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High-level Expression and One-step Purification of Chimeric Antigen Containing HTLV-I/-II Diagnostic Epitopes in *Escherichia coli*

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ABSTRACT

Purification and preparation of three diagnostic antigens used for the detection of human T-lymphotropic virus (HTLV)-I/-II infection in *E.coli* are different parts of a multi-step method. In this study, our aim was to design a chimeric protein for the simultaneous detection of HTLV-I and HTLV-II antibodies.

Immunodominant B cell linear epitopes of envelope and capsid proteins of HTLV-I/-II were selected and linked together; using a suitable amino acid linker and a chimeric antigen (CA). The codon-optimized synthetic DNA encoding the CA was subcloned into the pGS21a expression vector and CA expressed as His-GST fused protein in *E. coli* BL21 (DE3) cells. Then the recombinant CA was purified, using the Ni-NTA (Nickel Nitrilotriacetic acid) affinity chromatography under native conditions.

The Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and densitometric scanning results showed that CA accounted for 15% of the total cellular proteins and approximately 50% of the expressed histidine-glutathione s-transferase-chimeric antigen (His-GST-CA) proteins were soluble. The CA was successfully purified in one step with a purity of greater than 90%, which is suitable for antigenicity evaluations. Enzyme-linked immunosorbent assay (ELISA) results showed that the GST fused CA reacted in a concentration-dependent manner with HTLV-I/-II infected sera and was able to distinguish normal serum from HTLV-I/-II infected one with a proper sensitivity.

With further validation, CA, as described in the present study could be introduced as a novel reliable, cost-effective and easy alternative for the three separate HTLV-I/-II diagnostic peptide antigens, which is prepared as a fusion with GST.

Keywords: Antigenicity; Chimeric antigen; Enzyme-linked immunosorbent assay (ELISA); Human T-lymphotropic virus (HTLV); Protein purification

INTRODUCTION

The human T-lymphotropic virus type 1 (HTLV-1) and type-II (HTLV-2) viruses are human retroviruses

that have been reported endemic infection in the north eastern region of Iran and elsewhere worldwide.¹ In the world, about 10 to 20 million people are infected with these viruses. HTLV-I Associated Myelopathy/

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tropical spastic paraparesis (HAM/TSP) and Adult T cell Leukemia (ATLL) are the complications of infection with these viruses. Most people who carry these viruses, lack clear clinical symptoms and may not be aware of their infection. These people transmit the virus through sexual intercourse, breastfeeding or the use of common syringes in other healthy people.²

The best way to prevent the spread of HTLV-I/-II infections is by screening and identifying infected people. The presence of specific antibodies against the HTLV-I/-II viruses indicates the potential infection. Enzyme-linked immunosorbent assay (ELISA) is used for screening due to its high sensitivity and specificity. In individuals with positive ELISA, the Western Blot will be used to confirm the infection.³

Peptides are used to detect anti-HTLV-I/-II antibodies. MTA-I specifically reacts with HTLV-I antibodies, whereas K55 specifically targets anti-HTLV-II antibodies. The peptide GD-21 peptide reacts with the antibodies of both viruses.³ Because small peptides are not well expressed in bacteria, these peptides are usually expressed as a fusion with GST in *E. coli*.⁴ However, the expression and purification of the three peptides individually are difficult and time-consuming. Therefore a number of chimeric antigens are designed for HTLV-I/-II detection.⁵ Such chimeric antigens form the inclusion body when they are expressed in *E. coli*. Although purification of the inclusion body is simple, the refolding process is very complex and requires the optimization for each protein individually.⁶

The introduction of a solubility tag may increase the solubility of proteins. Glutathione S-transferase (GST) is soluble in the cytoplasm and is well established as a highly soluble fusion partner. Using GST-tags, many insoluble proteins have been successfully expressed as soluble forms in *E. coli*.⁷⁻⁹

In this study, our aim was to design a chimeric antigen, consisting of three peptides that can simultaneously detect HTLV-I and HTLV-II antibodies with sufficient sensitivity and specificity.

