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Molecular Cloning and Heterologous Expression of Human Interferon Alpha2b Gene

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ABSTRACT

Human alpha Interferons (hIFNα) have been shown to have antiviral, antiproliferative and immunomodulatory activities. The human interferon alpha2b (hIFNα2b), is one of the human interferon alpha2 sub variants, naturally synthesized as a polypeptide of 188 amino acid residues, the first 23 residues of which represents a signal peptide. In the present study, the hIFNα2b gene was expressed after being fused with Glutathione S-Transferase (GST) gene. The hIFNα2b gene was amplified from human genomic DNA by using a pair of specific primers, cloned into an *Escherichia coli* expression vector and expressed in *E. coli* cells under the direction of the *tac* promoter. The expressed protein was purified using a one-step affinity chromatography column containing immobilized gluthatione-bound resin. The purified protein was shown to react specifically with anti-human-interferon-alpha antibody, confirming that the protein was the human interferon alpha molecule. This strategy has the potential to be used as an alternative mean for production of pure human interferon α proteins for therapeutic purposes and for further studies on their molecular characterization and mechanism of action.

Keywords: Recombinant Human Interferon Alpha2b, *Escherichia coli*, Protein Expression, Protein Purification

1. INTRODUCTION

The Interferons (IFNs) are glycoproteins involved in antiviral, antiproliferative and immunoregulatory process. The human interferons have been classified into three major types, alpha (leucocytes), beta (fibroblasts) and gamma (immune). To variety of different threats, the diversity of IFN provides different immune response (Tan *et al*., 2009).

IFNs, namely recombinant human IFNα2a and IFNα2b, have been approved by the U.S Food and Drug Administration for treatment of malignant tumors and viral diseases. Interferon therapy is used (in combination with chemotherapy and radiation) as a treatment for many cancers, AIDS related Kaposi's sarcoma and chronic hepatitis B and C. More than half of hepatitis C patients treated with interferon respond with viral elimination (sustained virological response), better blood tests and better liver histology (Salunkhe *et al*., 2009).

Human alpha interferons belong to a family of homologous proteins coded by a multiple gene family. The human interferon α genes are located on the short arm of Homo sapiens chromosome 9. All these genes lack introns. Among 13 IFNα genes, a total of 28 different sequence variants have been described. These variants differ from each other in one to four amino acid positions, but share the same receptor system and exert similar biological activities (Gull *et al*., 2013).

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The molecular weight of individual interferon protein varies from 19 to 20 kDa. The newly synthesized polypeptide consists of 188 and 189 amino acids, the first 23 of which constitute the hydrophobic signal peptide. This polypeptide is split off yielding the active mature form of interferon α which consists of 165 or 166 amino acids (Gull *et al*., 2013; Ningrum *et al*., 2013).

The hIFNα2b is one of the most studied hIFNα. It has been used to treat a number of malignancies, with the ability to induce antiproliferative, antiviral, antineoplastic and immunomodulating activities. Substantial amounts of hIFN*α*2b are required for the pharmaceutical industry as well as therapeutic use (Azaman *et al*., 2010).

Recombinant DNA technology has now made possible the large-scale production of interferon proteins for pharmaceutical applications. Genes encoding human alpha interferons have been cloned and expressed in recombinant strains of microorganisms and their products have been isolated in a very pure form. *E. coli* has been the most widely used host cells for production of recombinant interferons. Apart from synthesis of human interferon in the cytoplamic space, production of hIFNα2b in the periplasmic space of *E. coli*, in order to facilitate its downstream processing, has also been studied and optimized (Tan *et al*., 2009).

The aim of the present study was the isolation, molecular cloning and heterologous expression in *E. coli* cells of hIFNα2b genes in the form of Glutathione S-Transferase (GST)-IFNα2b fusion proteins. This study was also intended to initiate development of strategy for large-scale production of pure human interferon α proteins for therapeutic purposes in Indonesia.

