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Research Article

Stability of Recombinant Human Interferon Alpha-2b Produced In Methylotropic Yeast *Pichia pastoris*

Syubbanul Wathon[1], Sri Budiarti[1], Popi Hadi Wisnuwardhani[2], Neng Herawati[2], Adi Santoso[2], Ratih Asmana Ningrum[2]*

[1]Graduate School, Bogor Agricultural University, Kampus IPB Dramaga, Bogor, West Java, Indonesia, 16680
[2]Indonesian Institute of Sciences, Jalan Raya Bogor km 46 Cibinong, West Java, Indonesia, 16911

ABSTRACT

The stability of recombinant human interferon alpha-2b (rhIFNα-2b) remains a great challenge for Pharmaceutical science. In previous research we constructed open reading frame encoding rhIFNα-2b and produced the protein in *Pichia pastoris* (*P. pastoris*). This research was aimed to study the stability of rhIFNα-2b in three parameters: temperature, pH and shelf life. The rhIFNα-2b was overproduced by using buffered methanol complex medium (BMMY) at 30 °C for 48 h with 2% of methanol as inducer. Filtration of protein was used by minimate^{TM} tangential flow filtration system with molecular weight cut off (MWCO) 5 kDa. Purification of rhIFNα-2b was performed by immobilized affinity chromatography column using AKTA purifier system. Colorimetric bicinchoninic acid as say informed that the yield of purified rhIFNα-2b was 10.92 mg/L (OD_{600} = 2.3). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blot analyses confirmed that the protein was rhIFNα-2b with 24 kDa in size. Matrix assisted laser desorption ionization-time of flight/time of flight (MALDI -TOF/TOF) mass spectrometry identified the protein as hIFNα-2b with 22% of amino acid coverage. Non reducing SDS-PAGE and Image J software analyses showed that temperature increment, acidic and basic pH as well as shelf life length caused protein aggregation and degradation. 3-[4.5-dimethylthiazol-2il]-2.5-diphenyltetrazolium bromide (MTT) assay informed that the aggregation and degradation reduced the anti -proliferative activity of rhIFNα-2b on human breast cancer MCF-7 cell line. To conclude, all parameters give an impact on rhIFNα-2b stability with the most influencing parameter was temperature at 25 °C. These data can be used to develop rhIFNα-2b formulations as therapeutic protein.

Keywords: rhIFNα-2b; *Pichia pastoris*; stability; aggregation; degradation.

INTRODUCTION

Interferon (IFN) is a cytokine secreted by eukaryotic cells as response to viral, bacterial, or mitogen stimuli. The IFNs are commonly classified into two clusters, type 1 which includes IFN-α, IFN-β, IFN-ω and IFN-τ while type 2 which has only one protein called IFN-γ (Kontsek and Kontsekova, 1997). Human interferon alpha (hIFN-α) has wide biological activities as anti-cancer, antiviral, and immune modulatory defense mechanisms (Cherbi-Alix and Wietzerbin, 2007). hIFN-α2 consists of three subtypes, including hIFNα-2a, hIFNα-2b, and hIFNα-2c. hIFNα-2b is being the predominant allele than other subtypes (Lee et al., 1995). hIFNα-2b is a polypeptide that consists of 188 amino acids (23 residues are signal peptide and 165 residues are mature protein) and O-glycosylation at threonine position 106 (Ningrum, 2014).

The human body can naturally produce IFN, however in a certain cases such as in cancer and hepatitis condition, its number is not sufficient to eliminate the disease agents that grow very rapidly. Currently, the requirement of IFN can be obtained from its recombinant form (Dingermann, 2008). Production of certain types of recombinant IFN has been performed, such as human recombinant IFNα-2b (rhIFNα-2b). rhIFNα-2b is a therapeutic protein, which is recommended by the United States Food and Drug Administration to treat hepatitis and several cancer treatments (Herawati et al., 2014).

rhIFNα-2b production can be carried out using several expression systems, including Tobacco BY2 (Xu et al., 2007), *Escherichia coli* (Srivastava et al., 2005), *Saccharomyces cerevisiae* (Tuite et al., 1982), *Streptomyces lividans* (Pimienta et al., 2002), *P. pastoris* (Shi et al., 2007; Li et al., 2007; Ghosalkar et al., 2008; Ningrum et al., 2013), avian eggs (Rapp et al., 2003; Patel et al., 2007) and mammalian cells (Rossmann et al., 1996; Loigon et al., 2008). However, all expression systems have some advantages and limitations. In our previous

* Corresponding Author
Email: ratih.asmana@gmail.com
Contact: +62-21-8754587, Fax: +62-21-8754587
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research, we developed rhIFNα-2b in *P. pastoris* expression system. Our protein has polyhistidine tag at C-terminus with 24 kDa in size. Biological activity assay shows that the protein has activity to inhibit MCF-7 breast cancer cell line (Ningrum et al., 2015).

