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Research Article

DEVELOPMENT AND CHARACTERIZATION OF POLYCLONAL ANTIBODY OF RECOMBINANT HUMAN INTERFERON A2B IN *NEW ZEALAND WHITE* RABBIT

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ABSTRACT

We have developed recombinant wild type and mutant human interferon α2b (rhIFNα2b) from synthetic gene in *Escherichia coli*. To identify the successful product of the proteins, immunology-based assay was suggested due to specificity for characterization. This work was aimed to develop and characterize rhIFNα2b polyclonal antibody generated in *White New Zealand* rabbits. The rhIFNα2b was overproduced in *Escherichia coli* BL21 containing rhIFNα2b synthetic gene in pET32b.The protein was obtained as inclusion bodies, refolded, purified using nickel affinity chromatography, and characterized using polyacrylamide gel electrophoresis. The purified rhIFNα2b protein was injected into rabbits for 21 days. Absorption of *E.coli* antibody was done using total *E. coli* protein to remove antibody againts host cell. The generation of antibody was monitored using dot blot and Western blot methods and quantified using Enzyme Linked Immunsorbant Assay (ELISA). To do so, rhIFNα2b was used as an antigen. The result showed that the rhIFNα2b was produced as a His-tag protein fusion of 33kDa in size. The results of dot blot and Western blot analyses strongly indicated that antibody against rhIFNα2b was generated and specifically recognized rhIFNα2b. ELISA showed that the titer of the polyclonal anti-rhIFNα2b was 1:10.000. In conclusion, polyclonal antibody spesifically against rhIFNα2b protein was successfully detected with high titer after 21 days after rabbit immunization.

Key words: polyclonal antibody, rhIFNα2b, *New Zealand White* rabbits.

INTRODUCTION

Interferon (IFN) is an endogenous cytokine generated as a response to virus, parasites, and mitogen. Based on its receptor, IFN is classified into type I (primary secreted by leukocytes and fibroblast) and type II (mainly produced by T-cells). IFN type I consists of IFNα, IFNβ, IFNκ, and IFNτ, while type II consists of IFNγ [Baldarrain *et al.*, 2001]. Currently, IFN has been developed for antiviral and anticancer treatments. However, the therapeutic use of IFN limited clinical application due to its short biological half life, its protease instability, and other side effects (Jonasch and Halusca, 2001; Thitinan and McConville, 2009).

IFNα2b is the mostly studied of IFN type I. It has been approved by World Health Organization for therapeutic use against hepatitis B and C virus infections. In our previous research, recombinant human IFNα2b (rhIFNα2b) coding region was synthesized using Thermodynamically Balanced Inside-Out method. An *Escherichia coli* BL21 carrying pET32b inserted by the rhIFNα2b coding region was used to produce rhIFNα2b. The rhIFNα2b was successfully purified and characterized (Retnoningrum *et.al.*, 2010).

Polyclonal antibody has been widely used in immunoassay, especially in research and clinical laboratories. Polyclonal antibody has wide application such as immunoblotting and immunostaining to identify and characterize antigens. It is also used for characterization of engineered protein and immunoprecipitation. Polyclonal antibody is commonly generated from immunized animals:rabbit, rat, mice, and horse. In the laboratory scale, rabbit is generally used because of its size, its easiness in handling,

and its ability to generate higher amount of antibody as compared to rat or mouse (Asai, 1993).

We reported here the successful development of antibody anti-rhifnα2b in *White New Zealand* rabbit. Our established rhifnα2b was used as an antibody inducer as well as to detect the antibody generation both qualitatively and quantitatively.

MATERIAL AND METHODS

Microorganism animal and growth medium

Escherichia coli harboring pET32b IFNα2b (*E. Coli* pET32b IFNα2b) was constructed in our previous work and was used as a source to obtain rhIFNα2b protein. Rabbit strain *White New Zealand* (12 weeks, 2 kg, female and male) was obtained from PT Bio Farma (Persero, Bandung, Indonesia). Luria Bertani agar and broth containing 100μg/mL ampicillin was used to growth recombinant *E. coli*.

Protein overproduction, purification and characterization.