MATERIALS AND METHODS

Materials

After approving the protocol of the study in Ethics board (IR.PNU.REC.1398.002), all materials were purchased from Sigma unless otherwise noted. Restriction enzymes were purchased from Takara

(Japan), Ni-NTA was obtained from Qiagen (Valencia, CA), and the standard molecular weight of the protein was purchased from Sinaclon (Iran).

Serum Samples

All serum samples used in this study belonged to those who referred to the Blood Transfusion Center of Mashhad, and in whom HTLV infection was confirmed, using ELISA and Western blotting. Samples used as controls were tested for HTLV, using ELISA (MP Diagnostics™ HTLV-I/II ELISA 4.0).

Structural Design

Sequences of gp46-I (Entry: P14075), gp-21 (Entry: P14075) and gp46-II (Entry:P03383) were selected from the Uniprot database (<https://www.uniprot.org/>). Initially, antigenic fragments of gp46-I (amino acids 160-220), gp21 (amino acid 360-410) and gp46-II (amino acids 160 to 210) were linked together; using an amino acid linker [sequence: (EAAK)₄]. Tertiary structures predicted by Phyre2 online tool (<http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index>). The three-dimensional structure of the protein was investigated in terms of exposure of epitopes using PyMol software. The amino acid sequence was then translated to the nucleotide sequence, using the reverse translator software (http://www.bioinformatics.org/sms2/rev_trans.html). The nucleotide sequence was optimized, using Optimizer software (<http://genomes.urv.es/OPTIMIZER/>). Optimization efficiency was analyzed; using rare codon analysis online tools (<https://www.genscript.com/tools/rare-codon-analysis>).

pGS21a-CA Construction

The DNA encoding the chimeric protein was synthesized by Biomatik and subcloned into the pGS21a vector according to standard protocols, using the *Bam*HI and *Eco*RI enzymes. The subcloning accuracy was verified by DNA sequencing. The vector was transformed into chemically competent *E. coli* BL21 (DE3) and was cultured on ampicillin-containing LB medium (100 µg/mL). Several colonies, survived on an antibiotic medium, were selected for further analysis.

His-GST-CA Expression

A colony containing pGS21a-CA was grown in a Lysogeny broth (LB) medium containing ampicillin

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(100 µg/mL) for one night with a consistent shaking at 250rpm. From overnight culture, 500 µL was transferred to 50 mL of fresh LB, containing ampicillin (100 µg/mL), and incubated with continuous shaking at 250rpm at 37°C, until OD₆₀₀ is reached to 0.6. Then, protein expression was induced by the addition of IPTG (final concentration 0.4 mM) and grown at 15°C for 18 h. The cells were harvested by centrifugation at 5000×g at 4°C for 15 min.

Protein Purification

One and a half milliliters of Ni-NTA resins were transferred to a column of 5 x 1 cm. This column was washed three times, using a binding buffer [sodium phosphate buffer (20 mM sodium phosphate; 500 mM NaCl), pH 7.4; 20 mM imidazole] and washed 5 times with a wash buffer [sodium phosphate buffer (20 mM sodium phosphate; 500 mM NaCl), pH 7.4; 100 mM imidazole]. The proteins absorbed into the column were eluted using elution buffer [sodium phosphate buffer (20 mM sodium phosphate; 500 mM NaCl), pH 7.4; 500 mM imidazole]. A flow rate of 0.5 milliliters per minute was used in all chromatography steps. The purified proteins were dialyzed overnight at 4°C against PBS.