P. pastoris is a methylotrophic yeast commonly used as an expression host to produce recombinant proteins (Fickers, 2014). The advantages of using the *P. pastoris* expression system are easiness of manipulation at molecular level, high level and expression of recombinant protein, easiness of transformation and selection, rapid growth ability in simple media, post-translational property, and lower production cost comparing to mammalian system (Cregg et al., 2000; Balamurugan et al., 2007).

The main problem in rhIFNα-2b production is low stability which is caused by physical and chemical degradation (Salmannejad et al., 2014). The instability of rhIFNα-2b may occur during preparation, formulation and storage (Diress et al., 2010). The stability of therapeutic protein has a pivotal role in clinical purposes since its degradation can decrease the biological activity, causing immunogenic effects as well as dosage changes during therapy (Ruiz et al., 2006). This research was aimed to study the stability of our rhIFNα-2b under three parameters, including temperature, pH, and shelf life. The stability information from these parameters can be used for further steps in therapeutic protein production, such as protein preparation, formulation and storage.

MATERIAL AND METHODS

Overproduction of rhIFNα-2b in *P. pastoris*

Single colony of *P. pastoris* X-33 harboring hIFNα-2b open reading frame (ORF) (Ningrum et al., 2013) from the YPDS + zeocin medium (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, 2% agar, 2000 µg of zeocin) (Invitrogen, USA) was grown in the 50 mL BMGY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6, 1.34% yeast nitrogen base, 4 x 10^{-5}% biotin, 1% glycerol) (Merck, Germany) at 30 °C and 250 rpm until reaching OD_{600} = 2-6. Cell harvesting was carried out by centrifugation at 1500 x *g* for 5 min at room temperature. Then, the pellet was resuspended into 100 mL BMMY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6, 1.34% yeast nitrogen base, 4 x 10^{-5}% biotin, 0.5% methanol) (Merck, Germany). Resuspended cells were incubated at 30 °C and 250 rpm. The induction was carried out after 24 h incubation by using methanol with final concentration 2% (Merck, Germany) into the cultures. Harvesting was performed after 48 h incubation by centrifugation at 1500 x *g* for 5 min at room temperature. Phenyl methyl sulfonyl fluoride (PMSF) (Sigma, USA) 1 mM was used as protease inhibitor (Herawati et al., 2014).

Filtration, purification and quantification of rhIFN-α2b

Minimate™ tangential flow filtration system with MWCO 5 kDa (Pall, USA) was used to concentrate the protein. The concentrated fraction was purified with AKTA purifier 10 system (GE Healthcare, Sweden) by using His trap column. The solution containing 20 mM of sodium phosphate, 20 mM of imidazole and 500 mM of sodium chloride was used as the binding buffer while 20 mM of sodium phosphate, 500 mM of imidazole and 500 mM of sodium chloride were used as elution buffer. The concentration of the purifi ed proteins were determined by bicinchoninic acid assay protein kit (Pierce, USA) at various concentrations of bovine serum albumin (25 to 2000 µg/mL) as standard. The absorption of the sample proteins was measured at 562 nm (Ningrum et al., 2015).

Characterization of rhIFN-α2b

Polyacrylamide gel (Bio-Rad, USA) comprised of separating gel (12%) and stacking gel (4%) was used in the SDS-PAGE analysis. Coomassie brilliant blue (Bio-Rad, USA) was used in gel staining. In Western Blot analysis, mouse anti human IFN-α (Calbiochem, USA) was used as primary antibody with dilution ratio 1:100 (v/v). Anti mouse IgG alkaline phosphatase conjugate (Promega, USA) with dilution ratio 1:7500 (v/v) was used as secondary antibody while nitro blue tetrazolium-[5-bromo-4-chloro-3-indolyl-phosphate] (NBT/BCIP) (Invitrogen, USA) used as substrate (Ningrum et al., 2015). Amino acid sequence determination was performed by MALDI-TOF/TOF mass spectrometry analysis (Proteomics International, Australia).

