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High-level Soluble Expression and One-step Purification of HTLV-I P19 Protein in *Escherichia coli* by Fusion Expression

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ABSTRACT

Expression of HTLV-I p19 protein in an *Escherichia coli* expression system always leads to the formation of inclusion body. Solubilisation and refolding of the inclusion bodies is complex, time consuming and difficult during large-scale preparation. This study aimed to express and purify a soluble form of recombinant HTLV-I p19 protein in an *E. coli* expression system.

The synthetic DNA encoding the p19 was subcloned into a pGS21a vector along with a His-GST solubility/purification tag. The recombinant pGS21a-p19 vector was then transformed into chemically competent *E. coli* BL21 (DE3) cells, and expression of the recombinant His-GST-p19 protein was induced by IPTG. Expression and distribution of the His-GST-p19 protein in soluble and insoluble fractions were evaluated using SDS-PAGE. Antigenicity of the His-GST-p19 protein was evaluated using ELISA after purifying the protein using Ni-NTA affinity chromatography, then compared to the results of synthetic immunodominant p19 peptide ELISA.

The fusion His-GST-p19 protein accounted for 30% of the total cellular proteins. The SDS-PAGE results indicated that approximately 50% of the expressed His-GST-p19 proteins were soluble and accounted for 50% of the total soluble proteins. ELISA showed that the His-GST tag did not impair the antigenicity of the p19 protein and that the fusion protein reacted with HTLV-I antibodies in a concentration-dependent manner. The results of His-GST-p19 ELISA indicated that specificity of p19 reactivity was compatible to the results of p19 peptide ELISA.

Combination of key strategies for the soluble expression of proteins, like fusion with solubility/purification tags, low IPTG concentration and induction at low temperature, provide an efficient and facile platform for producing soluble HTLV-I p19 protein.

Keywords: Antigenicity; His-GST-tag; HTLV-I capsid; Purification

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INTRODUCTION

HTLV-I infections are endemic to south Japan, parts of Africa, Melanesia, South America and northeast Iran. Most HTLV-I-infected individuals are asymptomatic and are not aware about their infection. Such carriers transmit the infection to other healthy individuals through blood transfusion, sexual contact and breast-feeding. Early detection of HTLV-I asymptomatic carriers using screening methods is an important part of HTLV-I prevention strategy.[1]

Because of its high sensitivity and specificity, enzyme-linked immunosorbent assay (ELISA) is recommended as a routine method for screening patients with HTLV-I infection. Western blotting is then performed to confirm HTLV-I infection in specimens yielding positive results during ELISA.[2] In western blotting, simultaneous presence of specific antibodies against HTLV-I diagnostic antigens GD21 or MTA-I and against core protein p19 or p24 confirms the presence of HTLV-I infection.[2,3] P19 that is used for preparing western blotting strips is extracted from the viral lysate through an expensive and complex procedure. Therefore, an easy method to prepare high-quality p19 using an expression system could facilitate the preparation of diagnostic reagents. *Escherichia coli* can express high levels of foreign proteins, which help in reducing the cost of producing diagnostic reagents. High growth rate and easy manipulation are other advantages of bacterial expression systems.[4] However, expression of p19 protein in bacterial expression systems is difficult because gag proteins result in the formation of inclusion bodies.[5] Introduction of a solubility tag may have a positive effect on the biochemical properties of p19. Glutathione *S*-transferase (GST; molecular weight, 26 kDa) is soluble in the cytoplasm and is well established as a highly soluble fusion partner for high-level expression.[6] Using GST-tags, many insoluble proteins have been successfully expressed in their soluble forms in *E. coli* expression systems.[7,8]

In this study, we modified and synthesised the gene encoding HTLV-I p19 according to *E. coli* codon usage bias without altering the protein sequence. The recombinant DNA was subcloned into pGS21a expression vector and was successfully used for expressing soluble p19 protein in a bacterial expression system. In addition, we evaluated the binding capacity of the recombinant His-GST-19 protein using ELISA.

MATERIALS AND METHODS

Serum Samples

Serum samples were collected from patients with HTLV- I infection (patients who were positive for anti-HTLV-I antibodies) who were referred to Blood Transfusion Center, Mashhad, Iran. All the serum samples yielded positive results when tested using commercial immunodiagnostic ELISA kits (PASTEO HTLV-I/II ELISA kit) and western blotting (MP biomedical). Blood samples from healthy volunteers were used as controls and were tested using ELISA.

