NUSANTARA BIOSCIENCE ISSN: 2087-3948
Vol. 9, No. 1, pp. 97-101 E-ISSN: 2087-3956
February 2017 DOI: 10.13057/nusbiosci/n090117

Overproduction, characterization and preliminary antiproliferative activity determination of non-tagged recombinant human interferon alpha-2a produced in *Pichia pastoris*

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Manuscript received: 29 August 2016. Revision accepted: 21 February 2017.

Abstract. *Herawati N, Wardiana A, Ningrum RA. 2017. Overproduction, characterization and antiproliferative activity determination of non-tagged recombinant human interferon alpha-2a produced in* Pichia pastoris*. Nusantara Bioscience 9: 97-101.* Recombinant human interferon alpha-2a (rhIFNα-2a) has been widely used for antiviral, anticancer as well as immunomodulatory clinical therapy. In our previous research, we constructed the Open Reading Frame (ORF) encoding synthetic rhIFNα-2a to be in framed with N-terminal alpha factor secretion system in pPICZαB shuttle vector. This research covered overproduction, characterization of the protein and preliminary determination of biological potency assay by using the estrogen positive cell line MCF-7. To overproduce the protein, cultivation was performed in Buffered complex medium containing glycerol (BMGY) for 24 h and production was performed in Buffered complex medium containing methanol (BMMY) during 48 hours at 30°C. The recombinant protein was purified by affinity chromatography using blue sepharose resin. Analysis of amino acid sequence by using MS/MS2 mass spectrometry covered 21% of the total amino acid residues. To determine the initial biological activity, the effect of rhIFNα-2a on MCF-7 breast cancer cell line was studied *in vitro*. The anti-proliferative activity of hIFNα-2a was performed by 3-[4.5-dimethyltiazol-2il]-2.5 diphenylltetrazolium bromide (MTT) assay. The result showed that the protein is able to inhibit MCF-7 proliferation and the reduction of cell growth was dose-dependently. To monitor the protein production, we performed stability expression assay as well. The result informed that the expression of ORF still stable until 60th generation

Keywords: Amino acid sequence, antiproliferative, human interferon alpha-2a, *Pichia pastoris*

INTRODUCTION

Interferon (IFN) is a protein secreted by eukaryotic cells as response to the exposure of virus, bacteria and different types of macromolecules. The secreted interferon stimulates surrounding cells to produce other proteins, which in turn regulate viral replication, the immune response, cell growth, and other cell functions. Interferon can be exactly detected after viral infection locally or systemically. Based on their receptor types on the cell membrane surface, IFN is classified into type I and type II. Type I consists of IFNα, IFNβ, IFNω, and IFNτ while type II consists of IFNγ (Ningrum 2014; Ningrum et al. 2016).

The Interferons (IFNs) are a family of cytokines that protect against disease by direct effects on targets cell and by activating immune responses (Parker et al. 2016). Interferon alpha has an important role in the immune system against viral infections. Human interferon alpha (hIFN-α) is a multigene family of inducible cytokines whose major therapeutic applications based on the antiviral, antiproliferative and immunomodulatory functions (Maeyer and Maeyer 1994; Goodbourn et al. 2000). The hIFN-α2 locus comprises three allelic variants, hIFN-α2a, hIFNα-2b and hIFNα-2c, being the first most widely used in the clinic for the treatment of chronic hepatitis B and C (Ferenci 1993) and several cancers such as melanoma, AIDS-related Kaposi's sarcoma, chronic myeloid lymphoma and angioblastoma (Borden et al. 2000; Goldstein and Laszlo 2000). The hIFN-α2a protein consists of 188 amino acids of which the first 23 residues code the signal peptide and 166 residues are contained in the mature protein (Huckans et al. 2007). In June 1986, hIFN-α2a has been approved as anti-tumor and anti-virus by U.S. Food and Drug Administration. Currently the recombinant form of hIFN-α2a has been produced in many types of expression system (Gutterman 1994).