Enzyme-linked Immunosorbent Assay (ELISA)

The purified His-GST-CA was dissolved in 50 mM sodium bicarbonate buffer (pH 9.6) with concentrations of 0, 1.25, 2.5, 5, 10, 20, and 25 µg/mL and coated in the wells of ELISA plate. The plates were incubated for one night at 4°C. The coated wells were washed twice with phosphate-buffered saline (PBS) and were blocked with 1% BSA at 37°C for 2 hours. After twice washing with PBS, 50 µL of pooled contaminated serum (consisted of 10 infected sera) was added to each well and incubated for one hour at 37°C. After twice of wash, 100 µL horseradish peroxidases (HRP)-conjugated antibody was added and incubated for one hour at 37°C. After washing six times by PBS with 1% Tween-20 (PBST), 100 µL of 3, 3', 5, 5'-tetramethylbenzidine (TMB) solution was added to each well and incubated for 15 minutes in darkness. At the end of the incubation period, the reaction was terminated by the addition of the stopping solution (H₂SO₄ 2N) and the absorbance was read at 450 nm by ELISA reader. His-GST was used as negative control under the same conditions. All experiments were repeated two times.

Sensitivity and Specificity Analysis

Three positive sera of different antibody levels were diluted from 1:100 to 1:500 and then detected by the peptide ELISA to analyze the sensitivity.

The specificity of peptide ELISA was determined with sera of patients positive to HCV, HBV and HAV and HTLV-I/II negative sera.

Statistical Analysis

All statistical analyses were performed using Stata software version 15 (Stata Crop LP, College Station, Texas). All results were given as mean±SD. Data were compared with ANOVA or an independent *t*-test. A significant level of 0.05 was considered for all analyses.

RESULTS

In Silico Design of Chimeric Antigen (CA)

Immunodominant fragments of gp-46, gp-21, and gp-46-II were selected and fused using linker sequences. The three-dimensional structure of the CA is shown in Figure 1. According to epitopes exposure analysis results; using PyMol software (data are not shown), epitopes are located at the surface and theoretically, they can react with HTLV-I/-II antibodies.

Computational Expression Optimization

After optimization of DNA, the codon adaptation index (CAI) value was 0.86 and the GC value was 55.98%, which, according to the software guide, shows that the sequence can be well expressed in *E.coli*. After the optimization, there was no rare codon in the optimized sequence of the construct.

Construction of the Expression Vector

The recombinant vector prepared in this study is shown in Figure 2. The gene encoding the CA is subcloned between the *Bam*HI and *Eco*RI sites of the pGS21a expression vector. The accuracy of subcloning was verified, using sequencing. This vector adds a His-GST tag, which increases the solubility of the expressed protein. Expression cassette in this vector is under the control of the T7 promoter and thus can be expressed at a high-level with IPTG induction. The theoretical molecular weight of the CA in the pGS21a vector was about 63 kDa (Figure 3).



Figure 1: Graphical display of the epitopes orientation in chimeric antigen (CA). This recombinant fragment is repeated two times in CA

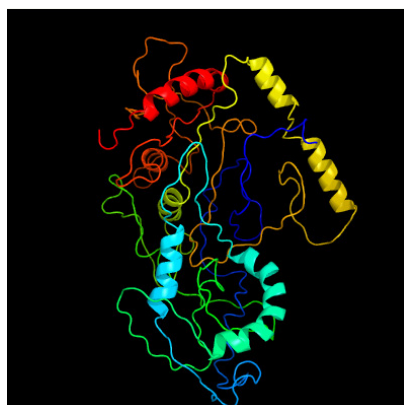


Figure 2. Modeled structure of the chimeric antigen (CA) predicted by Phyre2. The structure was represented in the ribbon

Small-scale Expression

To induce the His-GST-CA expression, IPTG (final concentration of 0.4 mM) was added to the medium and incubated at 15°C for 18 hours. The results of SDS-PAGE (Figure 4A) showed that the protein was successfully expressed in the induced cells. The same band was not observed in cells with a recombinant vector, not induced by IPTG. The His-GST-CA accounted for 15% of the total cellular proteins, as determined by protein scanning of coomassie blue-stained SDS-PAGE gels.

Soluble Fraction Preparation

To evaluate the presence of His-GST-CA in the soluble fraction, the supernatant was analyzed by SDS-PAGE which showed that His-GST-CA existed in the supernatant (Figure 4B). According to densitometric scanning results and evaluation of Bradford assay, approximately 50 mg of soluble forms were produced per liter of culture.