Computational Analysis of the Construct

The amino acid sequences of p19 were obtained from UniProt (http://www.uniprot.org/). As the amino acid sequences were identical, one sequence (Access No: P03345) was selected for subsequent analysis. The sequence was reverse translated to obtain the nucleotide sequence and codons optimised according *E. coli* codon usage using OPTIMIZER (http://genomes.urv.es/OPTIMIZER/). Molecular weight of the His-GST-p19 protein was predicted using ExPasy (http://web.expasy.org/protparam/).

Construction of Recombinant Plasmid Encoding N-terminally Tagged His-GST-p19 Protein

The DNA sequence encoding p19 was synthesised by Biomatik. The synthetic DNA and the pGS21a expression vector were digested with *BamHI* and *EcoRI*. After gel purification, the digestion products were incubated overnight with T4 ligase at 4°C. The ligation products were then transformed into chemically competent *E. coli* BL21 (DE3) cells, and the cells were grown on plates containing LB agar supplemented with 100 µg/ml ampicillin. Several surviving colonies were selected, and subcloning was verified by DNA sequencing.

Expression of the His-GST-p19 Protein

E. coli BL21 (DE3) colony harbouring the pGS21a-p19 expression vector was grown overnight at 37°C in 5 ml LB medium supplemented with 100 µg/ml ampicillin. The culture was inoculated into a baffled flask containing 100 ml LB medium supplemented with 100 µg/ml ampicillin and was incubated at 37°C until

optical density at 600 nm reached 0.6. Expression of the His-GST-p19 protein was induced by adding isopropyl β-D-1-thiogalactopyranoside (IPTG; final concentration, 0.4 mM) and by incubating the cells at 18°C for 12 h under constant shaking at 250 rpm. For expression analysis, cells present in 750 µl culture were harvested by centrifugation at 5000 rpm for 10 min, resuspended in 30 µl 2× sample loading buffer, heated at 100°C for 15 min and analysed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in 12% polyacrylamide gel. Non-induced cells (as negative controls) were analysed in parallel. Cells still present in the culture were harvested by centrifugation at 5000 rpm and 4°C for 10 min and were stored at -20°C for subsequent analysis.

Determination of His-GST-p19 Solubility

Cell pellets were resuspended in lysis buffer [sodium phosphate buffer (20 mM sodium phosphate; 500 mM NaCl), pH 7.4; 20 mM imidazole], and the cells were disrupted by sonication for 10 min, with 30-s intervals between the pulses. The cells were then centrifuged at 12000 rpm and 4°C for 20 min to segregated the cell lysate into two fractions supernatant and pellet that contained soluble and insoluble proteins, respectively. Both the supernatant and the pellet were analysed using SDS-PAGE in 12% polyacrylamide gel.

Protein Purification

The supernatant was centrifuged at 12,000 rpm and 4°C for 20 min to remove any insoluble material. Next, the supernatant was transferred to a 1 × 5-cm column packed with 1-ml nickel-nitriloacetic acid (Ni-NTA) resin. The resin was washed with three column volumes of binding buffer [sodium phosphate buffer (20 mM sodium phosphate; 500 mM NaCl), pH 7.4; 20 mM imidazole] and five column volumes of washing buffer [sodium phosphate buffer (20 mM sodium phosphate; 500 mM NaCl), pH 7.4; 100 mM imidazole]. Adsorbed His-GST-p19 protein was eluted from the resin using an imidazole gradient [sodium phosphate buffer (20 mM sodium phosphate; 500 mM NaCl), pH 7.4; 500 mM imidazole]. A flow rate of 0.5 ml/min was used in all the chromatographic steps.

Enzyme Linked Immunosorbent Assay (ELISA)

Wells of ELISA plate were coated with His-GST (1–25 µg/ml) or p19 immunodominant peptide (1-25 µg/ml) in 50 mM sodium bicarbonate buffer (pH 9.6) and were incubated overnight at 4°C. The coated wells were washed twice with phosphate-buffered saline (PBS) and were blocked with blocking buffer [3% bovine serum albumin in PBS with 1% Tween-20 (PBST)] for 2 h at room temperature. Next, 50 µl of the infected serum were added to the plates, and the plates were incubated for 1 h at 37°C. Secondary anti-human horseradish peroxidase-conjugated antibodies were added to the plates, and the plates were incubated for 1 h at 37°C. After washing six times with PBST, 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was added to the plates, and absorbance was read at 450 nm after 30 min. His-GST was used as the negative control under the same conditions.