Pichia pastoris (P. pastoris) is a methylotropic yeast that was genetically engineered as host cell to produce recombinant protein. It is suited for foreign protein expression due to two main reasons: (i) it can be easily manipulated at the molecular genetics level; and (ii) it can perform many of the 'higher eukaryotic protein modification such as glycosylation (Cregg et al. 2000). In addition, *P. pastoris* expression system offers several advantages for production of recombinant proteins, such as very high levels of expression of recombinant proteins, ease of doing scaling-up in the fermenter, cheaper production costs than mammalian cell culture, and ease of transformation. *P. pastoris* can be grown in a simple medium and grow to very high cell densities. The existence of inducible AOX1 as a very strong promoter makes the expression of recombinant proteins easier to control by means of induction (Skoko et al. 2003). AOX1 is a promoter that controls gene expression of alcohol oxidase

to metabolize methanol. Several publications used *Pichia pastoris* as host for hIFN-α2 production. For example, Shi et al. (2007) have successfully performed the expression and purification of hifnα 2b in *P. pastoris* strain GS115 with the number of 298 mg yield. The same protein also has been produced by Ayed et al. and published in 2007 with a yield of 600 mg/L. The process for the production of hifnα-2b using three different secretion signals has been successfully reported (Ghosalkar et al. 2008) .

hIFN-α2a has pleiotropic effects on cell functions (Pasquali and Mocellin 2010). The pleiotropic cellular activities are dependent on effective IFN-stimulated gene (ISG) expression. The IFNs has a broad activity and are involved in complex interactions. They display antiviral activity, impact cellular metabolism and differentiation, and have antitumor activity. Certain tumor cells may have lost genes necessary for responsiveness to growth regulatory cytokines, such as IFNs. Alternatively, tumor cells may have acquired mutations in genes that encode factors that interfere with the normal growth regulatory pathway (Linder and Borden, 1997). Liu et al. reported that IFN-α1b acts as a multifunctional antitumor agent in nasopharyngel carcinoma (NPC), which may have important therapeutic implications.

Many conventional chemotherapeutics, targeted anti-cancer agents, immunological adjuvants and oncolytic viruses are only fully efficient in the presence of intact type I IFN signalling. New anticancer immunotherapies are being developed that are based on recombinant type I IFNs, type I IFN-encoding vectors and type I IFN-expressing cells (Zitvogel et al. 2015).

The antitumor effects appear due to a combination of direct antiproliferative (Jonasch and Haluska 2001). The antiproliferative activity is initiated by activation of JAK STAT pathway which interferes with MAPK pathway that control a variety of processes in the cell, such as proliferation, differentiation, survival, and apoptosis (Ningrum 2014; Ningrum et al. 2015).

In our previous study we produced recombinant human interferon alpha2a (NAT-hIFNα2a) without an affinity tag in methilotropic yeast *P. pastoris* with 44 mg/L of total yield (Herawati et al. 2015). The absence of fusion tag of rhIFNα-2a was aimed to fit the compliance regulation for biosimilar products, since the reference product of this protein has no any affinity tag (Ventola 2013). This research aimed was to overproduce and characterize of NAT-hIFNα2a and to determine the antiproliferative activity using estrogen positive breast cancer cell line MCF-7.

MATERIALS AND METHODS

The overproduction of NAT-hIFNα2a in *P. pastoris*

P. pastoris strain GS115 carrying pPICZαB- hIFNα-2a recombinant plasmid form previous research was cultivated in 25 ml BMGY medium. The culture was incubated overnight at 30°C with vigorous shaking at 250 rpm. The cells were harvested (OD_{600}= 2-6) by centrifugation at 1500 x g for 5 mins then the pellet was resuspended in 50 mL BMMY medium (final OD_{600}= 1) and incubated for 24 h. Methanol (final concentration 1.5%) was added and the cells were reincubated for another 24 h in the same culture condition. Harvesting of the cell was performed by centrifugation at 10.000 rpm for 5 mins at room temperature to collect the supernatant.

Purification, quantification and characterization of NAT-hIFNα2a in *P. pastoris*

50 ml of supernatant was concentrated to a final volume of 5 ml by Minimate™ TFF system (PALL, USA) with 10 kDa cut-off. The concentrated fraction was applied onto a pre-washed Blue sepharose resin (GE Healthcare) using wash buffer containing PBS 1x pH 7.0. After 3 times sample loaded into the column, the resin was washed 5 times using wash buffer, then eluted with 5 mL elution buffer containing PBS 1x pH 7.0 and 1.5 M NaCl. The purified protein was filtered by Amicon® (Millipore) with 3 kDa cut-off and then characterized by SDS-PAGE and Western blot.