E. coli pET32b IFNα2b was inoculated in 30mL of Luria Bertani (LB) liquid medium containing100μg/mL ampicillin (Sigma, New York, USA). The growth of the bacteria was done at 37°C, 150 rotation per minutes (rpm) for 16h with rigorous shaking. 5% (v/v) of 16-hour inoculum was added into 320mL of LB broth containing 100μg/mL ampicillin for 3 hours. Culture was induced at OD600 ~ 0.5 using 0.5mM isopropyl β-D-1-thiogalacto-pyranoside (IPTG, Sigma) and culture was incubated for additional 3 hours in the same condition. Cells were collected by centrifugation at 1.300x g for 15min. The pellet was washed in 6mL of binding buffer without imidazole containing 1mM of phenyl-methyl-sulphonyl fluoride (PMSF, Sigma). The cells were disrupted by sonication at 4MHz for 10min. Cells were sonicated for 30s and then allowed to cool down for another 30s on ice. Suspension was centrifuged at 5.000x g for 15min. Most of the rhIFNα2b protein was produced as inclusion bodies (IB) and refolded in 6M urea for 60min. Soluble rhIFNα2b was obtained by centrifugation at 5.000x g for 30min.

Nickel affinity chromatography was used to purify rhIFNα2b according to manufacturer (Novagene). 5mL of total protein of 256μg/mL was loaded and eluted protein was collected in 6 fractions. Purified rhIFNα2b protein was measured using Bradford method. Total and purified proteins were characterized by 15% Sodium Dudocyl Sulphate Polyacrylamide Gel Eelectrophoresis (SDS-PAGE). 20μL of sampel protein from 6 fraction of purified protein (5.12μg; 5.64μg; 6.1μg; 3.40μg; 1.68μg) were mixed with 5μL of sample buffer. Electrophoresis was done at 125V for 60min.

Generation of polyclonal antibody in rabbit.

Purified rhIFNα2b was sterilized with 0.22 micron filter.Concentration of protein was measured with Bradford method. The sterilized rhIFNα2b was used to immunize 2 different rabbits (2.5 kg, 3 months, and specific pathogen free) subcutaneously at five spots on the back site of the animals. The first immunization was conducted using1 mL of solution I containing 100μg purified rhIFNα2b protein in 0.5mL of elute buffer and 0.5mL complete Freud adjuvant. Reimmunization was done three times with 1mL of solution II containing 200μg purified rhIFNα2b protein in 0.5mL of elute buffer and 0.5mL incomplete Freud adjuvant in 14days interval. Blood was collected 10days after each immunization and then incubated at 37°C for an hour. Serum was obtained after centrifugation at 900 x g for 10min and then was stored at -20°C until used.

Adsorption experiment.

This step was aimed to adsorp antibodies againts *E. coli* BL21 total proteins in rabbit serum after immunization. Protein totals of *E. coli* was obtained from *E. coli* BL21 grown in 50mL of LB broth. Cell pellet was disrupted with sonication and total proteins were separated by centrifugation at 124x g for 15min. Rabbit serum was mixed with total protein (256 μg/mL) at 7 (v/v) ratios, 1: 1.50; 1: 1.75; 1: 2.00; 1: 2.25; 1: 2.50; 1: 2.75; and 1: 3.00. The mixtures were incubated at room temperature for 30min and centrifuged at 966 x g for 2min. Supernatants were collected and stored at -20°C.

Characterization of polyclonal antibody.

Dot blot, Western blot and ELISA were used to characterize the antibody. In dot blot method, 1.2µg of purified rhIFNα2b protein was blotted onto nitrocellulose membrane. Alkaline phosphatase-conjugated rat anti-rabbit IgG and elute buffer were used as positive and negative controls, respectively. Blocking of unspecific proteins was done by incubating nitrocellulose membrane in Phospate Buffer Saline (PBS) containing 5% (w/v) of skim milk at room temperature for 60 min with gentle shaking. The membrane was then washed 3 times with TBST [0.1% (v/v) of Tween in Tris Buffer Saline (TBS)] then incubated with rabbit serum (dilution 1:2,500 using PBS containing 5% skim milk and 0.1% tween) at 4°C for two hours. The washing step was repeated 3 times with the same condition. The membrane was incubated with alkaline phospatase-conjugated rat anti-rabbit IgG (dilution 1:10,000 in TBS containing 5% skim milk and 0.1% tween) at room temperature for an hour and then washed. The staining was developed by incubation in 33µL of Nitro Blue Tetrazolium Chloride (NBT, Sigma, 50 mg/mL)] and 66µL of 5-Bromo-4-chloro-3-indolyl phosphate (BCIP, Sigma, 50mg/mL) in 10mL alkaline phospatase solution (100 mM sodium chloride; 5mM $MgCl_2$; 100mL trisHCl, pH 9.5) at dark room for 15min. Reaction was stopped by soaking membrane in 20mM EDTA solution.