RESULTS

Theoretical Evaluation of the Construct

To eliminate adverse effects of rare codons on p19 expression in *E. coli* cells, new sequence was designed and the preferential codons were employed using OPTMIZER tool (Figure 1). The TAATGA stop codons in series, which is the most efficient in translation termination in *E. coli* was added to the end of new sequence to increase translation termination efficiency. After codon optimisation, GC content increased from 55% to 58%, CAI (codon adaptation index) content increased from 0.79 to 0.90 and the most preferred codon frequency increased from 43% to 78%, indicating that the synthetic gene could be overexpressed in the *E. coli* expression system.

Construction of the Expression Vector

The DNA sequence encoding p19 was double digested and subcloned into the pGS21a expression vector. This construct was verified by DNA sequencing results. In this recombinant plasmid, the p19 was fused with His-GST tag under the control of T7 promoter (Figure 2). This solubility/purification tag preceding p19 coding sequence enable fast and easy purification of p19 using affinity chromatography. The enterokinase cleavage site between p19 and His-GST tag permits easy recovery of p19 from fusion protein after enterokinase treatment.

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Native          851 CGCTAGCCCTATTCCGCGGCCGCCCCGGGGGCTGGCCGCTCATCACTGGC  900
                    ||.||||||||.|||||.||.||.|||||.|||||.|||||||
Synthetic DNA     1 --------CCGATTCCGCGTCCGCCGCGTGGTCTGGCGGCTCACCACTGGC   43

Native          901 TTAACTTCCTCCAAGCGGCATATCGCCTAGAACCCGGTCCCTCCAGTTAC  950
                    |.||||||.||.||||||.||.||.||.||.||||||.|||||.||...||||
Synthetic DNA    44 TGAACTTTCTGCAAGCTGCGTACCGTCTGGAACCGGGTCCGTCTTCTTAC   93

Native          951 GATTTCCACCAGTTAAAAAAATTTCTTAAAATAGCTTTAGAAACACCAGT  1000
                    ||.||||||||||.|.||||||.||.||.||||||.||..|.||||| ||.|.
Synthetic DNA    94 GACTTCCACCAGCTGAAAAAGTTCCTGAAAATCGCGCTGGAAAC-CCCGG   142

Native          1001 CT-GGATCTGTCCCATTAACTACTCCCTCCTAGCCAGCCTACTCCCAAAA  1049
                     || |.||||||.||.||.||||||||.||.||.||...|||.||.||.|||
Synthetic DNA    143 CTCGTATCTGCCCGATCAACTACTCTCTGCTGGCGTCCCTGCTGCCGAAA   192

Native          1050 GGATACCCCGGCCGGGTGAATGAAATTTTACACATACTCATCCAAACCCA  1099
                     ||.||.||.||.||.||.||.||.||||||..|.||||||.||.||||||.||||||
Synthetic DNA    193 GGTTATCCGGGTCGTGTTAACGAAATCCTGCACATCCTGATCCAGACCCA   242

Native          1100 AGCCCAGATCCCGTCCCGTCCCGCGCCACCGCCGCCGTCATCCCCCACCC  1149
                     .||.||||||.||||||.||||||.||.||.||||||||||||.||.||.||||
Synthetic DNA    243 GGCGCAGATTCCGTCTCGTCCGGCACCGCCGCCGCCGTCTTCTCCGACCC   292

Native          1150 ACGACCCCCCGGATTCTGATCCACAAATCCCCCCTCCCTATGTTGAGCCT  1199
                     ||||.||.||||||.||||||.||.||.||||||.||.||.|||||||||.||.
Synthetic DNA    293 ACGATCCGCCGGACTCTGACCCGCAGATCCCGCCGCCGTATGTTGAACCG   342

Native          1200 ACGGCCCCCCAAGTCCT
                     ||.||.||.||.||.||
Synthetic DNA    343 ACCGCTCCGCAGGTTCT
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Figure 1. Comparison of nucleotide sequence of native and altered p19 gene.