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. Immunodetection was achieved by using anti-human IFNα monoclonal antibody (Merck-Calbiochem, Germany, Cat#407290) as primary antibody with 10x dilution and followed by the anti-mouse IgG alkaline phosphatase conjugate (Promega, USA) as secondary antibody. The membrane was stained with NBT/BCIP (4-nitro bluetetrazoliumchloride/ 5-Bromo-4-chloro-3-indolyl phosphate) solution (Invitrogen, USA).

Protein was quantified by using ELISA method. The human IFN alpha ELISA kit (VeriKine, pbl Science) was used to determine protein concentration. The detection was based on anti-detection antibody conjugated to horseradish proxidase (HRP). Tetramethyl benzidine (TMB) was applied as substrate. The range of IFN alpha standard concentration was 12.5 to 500 pg/mL. Absorbance was read at 450 nm. Further protein characterization was performed by LC MS/MS2 mass spectrometry analysis to determine amino acid sequencing (Proteomics International, Australia).

Antiproliferative assay

Cell cultification and treatments

MCF-7 cells were thawed and washed with 9 ml of DMEM medium containing penicillin. Cells were grown in the same medium containing 10% FBS at 37°C and 5% CO_2. After 90% confluency, the cells were transferred into a 96 well (3000 cells/well) and grown overnight in DMEM media containing penicillin-streptomycin with 5% FBS. The cells were washed with 100 µl of PBS and treated by 1 µM tamoxifen (Merck) overnight. Further treatment was applied by various concentration of hIFNα-2a for 5 days. The treatment conditions were based on a report by Ningrum et al. (2015).

Treated cells in 96 well plates were washed twice with 100 µL of PBS. 100µL of DMEM with 5% FBS containing MTT (with final concentration 0.5 mg/mL) was added to each well. Cells were then incubated for 3 hours and the medium was discarded. Formazan crystals formed at the bottom of the well were dissolved in 100 µL of SDS 10%. The cells were incubated for overnight. The reaction was stopped by 0.01 M HCl and dissolved formazan was measured at 570 nm. The percentage of viable cell was compared to control (untreated cells). The experiments were done in triplicates in three dependent experiments.

Stability expression assay

The replica plating method was used to check the stability expression of ORF encoding fusion protein until 60th generation. We used YPD media containing 100 µg/mL of zeozin. The expression was applied using 3 mL of BMMY as production media as previously described. Monitoring was performed for each 10th generation using SDS PAGE method. The supernatant was concentrated using membrane with 3 kDa molecular weight cut off.

RESULTS AND DISCUSSION

Overproduction, purification, characterization and quantification of NAT-hIFNα2a

Protein expression in *P. pastoris* controlled under strong inducible promoter Alcohol oxidase (AOX). There are two genes in *P. pastoris* that code for AOX—AOX1 and AOX2—but the AOX1 gene is responsible for the vast majority of alcohol oxidase activity in the cell. Expression of the AOX1 gene is tightly regulated and induced by methanol to high levels. Expression of the AOX1 gene is controlled at the level of transcription. In methanol-grown cells, approximately 5 percent of polyA+ RNA is from the AOX1 gene whereas in cells grown on other carbon sources, AOX1 message is undetectable. The regulation of the AOX1 gene is similar to the regulation of the GAL1 gene of *S. cerevisiae* in that control appears to involve two mechanisms: a repression/de-repression mechanism plus an induction mechanism. However, unlike GAL1 regulation, de-repressing conditions (e.g. the absence of a repressing carbon source such as glucose in the medium) do not result in substantial transcription of the AOX1 gene. The presence of methanol appears to be essential to induce high levels of transcription (Cregg et al. 2000).