Western blot.

The gel containing protein (5.12µg and 5.64µg) was transblotted onto nitrocellulose membrane in 1L transfer buffer [2.2g glycine; 5.8g tris base; 0.37g SDS; 200mL methanol (v/v); add aquadest up to 1L, pH 7,4] at 90Volt for 90min. Nitrocellulose membrane was treated with the same procedure for dot blot.

ELISA.

0.5ng/uL of purified rhIFNα2b in PBS was loaded into microtiter plate. The plate was incubated at 4°C overnight and washed with 200µL of PBST twice and 200µL of TBS once for each well. Blocking of unspecific binding was done with 5% of skim milk in PBS at room temperature for 60min. The plate was washed, incubated with rabbit's serum (dilution 1:1000-1:10.000) for an hour and washed again. Plate was incubated with 200µL alkaline phosphatase-conjugate rat anti-rabbit IgG (dilution 1:5000 in PBS) at 37°C for 60min and washed. The yellow color were developed by adding 200µL of PNPP (1mg/mL in 10% dietanolamin) into each well. The plate was incubated at 37°C for an hour. The adsorption was read by ELISA-reader (BioRad) at 405nm.

RESULT AND DISCUSSION

Recombinant human IFNα2b over-production and purification.

The amount of protein (total protein of *E. coli*, rhIFNα2b IB, and purified rhIFNα2b) (Table I). The result of SDS-PAGE methods for *E. coli* pET32b IFNα2b total soluble proteins from induced condition (Figure 1). Thick bands of 33 kDa (theoretical size of protein fusion of rhIFNα2b) were observed which are indicating the correct protein.

Table I. Total Protein and Product yield

Fraction	Volume (mL)	Total protein (µg)	Product Yield(%)
E.coli pET32b IFNα2b's total protein	320	3.073	100
IB	12	1.686	55
Purified IB	6	1.060	34

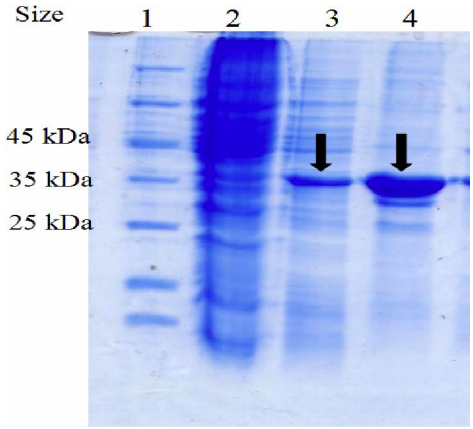

Figure 1. Coomassie briliant blue-stained 15% SDS–PAGE of protein overproduction and purification. 1 = protein marker, 2 = *E. coli* total soluble proteins from induced condition, 3 = refolded protein, 4 = purified rhIFNα2b from total refolded protein.