Protein Purification

Since the protein was expressed as a soluble form, all purification steps were carried out under native conditions at room temperature. His-GST-CA was

trapped on nickel columns and the unbound proteins were removed by washing steps. Afterward, the bound His-GST-CA was purified, using elution buffer, and dialyzed against PBS at 4°C for one night. SDS-PAGE results showed that the purity of the samples was more than 90%. This purity is acceptable for immunological examination (Figure 4C).

Result of ELISA

ELISA was used to evaluate the reactivity of His-GST-CA with serum antibodies against HTLV-I/-II. Initially, the interaction of different concentrations of antigen was investigated with the pooled serums, made of 10 infected serum samples and 10 healthy serum samples. ELISA data analysis showed that the purified antigens reacted in a concentration-dependent manner with serum antibodies in the pooled contaminated serum (Figure 5). In the next step, an antigen concentration of 1 µg / mL was coated on the bottom of the plate and reacted with different dilutions of the pooled contaminated serum. The result showed that up to a dilution of 1/700 antigens could detect contaminated serum (Figure 6). These results indicate that epitopes are at the surface and can react with antibodies. The results of the reaction of 19 infected

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serums and 19serums from healthy controls indicate that this antigen can well detect negative and positive serums (Table 1).

Specificity

Evaluation of antigen reaction with HBV, HCV, and HAV sera was shown that this antigen does not react with antibodies in these sera (Figure 7, 8).

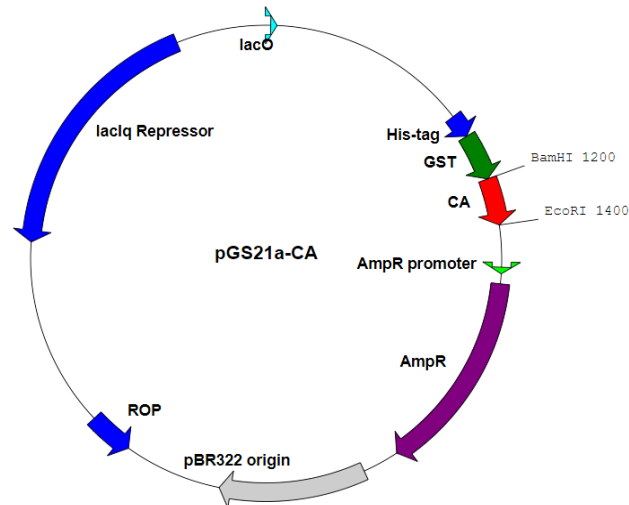


Figure 3. Schematic diagram of the construct used for chimeric antigen (CA) expression. The CA gene was cloned downstream of the His-GST tag in pGS21a expression vector, which also contained the gene for ampicillin resistance