Pichia pastoris strain GS115 carrying pPICZαB-hIFNα-2a recombinant plasmid (Clone#1) was chosen for over-expression of NAT-hIFNα-2a protein in yeast cell. Characterization of supernatant containing NAT-hIFNα-2a showed the target protein (Figure 1A) already quite pure. Tangential filtration system was used with an ultrafiltration membrane having a molecular weight cut- off (MWCO) that is substantially lower than the MW of molecules to be retained. Concentrated supernatant was purified on Blue sepharose 6 fast flow column.

Purification was carried out in two steps; firstly, affinity chromatography with Blue sepharose and secondly, protein concentration by membrane filtration. Molecular weight characterization of purified protein by SDS-PAGE showed a single band protein with about 19.2 kDa in size as theoretically (Figure 1 A). Protein identity was verified by Western-Blot with a specific anti-IFNα2 monoclonal antibody (Figure 1.B).

Protein quantification using ELISA method informed that the concentration was 272 ug/L. We used 10 ug of the protein to determine the amino acid sequence using LC MS/MS2. Sample proteins were digested with trypsin prior to sequencing analysis. There were three peptide fragments which matched with rhIFNα-2a sequence: K.EDSILAVR.K; R.TLMLLAQMR.K; and R.SFSLSTNLQESLR.S (Fig.2). These fragments covered 36 amino acid residues or 21% of the hIFNα-2a sequence. The low coverage of found sequence is likely attributable to the presence of contaminants in our protein target. Purity of the sample is one of the most important factors that greatly affected LC-MS/MS2 results (Egelhofer et al. 2002). In addition careful handling of protein sample is the key to obtain the maximum amino acid sequence coverage of the protein (s) of interest (Gundry et al. 2010).

Anti-proliferative activity

There are several anticancer drugs used in combination with interferon for cancer treatment. One of which is Tamoxifen. Tamoxifen is a nonsteroidal antiestrogen that has found successful applications for each stage of breast cancer in the treatment of selected patients. Combination interferon-α and tamoxifen shows synergistic effects on the inhibition human cancer cell lines (Lindner and Borden 1997; Porzsot et al. 1989; Jazieh et al. 2004).

The combination of NAT-hIFNα-2a and tamoxifen is reported to be very effective in inhibiting proliferation of estrogen positive MCF-7 cell line (Seymour and Bezweda 1993; Ningrum et al. 2015; Wathon et al. 2015). In this study we combined purified NAT-hIFNα-2a with various concentrations (2, 4, and 8 µg/mL) with tamoxifen (1µM) to investigate the effect of its antiproliferative activity by MTT colorimetric assay. The result showed that the antiproliverative activity of our protein was doses dependently (Figure 3).

This result is in line with other reports about antiproliferative activity of IFN. Several publications used various breast cancer cell line to determine the activity of IFN. The assay was performed as single or in combination with other cancer drugs. IFN (500 IU/mL) inhibited human breast cancer cells ZR-75-1 proliferation and the combination of this treatment with Tamoxifen (2 mM) had a greater anti-proliferative effect than either drug alone although there was no evidence of synergism (Van den Berg et al. 1987). Another study resulted in IFN-beta (with doses 10, 100, 500 IU/mL) displayed a dose-dependent inhibitory effect in four cell lines, i.e. MCF-7, T47D, MDA-MB231 and BT20, but the inhibitory was most evident in MCF-7 cells (Coradini et al. 1997).

Stabilization expression analysis

We monitored the ORF expression in *Pichia pastoris* until 60th generation since the expression stability is the most important thing in protein production. ORF

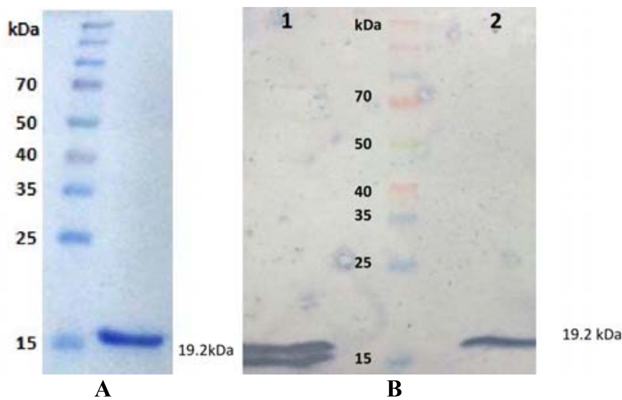

Figure 1. Characterization of NAT-hIFNα2a. A. Purified NAT-hIFNα2a characterized by SDS PAGE; B. Supernatant containing NAT-hIFNα2a (lane 1) and Purified NAT-hIFNα2a (lane 2) characterized by Western Blot