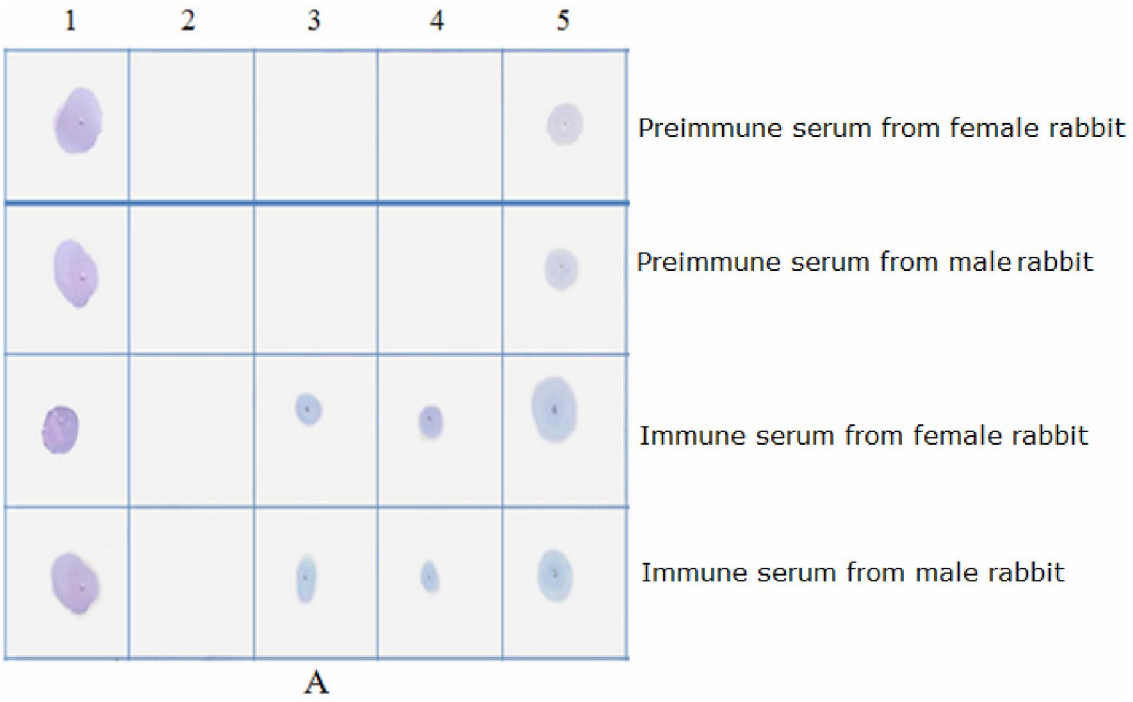

Figure 2. Dot blot of preimmune, immune and adsorbed rabbit sera. (A) Preimmune and immune sera. 1 = rat anti-rabbit IgG alkaline phosphate (positive control), 2 = elute buffer (negative control), 3 = 1.2 ng of purifiedrhIFNα2b, 4 = 0.645ng of purifiedrhIFNα2b, 5 = *E .coli* BL21 total protein. (B) Serum from female rabbit , (C) serum from male rabbit . 1 = 1.28µgof purified rhIFNα2b and *E. coli* BL21 total protein, 2 = 1.2µg purified rhIFNα2b (positive control), 3 = elute buffer (negative control), 4 =ratio volume of rabbit sera to *E. Coli* BL21 total protein.

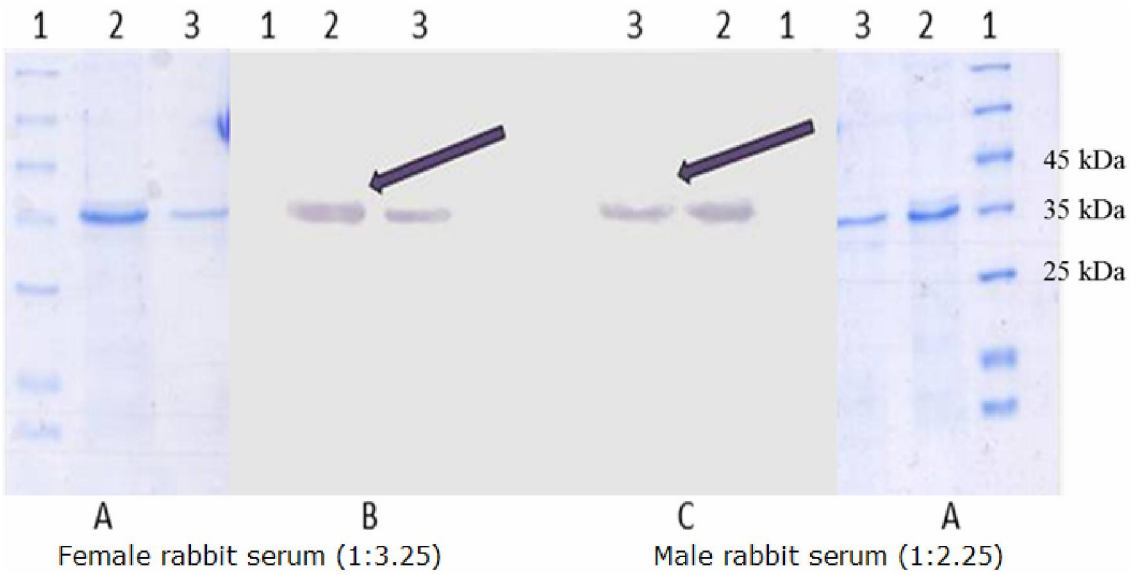

Figure 3. Characterization of rabbit sera by Western blot analysis using purified rhIFNα2b. 15% SDS-PAGE (A),Western blots of sera from female (B) and male (C) rabbits. 1 = marker protein, 2 = 4.2µg of purified rhIFNα2b from IB, 3 = 3.4µg of purified rhIFNα2b from supernatant.