2. MATERIALS AND METHODS

2.1. Isolation of Human Genomic DNA

Blood samples were obtained from a healthy donor. Genomic DNA was prepared from human white blood cells essentially according to the Puregene rapid DNA purification protocol for whole blood. As much as 5 mL blood was mixed with 15 mL red blood cell lysis buffer and incubated at room temperature for 10 min. The mixture was then centrifuged at 1500 rpm until a clear supernantant was observed. After discharging the supernatant, 1250 µL of white blood cell lysis buffer was added and the mixture was gently homogenized. Following the addition of 5 µL of RNAse (5 mg mL^{-1}), the mixture was homogenized and then incubated at 37°C for 15 min. Protein was precipitated by adding 833 µL of 5 M ammonium acetate. Following centrifugation at 3000 rpm, 4°C, for 15 min, the supernatant was collected and the DNA was then precipitated by adding 3850 µL of isopropanol. DNA was pelleted by centrifugation at 3000 rpm, 4°C, for 5 min. The pellet was washed with 4165 µL of 70 percent ethanol, dried and dissolved in Tris EDTA buffer. The purity of DNA was measured by spectrophotometry by determining the ratio of its absorbances at 260 and 280 nm (A_{260}/A_{280}).

2.2. Isolation and Nucleotide Sequencing of hIFNα2b Gene

The hIFNα2b gene was isolated by using the standard PCR amplification technique (Salunkhe *et al*., 2009) using the isolated human genomic DNA as a template. A pair of specific primers was employed. The nucleotide sequence of the forward primer was GGCTCACCCATTTCAACCAGTC and for the reverse primer it was GTCCTCTGTAAGGGACTAGTGC. The DNA fragment generated was purified using the QIA quick PCR purification kit, cloned into the pGEM-T vector and then sequenced using the ABI Prism 377 genetic analyzer. Nucleotide sequencing was carried out from forward and reverse directions, using T7 sequencing primer, TAATACGACTCACTATAGGGCGA and SP6 sequencing primer, ATTTAGGTGACACTATAGAATAC, respectively.

2.3. Cloning of hIFNα2b Gene into *E. coli* Expression Vector

The hIFNα2b gene was cloned into the pGEX-4T-2 *E. coli* expression vector using standard methods (Green and Sambrook, 2012). Two versions of hIFNα2b genes, one with and one without the signal peptide coding sequence, were cloned. In order to facilitate in-frame insertion of each gene into the expression vector, a pair of gene specific primers having restriction sites for BamHI and EcoRI was used to introduce these two restriction sites in the respective gene by using oligonucle-otide-directed PCR mutagenesis technique.

For introduction of BamHI and EcoRI sites into the hIFNα2b gene with signal peptide coding sequence, the nucleotide sequence of the forward primer was CAACATCTACAGGATCCATGGCCTTGAC and for the reverse primer was CTGGCATACGAATTCAATGAAAATC. The PCR amplified DNA fragment was digested with enzymes BamHI and EcoRI, fractionated by electrophoresis and the gel containing DNA fragment corresponded to the hIFNα2b gene plus signal peptide coding sequence was sliced out. The DNA fragment was then purified and ligated to pGEX-4T-2 previously digested with the same enzymes. Similarly, to introduce BamHI and EcoRI sites into the hIFNα2b gene without signal peptide coding sequence, the nucleotide sequences of the forward and reverse primers were GCTCTGTGGGATCCTGTGATCTG and CTGGCATACGAATTCAATGAAAATC, respectively. The PCR amplified DNA fragment was cut with BamHI and EcoRI enzymes, fractionated by electrophoresis and the gel containing DNA fragment corresponded to the hIFNα2b gene without signal peptide coding sequence was sliced out. Following purification, the DNA fragment was ligated to pGEX-4T-2 previously digested with the same enzymes. The correctness of each generated recombinant plasmid was analyzed by performing nucleotide sequencing of positive clones for each recombinant plasmid.

2.4. Expression and Purification of Recombinant hIFN-α2b Protein

Following the introduction of recombinant plasmids into *E. coli* BL21 host cells, protein expression experiments were carried out. Transformed BL21 cells were grown in 20 mL 2xYTAG medium containing 1% w/v yeast extract, 1.6% w/v tryptone, 100 mg mL^{-1} ampicillin, 2% w/v glucose and 0.5% w/v sodium chloride at 370C overnight. As much as 5 mL of this culture was used to inoculate 100 mL new 2xYTAG medium. The culture was incubated at 220C until it reached an Optical Density (OD_{600}) of 0.6-0.8. The expression of the interferon genes was induced by the addition of IPTG to give a final concentration of 0.1 mM followed by incubation at 22°C for 3 to 6 h. Cells were harvested by centrifugation and dissolved in PBS buffer containing 0.1 mM Phenylmethylsulfonyl Fluoride (PMSF). Suspension was sonicated for 1 min. Following the addition of 1% Tri-ton-X-100, the suspension was incubated on ice for 20 min followed by centrifugation. The supernatant was collected and passed through an affinity chromatography column containing an immobilized Glutathione-Separose-4B matrix. The flow through fraction was collected. The column was then washed several times with PBS buffer. The GST tagged-hIFNα2b proteins were eluted by using elution buffer containing 10 mM reduced glutathione in 50 mM Tris-HCl pH 8.0. The flow through and eluted fractions were analyzed using SDS-PAGE (Gasmi *et al*., 2011).