N-terminal amino acid sequence analysis

CDLPQTHSLGSRRTLMLLAQMRKISLFSCLKDRHDFG
FPQEEFGNQFQKAETIPVLHEMIQQIFNLFSTKDSSA
AWDETLLDKFYTELYQQLNDLEACVIQGVGVTETPLM
KEDSILAVRKYFQRITLYLKEKKYSPCAWEVVRAEIM
RSFSLSTNLQESLRSKE

Figure 2. The rhIFNα-2a amino acids sequence determination by LC-MS/MS2 mass spectrometry (Proteomis International, Australia). The identified residues were shown in red.

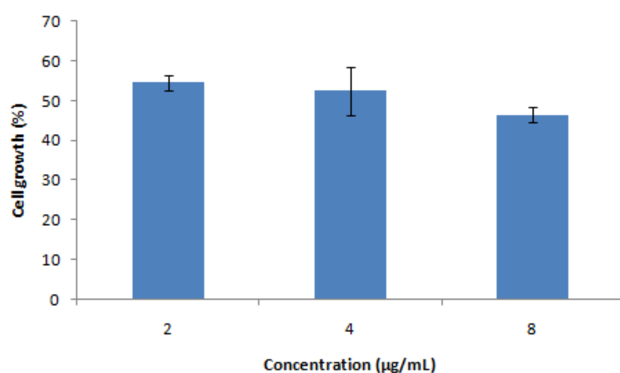

Figure 3. Antiproliverative activity NAT-rhIFNα-2a on MCF-7 cells line

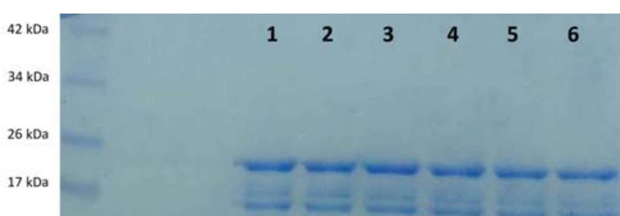

Figure 4. Stability expression of NAT-rhIFNα-2a. Line 1 to 6= 10 th to 60th transformant generation

expression stability was performed to monitor the stability integration of the ORF into *P. pastoris* genome. The stable transformants was generated by homologous recombination between the transforming ORF and regions of homology within the genome (Byme 2015). Such integrant show extreme stability in te absence of selective pressure even when present as multiple copies.

Gene insertion events at the AOX1 of GS115 loci arise from single crossover event between the loci and either of the two AOX1 regions on the pPICZα vector (AOX1 promoter and AOX1 transcription termination region). This may result in the insertion of one or even more copies of the vector upstream or downstream of the AOX1 (Cregg et al. 2000).

We found that there was no significant difference of expression among the generations until 60th generation. We observed the area under the curve (AUC) among the generation by using Imagej (data not shown). This result strongly indicated that the protein production of recombinant *P. pastoris* was stable (Figure 4). To further characterized, the pichia integrant may also copy number determined using southern hybridization or real time PCR. High copy number of integration results in high expression of ORF.

In conclusion, we have overproduced NAT-hIFNα-2a in *P. pastoris*. The protein was also characterized by SDS-PAGE and Western blot analyses as well as peptide mapping in combination with LC-MS. The protein production is also stable until 60th generation. The absence of fusion tag of rhIFNα-2a was aimed to fit the compliance regulation for biosimilar products, since the reference product of this protein has no any affinity tag.

ACKNOWLEDGEMENTS

The authors would like to thank to Adi Santoso, Ph.D. as head of the Laboratory of Therapeutic Proteins and Vaccines, Research Center for Biotechnology, Indonesian Institute of Sciences, Cibinong, West Java, Indonesia as well as to Syaipul Bahri and Syubbanul Wathon for their valuable assistance in this research. This research was funded by *Riset Unggulan* LIPI 2015-2016.

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