpha-interferons protein: structure, production, and biosimilar. *Preparative Biochemistry and Biotechnology, 45*(2), 109-127.

Ferenci, P. (1993). Historical treatment of chronic hepatitis B and chronic hepatitis C. *Gut* (*Supplement),* 34, S69-S73.

Herawati, N., Santoso, A., & Ningrum, R.A. (2014). The effect of non-nutritional factors of culture condition on human interferon alpha-2b production in methilotrophic yeast *Pichia pastoris. Journal of Pharmaceutical Bioanalitical Science, 3*, 1-5.

Invitrogen. (2010). Easyselect™ *Pichia* Expression Kit. Invitrogen.

Khan, F., Legler, P. M., Mease, R. M., Duncan, E. H., Bergmann-Leitner, E. S., & Angov, E. (2012). Histidine affinity tags affect MSP142 structural stability and immunodominance in mice. *Biotechnology Journal, 7*(1), 133-147.

Khatri, N. K., & Hoffmann, F. (2006). Impact of methanol concentration on secreted protein production in oxygen-limited cultures of recombinant *Pichia pastoris*. *Biotechnology and Bioengineering, 93*(5), 871-879.

Kim, D. C., & Jung, J. (2002). Purification of recombinant human alpha-2a interferon without using monoclonal antibodies. *Journal of Microbiology and Biotechnology, 12*(6), 916-920.

Klose, J., Wendt, N., Kubald, S., Krause, E., Fechner, K., Beyermann, M., ... & Rothemund, S. (2004). Hexa-histidin tag position influences disulfide structure but not binding behavior of *in vitro* folded N-terminal domain of rat corticotropin-releasing factor receptor type 2a. *Protein Science, 13*(9), 2470-2475.

Meager, A. (Ed.). (2006). *The interferons: characterization and application*. John Wiley & Sons.

Ningrum, R. A., Rahmatika, D. E., Retnoningrum, D. S., Wangsaatmadja, A. H., Sumirtapura, Y. C., & Rachmawati, H. (2012). Development of novel interferon alpha2b muteins and study the pharmacokinetic and biodistribution profiles in animal model. *Biomedical Science and Engineering*, *5*, 104-112.

Ningrum, R. A., Wisnuwardhani, P. H., Santoso, A., & Herawati, N. (2015). Antiproli-ferative activity of recombinant human interferon alpha2B on estrogen positive human breast cancer MCF-7 cell line. *Indonesian Journal of Pharmacy, 26*, 86-93.

Terpe, K. (2003). Overview of tag protein fusions: from molecular and biochemical fundamentals to commercial systems. *Applied Microbiology and Biotechnology, 60*(5), 523-533.

Tschopp, J. F., Brust, P. F., Cregg, J. M., Stillman, C. A., & Gingeras, T. R. (1987). Expression of the lacZ gene from two methanol-regulated promoters in *Pichia pastoris*. *Nucleic Acids Research, 15*(9), 3859-3876.

Villaverde, A., & Carrió, M. M. (2003). Protein aggregation in recombinant bacteria: biological role of inclusion bodies. *Biotechnology Letters, 25*(17), 1385-1395.