Stability assay of rhIFN-α2b

Protein stability assay was conducted at three parameters, including temperature, pH and shelf life. To minimize the various component caused by different buffer, we used phosphate buffer at all parameters. The treatment conditions were based on a previous report Ruiz et al. (2006) with some modifications. The untreated rhIFNα-2b was used as control. We used various temperatures ranging from 30 °C to 70 °C, various pH from 4 to 8 by using phosphate buffer system (Merck, Germany) and shelf life from 1st to 20th day. The incubation time for temperature and pH parameters was 3 days. We used phosphate buffer system pH 7 (Merck, Germany) at three temperatures on shelf life parameter: 4 °C, 25 °C and 37 °C for 20 days with sampling points every 2 days. The protein profile and antiproliferative activity was monitored by using non reducing SDS-PAGE and MTT assay respectively. The stability of the protein profile was also determined based on the primary area of the rhIFNα-2b band by using Image J software.

Antiproliferative activity assay of rhIFNα-2b in MCF-7 cells

Human breast cancer cell lines MCF-7 that used in this research were obtained from the Therapeutic Protein

and Vaccine Laboratory, Research Center for Biotechnology, Indonesian Institute of Sciences Cibinong-Bogor. The cells were thawed and washed with 9 mL dulbecco's modified eagle medium (DMEM) (Gibco, USA) with 1% solution containing penicillin (100 units/ mL) and streptomycin (100 mg/ mL) (Sigma, USA). The cells were grown in the same medium containing 10% fetal bovine serum (FBS) (Gibco, USA) at 37 °C and 5% CO_2. After 90% of confluence, the cells were washed with phosphate buffer saline (PBS) (Merck, Germany). Then, the cells were detached with 500 µL of Triple E (Gibco, USA) at 37 °C for 5 min. The cells were transferred into 96 well (3000 cells/well) and washed by using 100 µL PBS (Merck, Germany). The cells were grown overnight in 100 µL of complete medium (95% DMEM (Gibco, USA), 5% FBS (Gibco, USA) and 1% penicillin/ streptomycin (Sigma, USA)) containing 1 µM tamoxifen (Merck, Germany) at 37 °C and 5% CO_2. Medium without tamoxifen was used as blank and negative control. Then, the cells were washed and treated by rhIFNα-2b (4 µg/mL). The untreated rhIFNα-2b was used as control. The cells were incubated for 5 days. After incubation, the cells were washed and added with 100 µL of the complete medium containing 5 mg/mL of MTT (Invitrogen, USA). The cells were incubated for 3 h. The solution containing 100 µL of SDS 10% in 0.01 M of HCl (Merck, Germany) was added to stop the reaction and then the suspension was incubated overnight. The dissolved formazan was measured at 570 nm. The experiments were done in triplicates in three dependent experiments (Ningrum et al., 2015). The inhibition percentage of the cells was calculated by using the following formula:

$$\text{Cell viability}(\%) = \frac{\text{Average absorption of the tested cells} - \text{Average blank absorption}}{\text{Average absorption of the negative control} - \text{Average blank absorption}} \times 100\%$$

Analysis of variance of each parameter was processed by using SPSS version 21 software

RESULTS AND DISCUSSIONS

Overproduction of rhIFNα-2b in *P. pastoris*

The overproduction of rhIFNα-2b in the *P. pastoris* expression system consisted of two main stages: biomass increment and protein production. Biomass increment was achieved in BMGY medium. The medium served glycerol as the main carbon source and repressor of alcohol oxidase 1 (*AOX1*) promoter. *AOX1* promoter regulated 30% of total soluble protein expression in *P. pastoris* (Cereghino and Cregg, 2000; Fickers, 2014). BMMY medium provides methanol as carbon source. *P. pastoris* is methylotrophic yeasts, which uses methanol as a source of carbon and energy (Krainer et al., 2012). In our research, *P. pastoris* strain X-33 with methanol utilization plus (Mut^+) phenotype was used as an expression system to produce rhIFNα-2b. This strain could grow fast in the medium containing methanol and produced a great number of recombinant proteins (Kim et al ., 2009). *P. pastoris* is very sensitive to methanol, where the high methanol concentration is toxic and inhibits the growth of *P. pastoris* while the low concentration is unable to initiate the transcription process (Poutou-Pinales et al., 2010). The concentration of methanol in the medium determined the efficiency of the recombinant protein production. Herawati et al. (2014) claimed that the best concentration for methanol to induce rhIFN-α2b overproduction in *P. pastoris* strain X-33 was 2% (v/v).