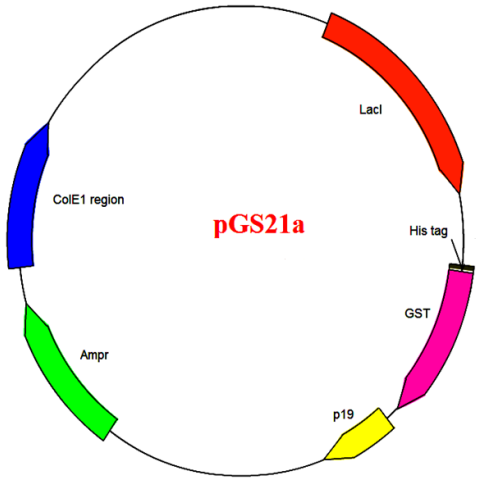

Figure 2. Schematic diagram of the construct used for fusion expression of recombinant p19. The p19 gene was cloned downstream of the His-GST tag in pGS21a expression vector, which also contained the gene for lac repressor (LacI) and ampicillin resistance.

Expression of the His-GST-p19 Protein

The recombinant plasmid was then transformed into chemically competent *E. coli* BL21 (DE3) cells, and the subcloning accuracy was validated by DNA sequencing. LB medium was used for seed preparation and target protein expression. After IPTG induction for 12 h at 18°C, the lysate of E. *coli* BL21 (DE3) cells harbouring the His-GST-p19-encoding plasmid was analysed by performing SDS-PAGE in 12% polyacrylamide gel. SDS-PAGE showed a 48-kDa band, which was approximately similar to the predicted molecular weight of the His-GST-p19 protein. This band was not observed in the SDS-PAGE analysis of the cell lysate of non-induced *E. coli* BL21 (DE3) cells. The fusion protein accounted for 30% of the total cellular proteins, as determined by protein scanning of coomassie blue-stained SDS-PAGE gels (Figure 3).

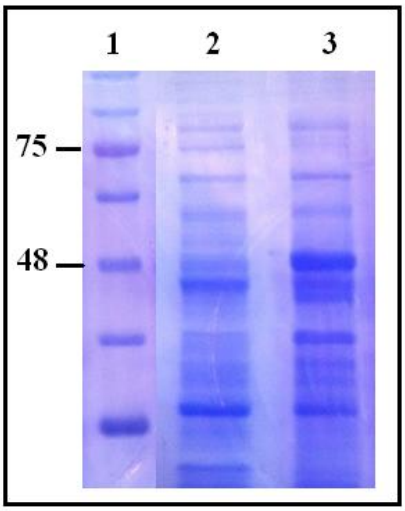

Figure 3. Expression analysis of the His-GST-p19 protein by SDS-PAGE in 12% polyacrylamide gel. Non-induced *E. coli* BL21 (DE3) cells harbouring the recombinant plasmid were used as negative controls. Lane 1, protein marker; lane 2, non-induced control cells containing pGS21a and lane 3, induced bacterial cells containing pGS21a-p19. His-GST was expressed at a molecular weight of approximately 48kDa.

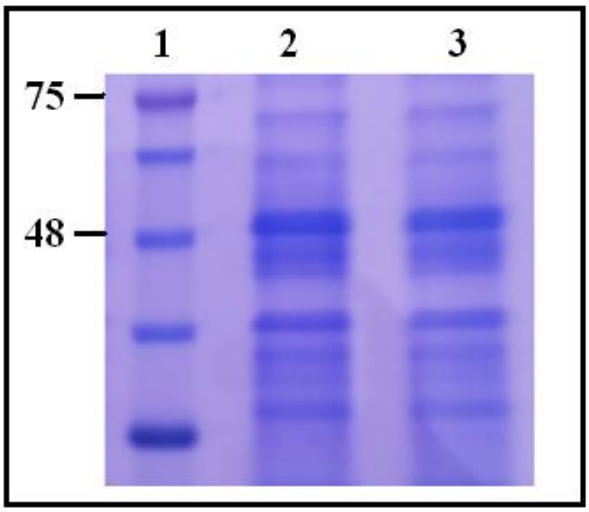

Figure 4. Determination of the solubility of the His-GST-p19 protein. Solubility was analysed by performing SDS-PAGE in 12% polyacrylamide gel. Lane 1, protein marker; lane 2, supernatant and lane 3, pellet.