2.5. Western Blot Analysis

The protein bands generated from SDS-PAGE analysis were translated onto Polyvinylidene Fluoride (PVDF) membrane. The membrane was blocked by incubation 3 times in blotto solution (PBS buffer containing 5% skim milk) for 15 min. The membrane was incubated overnight in blotto solution containing anti-human interferon alpha2 monoclonal antibody as the primary antibody. Following removal of unbound primary antibody, the membrane was blocked as before. The membrane was then incubated in blotto solution containing secondary antibody rabbit-anti-mouse-IgG conjugated to horseradish peroxidase at room temperature for 1 h. The membrane was then washed and resulting protein bands were developed.

3. RESULTS

3.1. Human Genomic DNA and hIFNα2b Gene

The isolated human genomic DNA was of good quality having a DNA concentration of 840 ng μL^{-1}. Ratio of its absorbance's at 260 and 280 nm (A_{260}/A_{280}) was 1.8, indicating that the DNA was pure. The genomic DNA was used as a template for PCR amplification of the hIFNα2b gene. As expected, the PCR process resulted in a DNA fragment of 797 bp (**Fig. 1**). This fragment encompassed the putative 45 bp of upstream region of hIFNα2b gene, 567 bp of hIFNα2b coding sequence and 185 bp of downstream region of hIFNα2b gene. Following its nucleotide sequence analysis, the generated sequence was aligned with nucleotide sequence of interferon genes in the GenBank using standard methods. Results showed that the PCR-amplified-DNA fragment harbor the hIFNα2b gene (**Table 1**) marked by Guanine (G) at position 137 and Adenine (A) at position 170.

Table 1. The nucleotide sequence of human interferon α2b gene and the sequence of predicted encoded amino acids*

1	atg	gcc	ttg	acc	ttt	gct	tta	ctg	gtg	gcc	ctc	ctg	gtg	ctc	agc	tgc	aag	tca	agc	tgc
	M	A	L	T	F	A	L	L	V	A	L	L	V	L	S	C	K	S	S	C
61	tct	gtg	ggc	tgt	gat	ctg	cct	caa	acc	cac	agc	ctg	ggt	agc	agg	agg	acc	ttg	atg	ctc
	S	V	G	C	D	L	P	Q	T	H	S	L	G	S	R	R	T	L	M	L
121	ctg	gca	cag	atg	agg	aga	atc	tct	ctt	ttc	tcc	tgc	ttg	aag	gac	aga	cat	gac	ttt	gga
	L	A	Q	M	R	R	I	S	L	F	S	C	L	K	D	R	H	D	F	G
181	ttt	ccc	cag	gag	gag	ttt	ggc	aac	cag	ttc	caa	aag	gct	gaa	acc	atc	cct	gtc	ctc	cat
	F	P	Q	E	E	F	G	N	Q	F	Q	K	A	E	T	I	P	V	L	H
241	gag	atg	atc	cag	cag	atc	ttc	aat	ctc	ttc	agc	aca	aag	gac	tca	tct	gct	gct	tgg	gat
	E	M	I	Q	Q	I	F	N	L	F	S	T	K	D	S	S	A	A	W	D
301	gag	acc	ctc	cta	gac	aaa	ttc	tac	act	gaa	ctc	tac	cag	cag	ctg	aat	gac	ctg	gaa	gcc
	E	T	L	L	D	K	F	Y	T	E	L	Y	Q	Q	L	N	D	L	E	A
361	tgt	gtg	ata	cag	ggg	gtg	ggg	gtg	aca	gag	act	ccc	ctg	atg	aag	gag	gac	tcc	att	ctg
	C	V	I	Q	G	V	G	V	T	E	T	P	L	M	K	E	D	S	I	L
421	gct	gtg	agg	aaa	tac	ttc	caa	aga	atc	act	ctc	tat	ctg	aaa	gag	aag	aaa	tac	agc	cct
	A	V	R	K	Y	F	Q	R	I	T	L	Y	L	K	E	K	K	Y	S	P
481	tgt	gcc	tgg	gag	gtt	gtc	aga	gca	gaa	atc	atg	aga	tct	ttt	tct	ttg	tca	aca	aac	ttg
	C	A	W	E	V	V	R	A	E	I	M	R	S	F	S	L	S	T	N	L
541	caa	gaa	agt	tta	aga	agt	aag	gaa	tga											
	Q	E	S	L	R	S	K	E	*											