Overproduction of rhIFNα-2b in *P. pastoris* is depends on biosynthesis of alcohol oxidase (AOX) which is regulated by *AOX1* promoter. *P. pastoris* produces AOX as response to the presence of methanol in the media. Methanol causes the accumulation of AOX (Cos et al ., 2006). We constructed ORF encoding hIFNα-2b in the downstream of the *AOX1* promoter, therefore its expressions would be in line with AOX. Some promoters have been developed in *P. pastoris* expression system, for example *GAP*, *FLD1*, *PEX8*, and *YPT1*. *GAP* is a constitutive promoter continuously producing target protein so it could not use in toxic protein production. *FLD1*, *PEX8* and *YPT1* are inducible promoters that require more complex inducers compare to *AOX1* promoter (Cereghino and Cregg, 2000).

P. pastoris has ability to perform post-translational modifications, such as the glycosylation and disulfide bond formation. The glycosylation pattern affects the biological activity of recombinant proteins, especially therapeutic proteins (Li et al., 2012). rhIFNα-2b has O-glycosylation at threonine position 106 (Ningrum et al., 2013). The O-glycosylation formation increases the half-life of rhIFNα-2b. Macauley-Patrick et al. (2005) reported that the *P. pastoris* expression system had successfully produced proteins with disulfide bond patterns. Disulfide bonds are also important for maintaining the biological activity of proteins. rhIFNα-2b has two disulfide bonds, formed by cysteines 29 and 138 as well as cysteines 1 and 98 (Bae et al., 1995). Disulfide bond at position 1 and 98 is not important in biological activity of rhIFNα-2b (Morehead et al., 1984). *P. pastoris* can produce proteins intracellularly and extracellularly. In this study, rhIFNα-2b was produced extracellularly. We constructed rhIFNα-2b which contains α-factor prepo of signal peptides (Ningrum et al., 2013). Ghosalkar et al. (2008) stated that α-factor prepo of signal peptide was more efficient in the secretion of rhIFNα-2b.

Protein quantification informed that the yield of our purified rhIFNα-2b was 10.92 mg/L (at OD_{600} = 2.3). This result was higher than other expression systems, for example Tobacco BY2 cells (0.02 mg/L) (Xu et al., 2007), *Streptomyces lividans* (0.1 mg/L) (Pimienta et al., 2002) and avian eggs (2 mg/L) (Rapp et al., 2003). Other expression systems have been reported can produce rhIFNα-2b with higher yield. For instance, *Escherichia coli* produced 3000 mg/L (Srivastava et al., 2005). How-

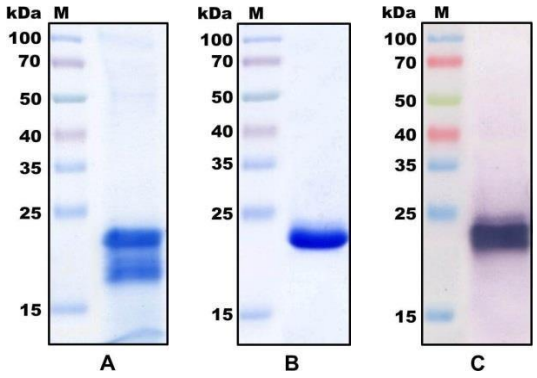

Figure 1: Characterization of rhIFNα-2b: (A) unpurified; (B) purified; (C) Western Blot of purified; M = spectra multicolor broad range protein ladder (Thermo Scientific, USA).

CDLPQTHSLGSRRTLMLLAQMRKISLFSCLKDRHDFGFPQEEFGNQFQKAETIPVLHEMI QQIFNLFSTKDSSAAWDETLLDKFYTELYQQLNDLEACVIQGVGVTETPLMKEDSILAVRK YFQRITLYLKEKKYSPCAWEVVRAEIMRSFSLSTNLQESLRSKE

Figure 2: rhIFNα-2b amino acid sequence determination by MALDI-TOF/TOF mass spectrometry (Proteomic International, Australia).