Solubility Determination

To evaluate the distribution of the His-GST-p19 protein in the soluble and insoluble fractions, the supernatant and pellet were analysed by SDS-PAGE which showed that even after the incorporation of the GST solubility tag, the p19 protein existed in both the supernatant and the pellet (Figure 4). These results clearly showed that the E. coli strain BL21 (DE3) is suitable host for soluble expression of p19.

The SDS-PAGE analysis revealed that the recombinant proteins were most soluble at 18 °C with solubility of about 50% or higher. According to densitometric scanning results and evaluation of Bradford assay, His-GST-p19 accumulated up to about 35% of soluble proteins and approximately 30mg of soluble forms was produced per litter of culture.

Purification of the His-GST-p19 Protein

For purifying the His-GST-p19 protein, the soluble fraction of the cell lysate was passed through a column containing 1.5 ml Ni-NTA resin. SDS-PAGE in 12% polyacrylamide gel was used to analyse the eluted fractions and showed that the supernatant primarily contained the His-GST-p19 protein and lower concentrations of other proteins. The purity of His-GST-p19 fusion protein in affinity purification step increased from 30% to 85% (Figure 5). These results indicated that purification of the His-GST-p19 protein required further optimisation.

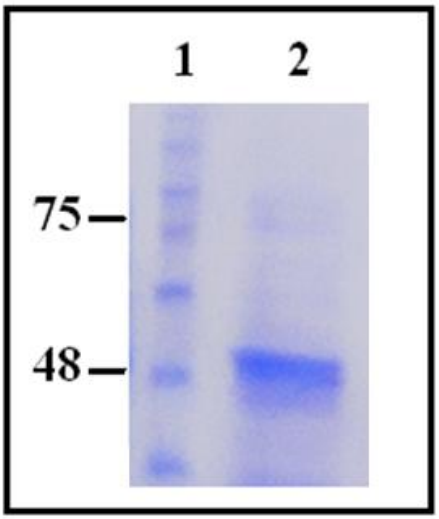

Figure 5. SDS-PAGE analyses of the His-GST-p19 protein purified using Ni-NTA agarose column. Lane 1, protein marker and lane 2, purified His-GST-p19 from different elution.

ELISA

Binding activity of the His-GST-p19 protein to antibodies present in HTLV-I-infected serum was determined using ELISA. The results showed that the His-GST-p19 protein could bind to anti-HTLV-I antibodies in concentration-dependent manner (Figure 6, 7). Also; comparison of P19 peptides ELISA results with fusion His-GST-p19 indicated that these interactions were highly specific (Table 1). ELISA results indicated that peptide show high OD450nm absorbance in comparison to commercial kit and His-GST-p19. This may to be due to high immunodominant epitope present on antigen

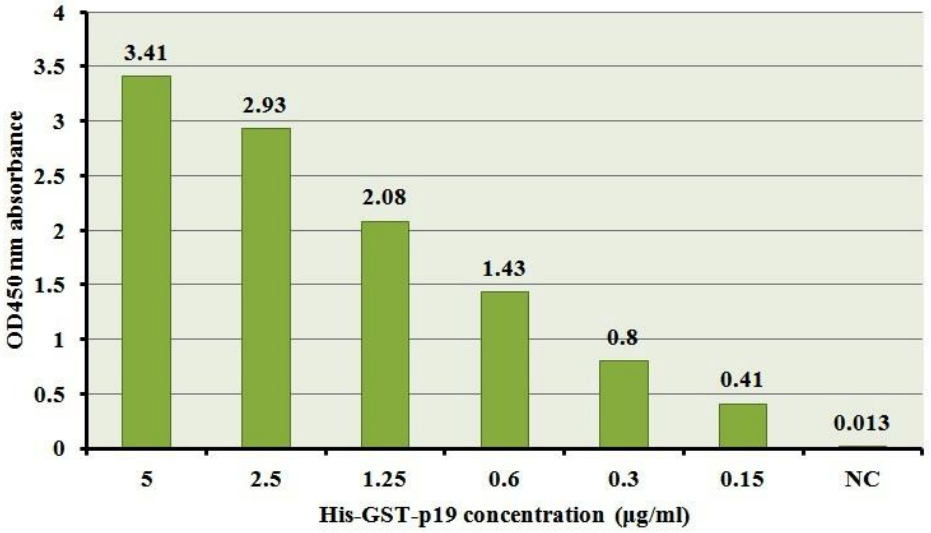

Figure 6. Binding activity of the His-GST-p19 protein towards pooled infected sera. Results of ELISA showed that the His-GST-p19 protein reacted with antibodies present in the pooled infected serum (fixed level) in a concentration-dependent manner. His-GST was used as the negative control at the same condition.