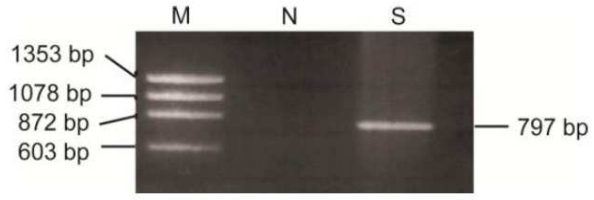

Fig. 1. PCR amplification of hIFNα2b gene. PCR was carried out using a pair of specific primers for human interferon α2. M, DNA marker; N, negative control; S, sample

3.2. Recombinant Plasmids pGEX-4T-2-hIFNα2b and pGEX-4T-2-hIFNα2b-w

Two versions of hIFNα2b genes, with and without the DNA segment encoding the signal peptide, were inserted into the *E. coli* expression vector pGEX-4T-2. PCR amplification using the primers to introduce BamHI and EcoRI sites into the hIFNα2b gene with signal pep-tide coding sequence, resulted in a DNA fragment of 630 bp. Following digestion with enzymes BamHI and EcoRI, a DNA fragment of 608 bp was generated. This fragment encompassed 567 bp of hIFNα2b coding sequence and 41 bp of its flanking sequences. The DNA fragment was then ligated to pGEX-4T-2 previously digested with the same enzymes (4960 bp) resulted in recombinant plasmid pGEX-4T-2-hIFNα2b (5568 bp). Similarly, PCR amplification to introduce BamHI and EcoRI sites into the hIFNα2b gene without signal pep-tide coding sequence, resulted in a DNA fragment of 558 bp. Digestion of the fragment with BamHI and EcoRI enzymes resulted in a DNA fragment of 539 bp. This fragment encompassed 498 bp of hIFNα2b coding sequence and 41 bp of its flanking sequences. Insertion of the fragment into pGEX-4T-2 previously digested with the same enzymes (4960 bp) resulted in recombinant plasmid pGEX-4T-2-hIFNα2b-w (5499 bp). The schematic diagram of the recombinant constructs is shown in **Fig. 2**. Nucleotide sequence analysis showed that the inserted hIFNα2b genes were in frame.

3.3. Heterologously Expressed hIFNα2b Protein

The two versions of hIFNα2b genes, with and without DNA segment encoding signal peptide, were heterologously expressed as fusion proteins with GST. As expected, protein bands of about 45 kDa and 43 kDa (**Fig. 3**) were generated following SDS-PAGE analysis. The protein bands of 45 kDa and 43 kDa corresponded to fusion proteins GST-hIFNα2b, with and without signal peptide, respectively. Western blot analysis of the 43 kDa protein (**Fig. 4**) showed that the protein reacted specifically to anti-human interferon alpha antibody confirming that the heterologously expressed protein was human interferon alpha.

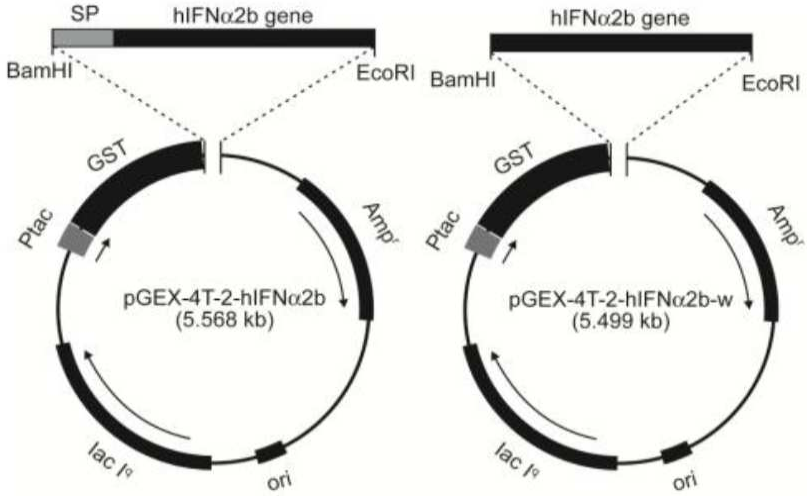

Fig. 2. Map for cloning of the human interferon α2b genes into the *Eschericia coli* expression vector. Schematic diagram of construction of recombinan plasmids pGEX-4T-2-hIFNα2b and pGEX-4T-2-hIFNα2b-w is shown. The relevant restriction sites are indicated. SP = DNA segment encoding signal peptide, hIFNα2b gene = the human interferon al-pha2b gene, GST = Glutathione S-Transferase gene, Amp^r = Ampicillin resistant gene, ori = origin of replication, lac I^q = lactose repressor gene, Ptac = the *tac* promoter