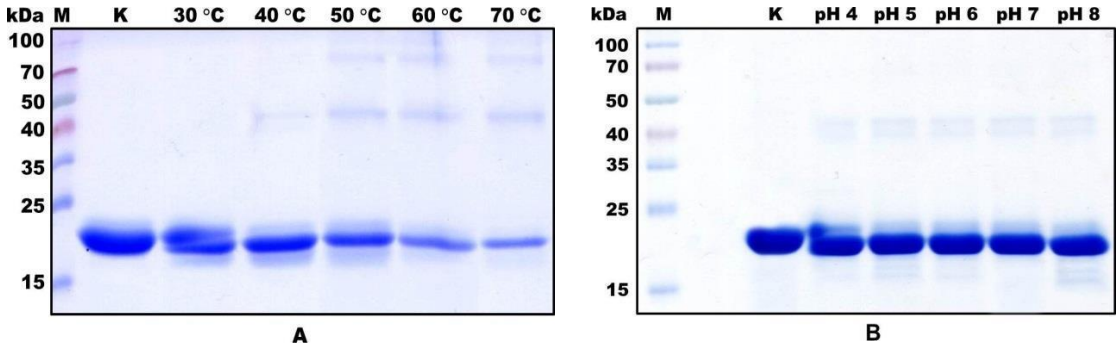

Figure 3: rhIFNα-2b profiles at: (A) various temperature; (B) various pH; K = control; M = spectra multicolor broad range protein ladder (Thermo Scientific, USA).

ever, the protein was unglycosylated and produced as inclusion bodies that need to refold into its native conformation. Unfolding protein could reduce biological activity and unglycosylation may cause shorter half-life of rhIFNα-2b. *Saccharomyces cerevisiae* produced 15 mg/L (Tuite et al., 1982), nevertheless the protein was hyper glycosylated and may cause immunogenic effects. HEK292 mammalian cells produced 200 mg/L (Loigon et al., 2008) and NS0 myeloma cells produced 120 mg/L (Rossmann et al*.,* 1996). However, mammalian cell expression systems require expensive production costs, long production time, difficult scale-up and purification (Li et al., 2012).

Characterization of rhIFNα-2b

The unpurified fraction showed a number of protein bands with different molecular weights (Figure 1 A). Purified protein was shown in Figure 1 B and 1 C. Our protein had different sizes with its native form (21.5 kDa). The rhIFNα-2b was 24 kDa which contains polyhistidine tag and c-myc epitop in its C-terminus.

Protein characterization by using MALDI -TOF/TOF mass spectrometry showed three peptide fragments (underline) which is match with hIFNα-2b sequence: R.TLMLLAQMR.K; K.EDSILAVR.K; and R.SFSLSTNLQESLR.S (Figure 2). These three peptide fragments covered 36 amino acid residues or 22% of the total hIFNα-2b amino acid sequence. This low amino acid sequence coverage might be caused by the existence of impurities in protein sample. Purity of the sample protein was a factor that greatly affected the MALDI-TOF/TOF results (Egelhofer et al., 2002).

Stability of rhIFNα-2b

Environmental stress condition, such as temperature increment, more acidic or basic pH condition, and shelf life condition gave information related to therapeutic protein stability (Diress et al., 2010). The stability of rhIFNα-2b was evaluated based on its SDS-PAGE profile and antiproliferative activity in MCF-7 cells. We used non-reducing SDS-PAGE to monitor the protein profile which is more representative in showing aggregation and degradation of protein. Ruiz et al. (2006) stated