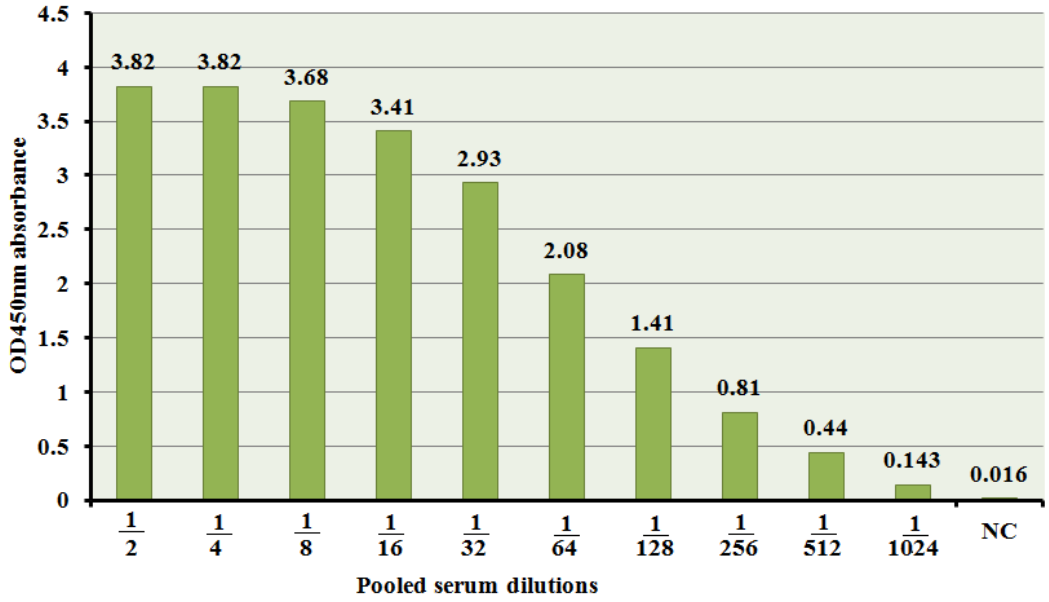

Figure 7. Serial dilution of pooled serum for evaluation of His-GST-p19 antigen- anti-HTLV-I antibodies interaction. Pooled sera of HTLV-I infected diluted using PBS buffer. Pooled healthy serum samples were used as negative control (NC: negative control).

Table 1. Results of HTLV-I detection in sera of 30 HTLV-I carrier blood donors.

Number	Commercial Kit	P19 peptide ELISA	His-GST-p19 ELISA
1	1.850	2.341	1.327
2	2.447	2.136	1.972
3	1.892	1.919	1.302
4	2.704	2.596	1.797
5	1.527	1.899	0.814
6	1.890	1.878	1.281
7	1.757	1.918	1.312
8	1.220	2.150	0.732
9	1.665	2.097	0.838
10	1.926	2.158	1.126
11	1.353	1.066	1.276
12	2.035	0.762	1.287
13	2.058	2.412	1.039
14	1.133	1.612	1.209
15	1.470	1.957	0.595
16	1.981	2.302	1.347
17	1.909	2.219	0.710
18	1.826	2.021	1.084
19	1.635	2.231	0.933
20	2.055	2.066	1.095
21	1.429	1.755	0.516
22	1.547	2.047	1.132
23	1.469	2.151	0.460
24	2.058	2.185	1.417
25	1.432	1.773	0.630
26	1.775	2.078	0.575
27	2.138	1.945	0.953
28	1.423	1.907	0.724
29	1.158	1.333	0.433
30	1.150	2.267	0.830