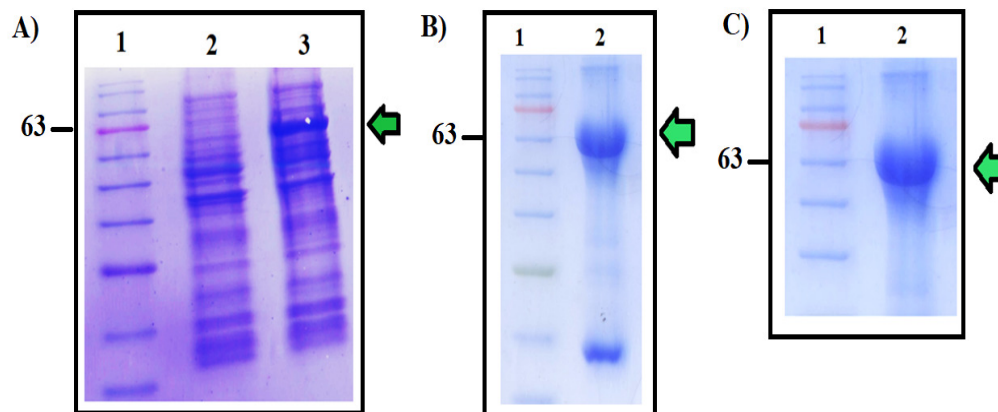


Figure 4. His-GST-CA expression and purification. A) Expression analysis of the His-GST-CA by SDS-PAGE in 12% polyacrylamide gel. Non-induced *E. coli* BL21 (DE3) cells harboring the recombinant plasmid were used as negative controls. Lane 1, protein marker; lane 2, non-induced control cells containing pGS21a-CA and lane 3, induced bacterial cells containing pGS21a-CA. His-GST-CA was expressed at a molecular weight of approximately 63kDa. B) Determination of the solubility of the His-GST-CA protein. Solubility was analyzed by performing SDS-PAGE in 12% polyacrylamide gel. Lane 1, protein marker; lane 2, supernatant. C) SDS-PAGE analyses of the His-GST-CA purified using Ni-NTA agarose column. Lane 1, protein marker and lane 2, purified His-GST-CA

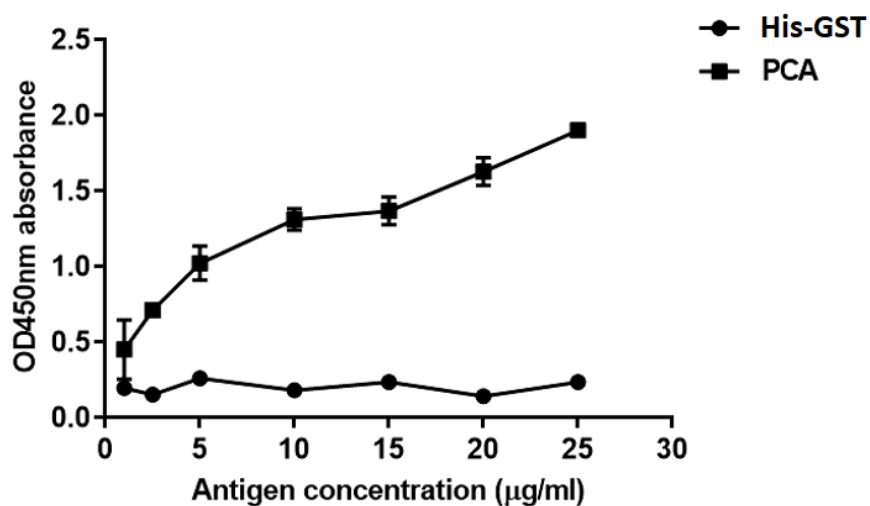


Figure 5. Binding activity of the His-GST-CA towards pooled infected sera. Results of ELISA showed that the His-CA reacted with antibodies present in the pooled infected serum (fixed level) in a concentration-dependent manner. His-GST was used as the negative control of the same condition.

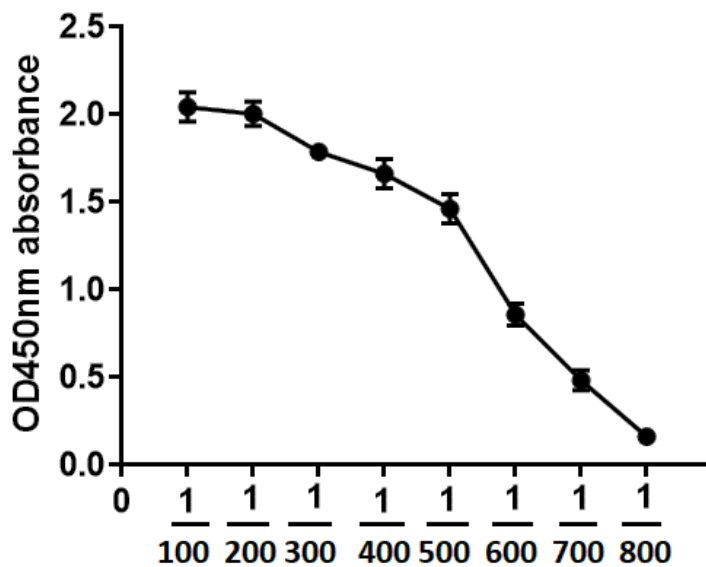


Figure 6. Serial dilution of pooled serum for evaluation of His-GST-CA antigen- anti-HTLV-I/-II antibodies interaction. Pooled sera of HTLV-I/-II infected diluted using PBS buffer. Pooled healthy serum samples were used as the negative control (NC: negative control)