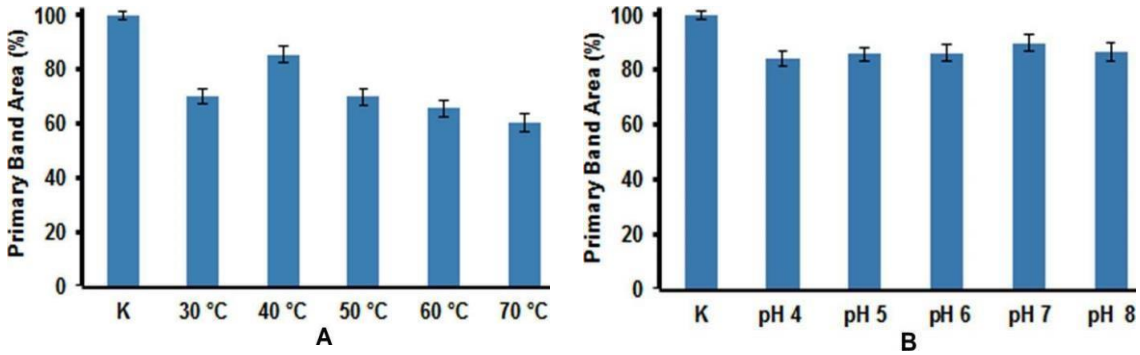

Figure 4: Analysis of rhIFNα-2b band primary area by using Image J software at: (A) various temperature; (B) various pH; K = control.

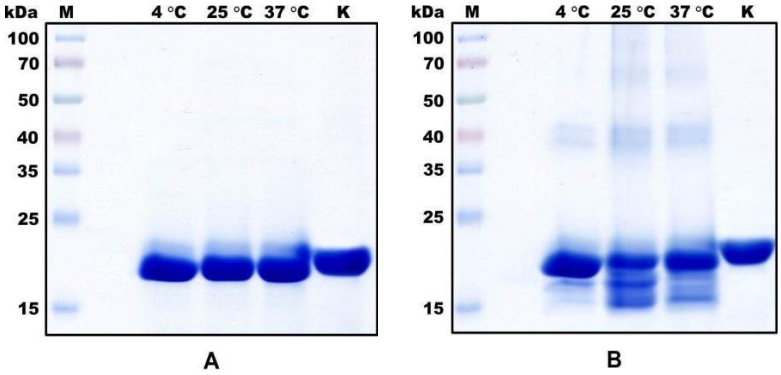

Figure 5: rhIFNα-2b profiles after: (A) 2 day shelf life; (B) 20 day shelf life; K = control; M = spectra multicolor broad range protein ladder (Thermo Scientific, USA).

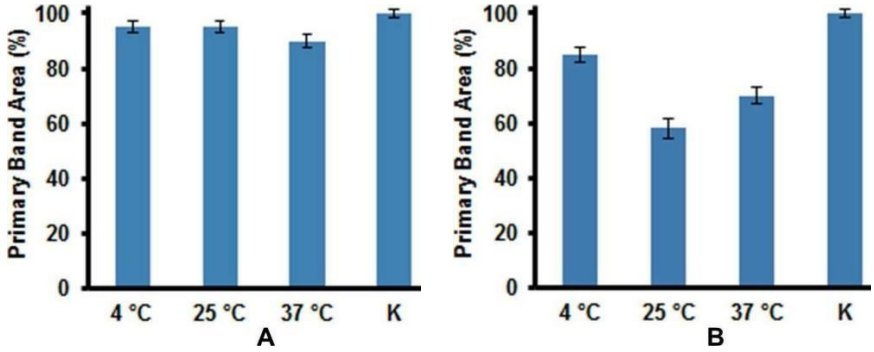

Figure 6: Analysis of rhIFNα-2b band primary area by using Image J software after: (A) 2 day shelf life; (B) 20 day shelf life; K = control.

that the sensitivity of aggregation monitoring under the reducing condition was lower than the non-reducing condition.

Figure 3 A shows different protein profiles after incubation at various temperature: 40 °C, 50 °C, 60 °C and 70 °C. The result informed that temperature increment cause aggregation. The aggregation can be formed by unfolded protein. The opened hydrophobic clusters interacted with the surrounding environment and forms aggregation (Diress et al., 2010). Wang et al. (2010) mentioned that the formation of protein aggregates under the increasing temperature was caused by the increment of molecular collisions and interactions among the hydrophobic clusters. Figure 3A informs that degradation was occurred at 30 °C. Previous publication stated that it was caused by protease activities. The proteases secreted by *P. pastoris* were aminopeptidases which are active at 25 °C to 30 °C (Sinha et al., 2005). We used Image J software analysis to confirm the aggregation and degradation on protein bands. Figure 4A shows the decrement of rhIFNα-2b that corresponds to active protein band primary area at various temperatures. This strongly indicated that the temperature increment affected protein stability.