DISCUSION

E. coli is the most commonly used expression system because of its high growth rate and easy to manipulation.[4] However, two major barriers exist in p19 expression using *E. coli* as host cell: inclusion bodies formation and codon preference.[9] GST is a highly soluble protein; therefore, expression of proteins by fusion with GST is an accepted strategy for expressing soluble proteins.[10] GST-tag is more efficient when positioned at the N-terminal rather than at the C-terminal end.[11] Many aggregation prone proteins have been successfully expressed in their soluble forms in *E. coli* using GST-tag as the fusion partner.[12-14] In addition to expressing the proteins in their soluble form, GST-tag mildly enhances the expression of the recombinant protein.[11] Generally, DNA sequences encoding viral proteins often contain codons that are rarely used in prokaryotic cells. It is difficult to overproduce proteins from sequences containing rare codons because tRNAs involved in translation are rapidly depleted during protein expression.[15] Hence, new DNA sequence encoding p19 was synthesized with perfereed codons in *E.coli* and fused with His-GST tag. High expression of soluble p19 fusion protein was achieved. Results of densitometry scanning assay showed that the His-GST-p19 protein accounted for 30% of the proteins in the cell lysate. We believe that this high expression may be

because of codon optimisation.

It has been suggested that low induction temperature and inducer concentration could further improve the solubility of the His-GST-p19 protein.[16] In this study, we use 0.4mM IPTG and 18 °C for induction of p19 expression. As indicated by SDS-PAGE, under this condition *E. coli* expressed the fusion protein in both soluble and insoluble forms. Because p19 expression leads to the formation of an inclusion body,[9] expression of the soluble p19 might be because of the presence of the GST fusion. However, high concentration of the His-GST-p19 protein was still present in the insoluble form. This finding was consistent with that of other reports.[17-19] In some cases, GST fusion may cause some of the target protein to form inclusion bodies.

Proteins without the His-tag were removed from the Ni-NTA resin by the washing buffer, and the His-GST-p19 protein was eluted using the elution buffer. SDS-PAGE analysis of the eluted proteins primarily showed a single 48-kDa band corresponding to the His-GST-p19 protein. Using this method, approximately 40mg protein could be purified from 1 lit baffled flask, followed by 30% recovery.

The His tag GST fusion was used as a negative control during ELISA. Previous data about GST tag immunoreactivity are controversial. A few studies have shown that the GST-tag interferes with antibodies during ELISA. Different studies have shown that the GST-fused does not react with sera from healthy humans.[20-22] Li et al showed that although GST was derived from Schistosoma japonicum, recombinant GST-tagged proteins reacted with only one of 88 sera from patients with schistosomiasis.[23] Thus, we used the GST-His-tag as a control during ELISA to negate the any reaction antibodies with this fusion protein.

Both fusion p19 and C-terminal peptide showed similar binding activity to HTLV-I infected serums. As demonstrated, the purified p19 protein could specifically react with antibodies in HTLV-I-infected serum.

Expression of soluble proteins in *E. coli* has been previously performed using thioredoxin tag that enhances the solubility of the recombinant proteins. However, GST-fusion has several advantages over thioredoxin fusion. GST catalyses the addition of thiol from glutathione to various hydrophobic and electrophilic molecules. Thus, GST is useful for monitoring protein production and purification because of its catalytic activity. A major advantage for using GST-fusion is that it can be coupled with other affinity strategies, e.g. His6 tag, to improve protein purification. Moreover, Ding et al suggested that greater solubility facilitates the folding and enhances the immunogenicity of fusion proteins. The finding of this study supports our finding of strong reactivity of coated p19 proteins observed during ELISA.

Results of 60 clinical serum samples detection by p19-ELISA showed 100% concordance rate with commercial kit. No cross reactivity was detected with positive sera against healthy controls. These data indicated that the coating antigen processed good specificity.

As shown in table 1, peptide based ELISA gave slightly higher antibody-antigen interaction. A possible explanation might be conformational hindrance of main epitope in full length protein.

In summary, the *E. coli* expression system producing active p19 protein has been developed. In comparison to previous study,[9] codon optimisation of the p19 DNA sequence and GST fusion resulted in high expression, high solubility and facile purification of the fusion protein. This recombinant protein has high sensitivity and specificity for detecting anti-HTLV-I antibodies.

In this study, we described the preparation of soluble HTLV-I p19 protein from *E. coli* as an alternative to the expensive production of the p19 protein from viral lysates. Integration of the GST-fusion and induction of protein expression at low temperature are excellent strategies for expressing active soluble p19 protein in the *E. coli* expression system.

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