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Table 1. Evaluation of HTLV-I/-II infected and healthy control sera using His-GST-CA ELISA. Dash line is cut-off which above those serums considered HTLV-I positive. A cut-off point above which a sample was considered positive was calculated as the mean OD (Optical Density) of negative population plus three standard deviations.

Number	Absorbance	Number	Absorbance
1	2.72 ± 0.12	20	0.013 ± 0.00
2	2.52 ± 0.22	21	0.010 ± 0.00
3	2.08 ± 0.21	22	0.012 ± 0.00
4	1.46 ± 0.13	23	0.014 ± 0.00
5	2.42 ± 0.31	24	0.009 ± 0.00
6	0.82 ± 0.11	25	0.037 ± 0.00
7	1.86 ± 0.19	26	0.035 ± 0.00
8	1.98 ± 0.14	27	0.043 ± 0.00
9	2.39 ± 0.12	28	0.037 ± 0.00
10	1.56 ± 0.15	29	0.027 ± 0.00
11	1.95 ± 0.12	30	0.023 ± 0.00
12	1.81 ± 0.18	31	0.023 ± 0.00
13	2.37 ± 0.13	32	0.014 ± 0.00
14	1.66 ± 0.10	33	0.010 ± 0.00
15	2.14 ± 0.11	34	0.033 ± 0.00
16	2.03 ± 0.17	35	0.035 ± 0.00
17	1.74 ± 0.16	36	0.049 ± 0.00
18	2.53 ± 0.12	37	0.044 ± 0.00
19	2.67 ± 0.06	38	0.013 ± 0.00

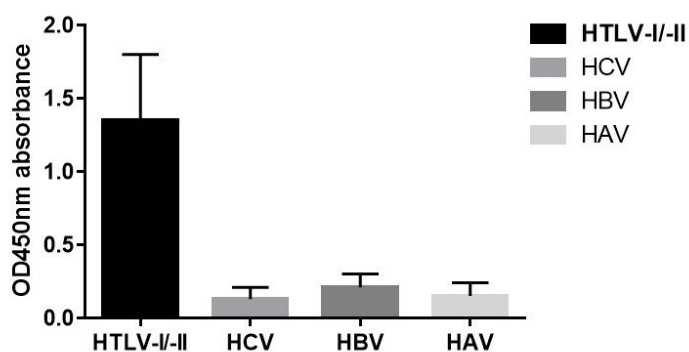


Figure 7. Specificity evaluation of HTLV-I/II ELISA assay. Different sera collected from hepatitis C virus (HCV), hepatitis B virus (HBV) and hepatitis A virus (HAV)-infected patients tested by HTLV-I/II ELISA. Each column represents a corresponding serum

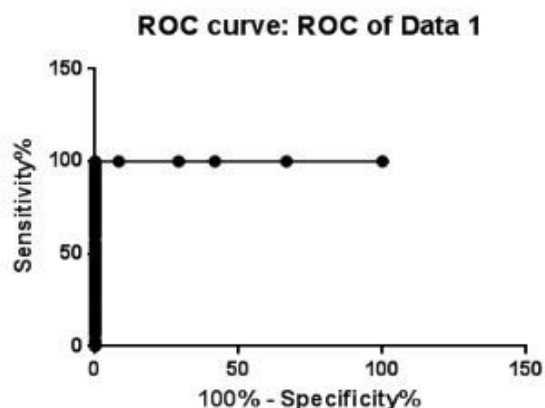


Figure 8: Sensitivity and specificity 100 % and cutoff 0.09

DISCUSSION

In this study, our aim was to produce a soluble chimeric antigen for the specific detection of HTLV-I/-II detection. A chimeric antigen was designed using three linear immunodominant epitopes of HTLV-I/-II diagnostic antigens and evaluated using computational tools. After expression and purification in the *E.coli*, it was evaluated by ELISA.