In addition, instability of protein was occured in more acidic or basic pH condition. Figure 3 B shows that incubation at pH 4, 5, 6 and 8 caused aggregation as well as degradation. However, protein aggregation and degradation was not produced at pH 7. Therefore, protein degradation can be caused by protease activity at a certain pH. We used one-step purification by using affinity chromatography which has possibility to have

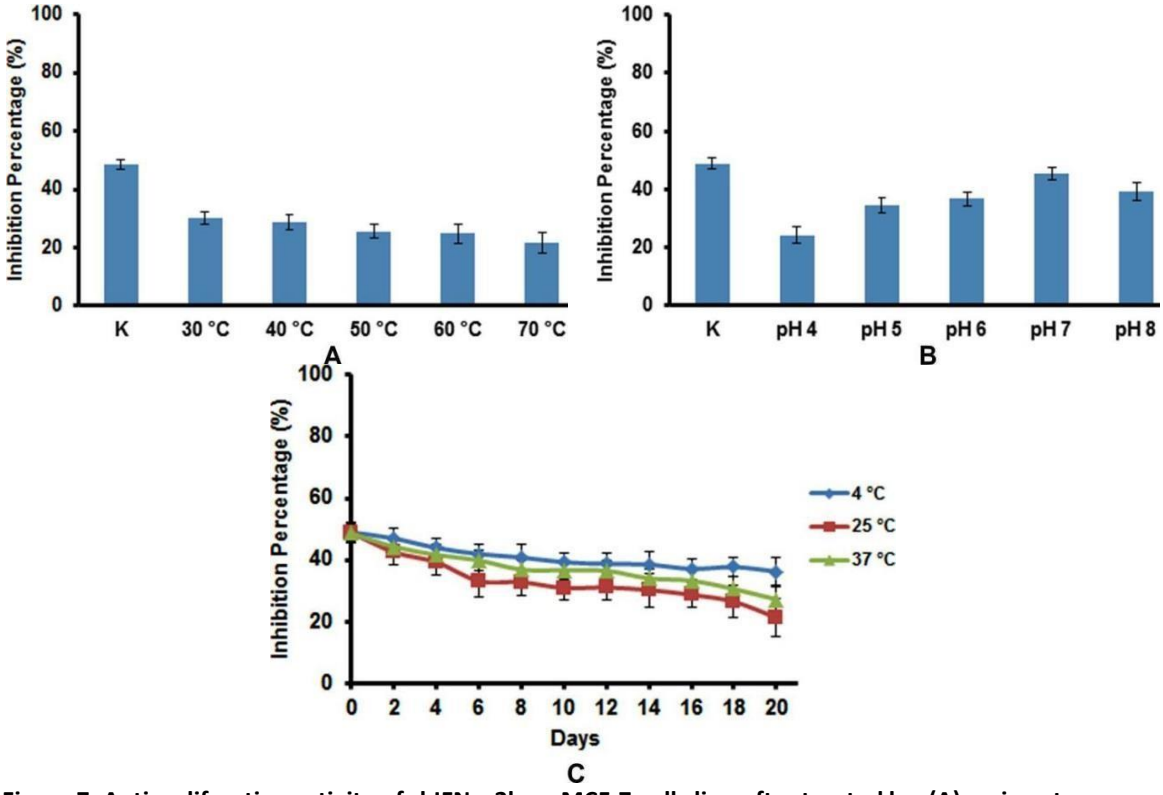

Figure 7: Antiproliferative activity of rhIFNα-2b on MCF-7 cells line after treated by: (A) various temperature; (B) various pH; and (C) various shelf life; K = control.

proteases as impurities. Some proteases had similar physicochemical properties and affinity characteristics to a recombinant protein, so that they became impurities (Macauley-Patrick et al., 2005). *P. pastoris* produces several protease, they are proteinase A, proteinase B, carboxypeptidase and aminopeptidase (Sinha et al ., 2005). rhIFNα-2b degradation at pH 4, 5 and 6 might be caused by acidic protease such as proteinase A while degradation at pH 8 might be caused by alkaline protease such as proteinase B (Zhang et al., 2007).