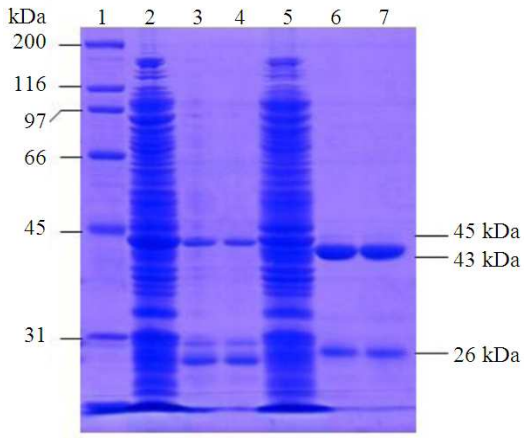

Fig. 3. SDS-PAGE analysis of GST-hIFNα2b protein expressed in *Escherichia coli* cells. The GST-hIFNα2b protein expression was carried out at 22°C, induced by addition of IPTG for 3 to 6 h. Cells were harvested and sonicated. The protein in the lysate was purified using affinity chromatog-raphy column containing immobilized Glutathione. The flowthrough and eluted fractions were analyzed. Lane 1 = protein marker, lane 2 = flowtrough of GST-hIFNα2b with signal peptide, lane 3 and 4 = eluted GST-hIFNα2b with signal peptide, lane 5 = flowtrough of GST-hIFNα2b without signal peptide, lane 6 and 7 = eluted GST-hIFNα2b without signal peptide

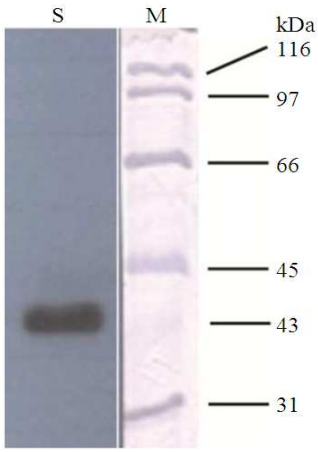

Fig. 4. Western blot analysis of GST-hIFNα2b protein. The protein bands generated from SDS-PAGE analysis were transblotted onto poliyvinylidene flouride (PVDF) membrane. The membrane was blocked and incubated overnight in solution containing anti-human interferon alpha2 monoclonal anti-body as primary antibody. Following removal of unbound primary antibody, membrane was blocked and incubated in solution containing secondary antibody rabbit anti-mouse-IgG conjugated to horseradish peroxidase at room temperature for 1 h. Membrane was washed and protein bands were developed. S = sampel, M = protein marker

4. DISCUSSION

In order to isolate the hIFNα2b gene from human genomic DNA to be cloned and heterologously expressed, the human genomic DNA was firstly isolated from human whole blood. Because the genomic DNA is only present in the nucleus of the white blood cells (lymphocytes), for genomic DNA isolation from whole blood, it is first necessary to remove erythrocytes using red-blood-cell lysis buffer. The genomic DNA was then released from white blood cells by using white-blood-cell lysis buffer. The white-blood-cell lysis buffer contained two major components, ethylene-Diamine-Tetraacetic Acid (EDTA) and Sodium-Dodecyl-Sulphate (SDS), which destroy membrane structure. EDTA removes magnesium ions that are important for maintaining the integrity of the cell membranes. It also inhibits the cellular enzymes which could degrade DNA. SDS aids the process of lysis by removing lipid molecules, thereby causing disruption of the cell membranes (Brown, 2010). The released genomic DNA was then purified by removing the two major contaminants, RNA and protein molecules. RNA was degraded using RNase and protein was removed by precipitation using ammonium acetate. Following precipitation using isopropanol, the genomic DNA was washed using 70% v/v ethanol to remove salt and small organic molecules. The resulting genomic DNA was then used as a template for PCR amplification of hIFNα2b gene. As the hIFNα2b gene contains no introns, the PCR-amplified hIFNα2b gene could directly be cloned into the pGEX-4T-2 expression vector following the introduction of flanking compatible restriction sites, BamHI and EcoRI sites. The hIFNα2b gene is marked by Guanine (G) at position 137 and adenine (A) at position 170.