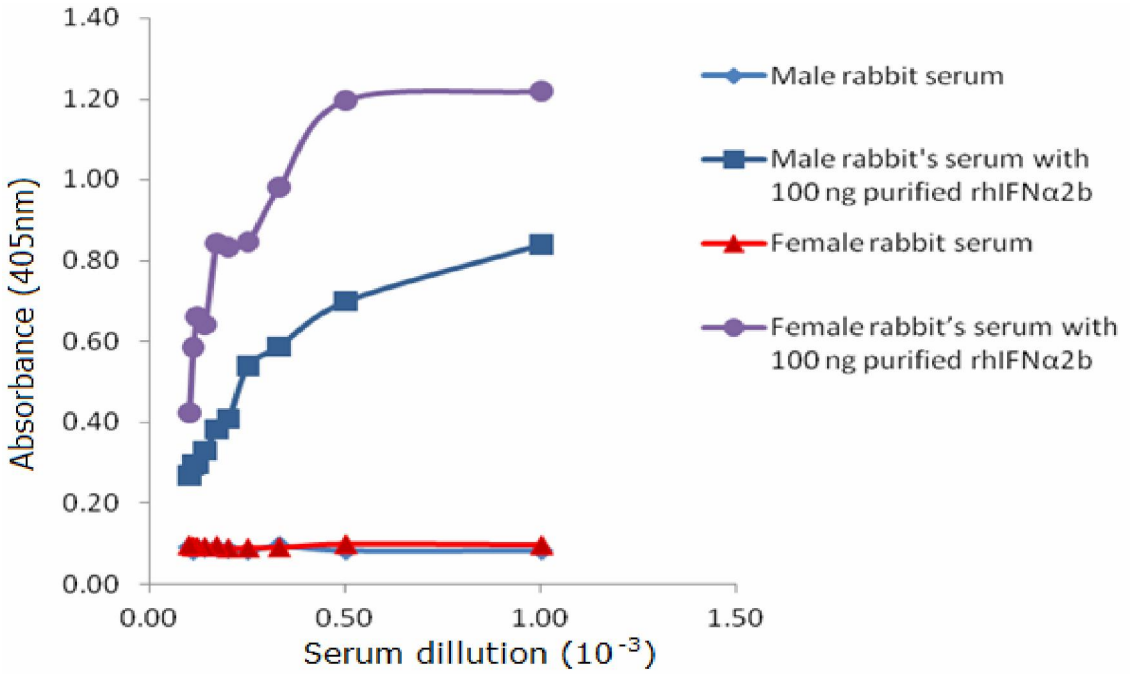

Figure 4.Titer assay of adsorbed polyclonal antibody against rhIFNα2b using ELISA.(▲) female rabbit serum without rhIFNα2b, (♦) male rabbit serum without rhIFNα2b, (■) male rabbit serum with 100 ngpurifiedrhIFNα2b; (●) female rabbit's serum with 100 ng purified rhIFNα2b.

The rhIFNα2b protein which is expressed in *E. coli* mostly will form inclusion bodies (IB) and only a little was obtained from cytoplasm [Srivastava *et al.*, 2005]. rhIFNα2b protein as IB can be refolded by 6 M urea [Tsumoto, Ejima, and Kumagai, 2003]. rhIFNα2b protein from cytoplasm did not used because the concentration was too low.

Production of polyclonal anti-recombinant human IFNα2b.

Dot blot result from serum before immunization and after 10 days of the first immunization are shown in figure 2. No positive colour was produced from serum taken before immunization, indicating no antibody produced yet.In contrast, serum taken after 10 days of the first immunization showed positive result for both female and male rabbit's sera. In order to remove the antibodies against *E.coli,* the adsorption of anti-total protein was done at various concentrations. The optimum adsorption from female rabbit's serum was 1:3.00, while the male rabbit's serum was 1:2.25 (no colour produced at those ratios).Dot blot results from adsorption anti-total protein of *E.coli* BL21 are shown in figure 2.