Aggregation can be caused by influence of the buffer system which was used in the protein preservation (Ruiz et al., 2006). Protein stability is obtained by the presence of negative charge in buffer system, which interacts with positive amino acid clusters on proteins. Sodium phosphate buffer contains negative charges from phosphate ions which should be able to stabilize the conformation of rhIFNα-2b. In this research the aggregation might be caused by trace amounts of metal ions (Fe^{2+}, Ca^{2+}, Cu^{2+}, Mg^{2+} and Zn^{2+}) that contaminate the buffer salts and participated in the oxidation reactions. Protein oxidation occurs mainly in unstable amino acid residues, such as cysteine, histidine, tryptophan, tyrosine, proline, arginine, lysine and threonine (Wang, 1999). The oxidation reaction causes conformational change of proteins, such as disulfide bonds breakage. This condition caused protein conformation became unstable and tended to form aggregates. The aggregation and degradation on protein bands was confirmed by Image J software analysis. Figure 4 B shows the decrement of primary area rhIFNα-2b band at pH 4, 5, 6, 7 and 8 when compared to the control.

The 20 day shelf life period was chosen based on IFN-α activity half-life which is 19 days (Ruiz et al., 2006). The proper storage temperature is very important to maintain the stability of the therapeutic protein during storage periods. We used three different temperatures which commonly happened during shelf life. Figure 5 A shows that after 2 days, the rhIFNα-2b profile was not much different with control. It was confirmed by Image J software analysis (Figure 6 A). After 20 days of storage, the aggregation and degradation occurred (Figure 5 B). The aggregation might also be caused by the contamination of metal ions on buffer system as described previously. The highest degradation level occurred at 25 °C. This indicated that proteases were more active at room temperature. Some of proteases in *P. pastoris* were active at room temperature and secreted in high quantities, such as amino peptidase (Sinha et al., 2005). A cluster protease was difficult to identify and was not easily removed by using certain anti-protease (Kobayashi et al., 2000). PMSF did not completely work as anti-protease because it only targets serine protease (Sinha et al., 2005). Image J software analysis showed the decrement of primary area rhIFNα-2b band after 20 days of storage when it was compared to the control (Figure 6 B). This indicated that temperature incubation, protease activity and contamination of metal ions on buffer system affected protein stability.

Antiproliferative activity assay of rhIFNα-2b in MCF-7 cells

Anti-proliferative activity of rhIFNα-2b fractions from the stability assay were tested in estrogen positive

breast cancer cell line MCF-7. We used MCF-7 as model because the cell was responsive to IFN-α (Lindner and Borden, 1997; Porta et al ., 2005). We combined rhIFNα-2b with tamoxifen an anticancer drug that has synergistic activity with rhIFNα-2b (Jonasch and Frank, 2001). Ningrum et al. (2015) claimed that the inhibitory concentration 50% (1C50) was achieved while 1 µM of tamoxifen was combined with 4 µg/mL of rhIFN-α2b.

The analysis of variance showed that treatments in all parameters, including temperature, pH and shelf life had significant effect on decreasing inhibition percentage of the MCF-7 cells proliferation (P<0.05). The temperature increment reduced inhibition percentage about 20% from IC50 (Figure 7 A). The more acidic and basic pH condition also decreased inhibition percentage (Figure 7 B). However, a stable anti-proliferative activity was achieved at storage temperature of 4 °C as predicted and lowest anti-proliferative activity was resulted at 25 °C (Figure 7 C). This result correlates and in line with its protein band profile that showed highest aggregation and degradation level at 25 °C. It was strongly indicated that temperature at 25 °C is the most influencing factor on rhIFNα-2b stability.

The active site and conformation of rhIFNα-2b were unique and susceptible to change during the preparation, formulation and storage (Ruiz et al., 2006). Disulfide bond has an important role in biological activities of rhIFNα-2b. Disulfide bond alteration causes conformation modification that induce aggregation and degradation. The aggregation and degradation could reduce the amount of active rhIFNα-2b and lowering its anti-proliferative activity (Diress et al., 2010).

CONCLUSION

Temperature increment, variation on pH as well as shelf life gives an impact on rhIFNα-2b stability by generating protein aggregation and degradation. Moreover, temperature at 25 °C is the prominent factor influencing the protein stability. Aggregation and degradation reduce rhIFNα-2b anti-proliverative activity on MCF-7 cell lines by decreasing the quantity of protein active form.

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