For many purposes, it is important to produce interferon α2b in a highly purified form. In the present study, two versions of hIFNα2b genes, with and without the DNA segment encoding the signal peptide, were heterologously expressed in order to obtain hIFNα2b molecules corresponding to both the newly synthsized and the mature forms. To facilitate their purification and detection, a strategy to express interferon α2b in the form of recombinant hybrid containing a polypeptide fusion partner termed a GST tag was developed. The main purpose of tagging the interferon proteins with GST is to facilitate their purification from whole cell extracts by affinity chromatography. Binding of proteins is based on the selective interaction between the GST tag and the immobilized glutathione of the affinity resin. The GST tag is a large affinity tag consisting of 220 amino acids (26 kDa) forming dimer structures. Elution of the GST-tagged interferon protein was performed with free glutathione in the elution buffer. The affinity of GST for free glutathione is higher than the one for the immobilized glutathione bound to the agarose matrix. Therefore free glutathione replaces the immobilized glutathione which leads to release of the GST-hIFNα2b proteins from the matrix. The GST-tag can be detected using an enzyme assay or an immunoassay. Therefore, the GST-hIFNα2b proteins generated in the present study might be useful for preparation of pure hIFNα2b for therapeutic purposes. In addition, they may be of use for further molecular studies of hIFNα2b such as DNA-protein and protein-protein interactions to further elucidate their mechanism of action. When required, it is recommended to cleave the GST-tag from the fusion proteins by using a site-specific protease such as thrombin or factor Xa (Salunkhe *et al*., 2009).

The GST tagging was also intended to help in stabilizing the interferon protein and enhancing its solubility. The most challenging tasks in bacterial expression of foreign protein is the prevention of proteolytic degradation and accumulation of misfolded protein. Expression of proteolitically sensitive polypeptides by their fusion to other stable proteins has been shown to suppress degradation, although the mechanism responsible for the stabilization is not well understood (Salunkhe *et al*., 2009).

The insertion of the GST-hIFNα2b gene under the *tac* promoter was intended to yield high levels of transcription products which leads to high levels of synthesis of GST-hIFNα2b fusion protein. The *tac* promoter is a strong inducible promoter that gives very high rate of transcription initiation (Demain and Vaishnav, 2009). The *tac* promoter contains the *lac* operator sequence which is responsible for its transcription regulation. Synthesis of GST-hIFNα2b fusion protein was induced by addition of the lactose analog, IPTG, to the fermentation medium. The IPTG derepressed the binding of the *lac* repressor to the *lac* operator. IPTG is a convenient inducer for GST-hIFNα2b protein synthesis on the laboratory scale, but

it may not be suitable for industrial fermentation as it is prohibitively expensive. Lactose can be used as an inexpensive inducer but it is somewhat weaker. Although the *tac* promoter is a "leaky promoter" because it always has a basal level of transcription, even in the absence of inducer, the *E. coli* BL21 host cells grew well under both noninducing and inducing conditions. This indicates that the GST-hIFNα2b protein may not be toxic to the BL21.

Production of interferons by recombinant techniques is favoured by the biotechnological industry because of its homogeneity and better productive yields. In the pre-sent study, *E. coli* BL21 was used as a host cell for expression of GST-hIFNα2b protein. The primary reasons were that it is easier to manipulate and grow than the cells of higher life forms. The BL21 is the most common bacterial host and this has proven to be outstanding in used in recombinant expression. In addition, it is deficient in ompT and lon, two proteases which may be harmful to the GST-hIFNα2b protein being expressed, hence these may interfere with the isolation of intact GST-hIFNα2b protein. Although the optimal growth temperature for the *E. coli* BL21 cell is 37°C, expression of the GST-hIFNα2b protein was carried out here at 23°C. This lower temperature was employed so as to reduce proteolysis that may degrade the nascent GST-hIFNα2b proteins and to avoid protein misfolding. Further studies are needed to test the activity of the expressed GST-hIFNα2b protein and to explore strategy to upscale its production in Indonesia.

5. CONCLUSION

The results presented show that a strategy has been develop to isolate, clone and heterologously express the human interferon α2b gene. The expressed proteins can be purified using a one-step affinity chromatography column. The strategy, therefore, has the potential to be used as a mean for production human interferon α2b proteins for various purposes.

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