Polyclonal anti-recombinant human IFNα2b characterization.

The antibody produced was analyzed semi quantitatively with Western blot which is a specific method. Western blot analyses are shown in figure 3. Purified rhIFNα2b were run at 15% SDS-PAGE. These membranes (Figure 3.B and C) show thick and single purple bands at 33 kDa. The result of molecular weight by SDS PAGE was shown as a 33 kDa band. Theoritically, the size of human IFNα2b protein is 19 kDa. From our previous research, rhIFNα2b protein was generated as fusion protein with 33 kDa in size. This additional size was originated from pET32b expression vector (Retnoningrum, *et. al.*, 2010). The purpose of constructing the fusion protein containing six histidine amino acid was for purification using nickel affinity chromatography.

The proteins were transferred to nitrocellulose membrane. The nitrocellulose membranes, figure 3, show bands at 33 kDa

confirming the presence of anti-rhIFNα2b in rabbit's serum. The result from Western blot showed that anti-rhIFNα2b recognized rhIFNα2b. A single band at 33 kDa, means that adsorption of anti-total protein of *E. coli* was optimum.

ELISA was used to determine antibody titer in female and male rabbit's serum. To find out the influence of serum dilution, a serial dilution of serum was made (1:1000-1:10.000) without addition of rhIFNα2b. The result from this can be seen at Figure 4 and showed that the serum dilution did not have any influence in ELISA. The titer of both female and male rabbit's serum was 1:10.000. Absorbance from female rabbit's serum was higher than male. Generally, female mammalian generates higher antibody than male. This caused by esterogen hormone in female that increases inflammation response and antigen presenting process to T and B cells [Bilbo and Nelson, 2001]. The findings we reported here is meaningful in the area of analytical method in particular for detection of rhifnα2b. This protein is routinely produced in our laboratory for many purposes such as to study the pharmacokinetic profile, activity study in various model of immune-related diseases. As rhifnα2b is a potent therapeutic protein for many chronic diseases, the successful production of this macromolecules confirmed by immunology based-analysis such as Western blot and ELISA opens way to explore more about potential function of rhifnα2b as a drug.

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CONCLUSION

rhIFNα2b protein was successfully overproduced from *E. coli* pET32b IFNα2b. The protein was refolded and purified with nickel affinity chromatography. Purified rhIFNα2b protein was used to generate rhIFNα2b polyclonal antibody in *New Zealand white rabbits*. Polyclonal antibody was successfully generated confirmed with dot blot analysis.This antibody recognized protein at 33 kD, i.e the size of rhIFNα2b. Using protocol we developed, antibody was generated with high titer of 1:10,000 measured with ELISA.

REFERENCES

Asai, D.J. 1993. Antibodies in cell Biology**.** *Academic Press*. 16-50.

Bilbo, S.C. and R. J. Nelson. 2001. Sex Steroid Hormones Enhance Immune System in Male and Female Siberian Hamster. *Am. J. Physiol. Regul. Integr.*207-213.

Baldarrain, A., Y., Cruz, O., Navarro, and Gil, M. 2001. Purification and conformational properties of a human interferon α2b produced in *Escherichia coli*. *Biotechnol. Appl. Biochem.* 173-182.

Jonasch, E., F.G. Haluska**.** 2001**.** Interferon in Oncological Practice: Review of Interferon Biology, Clinical Applications, and Toxicities, *Oncology*, 6: 34-55.

Retnoningrum, D.S, Ningrum, R.A., Kurniawan, Y.N., Indrayati, A. and Rachmawati, H. 2010. Construction of synthetic open reading frame encoding human interferon alpha 2b for high expression in E.coli and characterization of its gene product, *J Biotech*. 145, 193-198.

Srivastava, P., P. Battacharaya, G. Pandey, and K.J. Mukherjee. 2005, Overexpression and purification of recombinant Human Interferon Alpha 2b in *Escherichia col.Protein Expr. Purif*. 41: 313-322.

Thitinan, S., and J. McConville. 2009. Interferon Alpha Delivery System for The Treatment for Hepatitis C, *Int. J. Pharm*. 369: 121-135.

Tsumoto, K., D. Ejima, and I. Kumagai. 2003. Practical consideration in refolding protein from inclusion bodies. *Protein Expr. Purif*. 1-8.