In previous studies, it was shown that using of MTA-I, GD-21, and gp46-I, all HTLV-I/-II infected serum can be detected.³ These three peptides are used in the form of synthetic peptides,¹⁰ chimeric peptides,^{11,12} fusion proteins³ or chimeric proteins⁵ to detect anti-HTLV-I/-II antibodies. Chimeric antigens are superior to synthetic peptides, chimeric peptides, and fusion peptides. Coating the synthetic peptides on the plate is the biggest challenge in their application for diagnostic methods.¹³ The purification of three fusion peptides is also time-consuming. However, the chimeric antigens are very suitable for detecting HTLV-I/-II antibodies because those do not have the mentioned limitations.²²

In previous studies, Heidari et al suggested a chimeric antigen to detect HTLV-I antibodies.^{5,14} However, they don't incorporate HTLV-II diagnostic epitope in their chimeric antigen. Also, they use the Trx tag to express chimeric protein as the soluble form. However, GST has several advantages over the Trx tag. GST tag can be coupled with other affinity strategies, e.g. His tag, to improve protein purification. GST-tag is

more efficient when positioned at the N-terminal rather than at the C-terminal end.¹⁵

DNA encoding viral proteins often contain rare codons that are rarely used. So high expression of viral proteins in *E. coli*, is often difficult. The tRNA molecules that identify these codons are less frequent compared to other tRNAs in bacteria.¹⁶ Hence, the presence of such codons reduces the rate of protein synthesis. It has been suggested that the optimization of sequences can increase the translation rate. Several studies showed that by optimizing the sequence, the rate of protein synthesis can be increased.¹⁷⁻¹⁹ Hence, new DNA sequence encoding CA was synthesized with preferred codons in *E.coli*. Results of densitometry scanning assay showed that the His-GST-CA protein accounted for 15% of the proteins in the cell lysate. We speculate that the relatively good protein expression in this study is partly due to the optimization of sequences.

It has been suggested that the protein expression at low temperatures and low inducer concentrations, increases the soluble expression of proteins. In the cold protein expression, hydrophobic interactions may decrease, as a result of which, the accumulation of proteins decreases.²⁰ Therefore, we used 0.4mM IPTG and 15°C to induce the expression of the CA. As SDS-PAGE results indicated at this condition CA was expressed as a soluble form. Because HTLV-I/-II proteins form the inclusion body when expressed in *E. coli*,⁷ expressions of the soluble CA might be because of the presence of the GST fusion, low temperature and

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low inducer concentration.

Proteins were purified using Ni-NTA resin. The SDS-PAGE analysis of the purified proteins primarily showed a single 63-kDa band corresponding to the His-GST-CA protein. Using this method, approximately 30mg protein could be purified from 1 lit baffled flask.

Previous data about the GST tag interferes with antibodies during ELISA are controversial. A few studies have shown that the GST-fused does not react with sera from healthy humans. On the other hand, a few studies shown that the GST-tag interferes with antibodies during ELISA.²¹ Thus, we used the GST-His-tag as a control during ELISA to negate any reaction antibodies with this solubility tag. As demonstrated, the purified CA could specifically react with antibodies present in HTLV-I/-II infected serum. Results of 19 clinical serum samples detection by CA-ELISA showed a 100% concordance rate with a commercial kit. No cross-reactivity was detected with positive sera against healthy controls. These data indicated that the coating antigen processed good specificity.

In summary, the *E. coli* expression system producing soluble CA for HTLV-I/-II antibodies detection has been developed. In comparison to the previous study, the incorporation of the HTLV-II linear epitope results in the simultaneous detection of HTLV-I/-II antibodies. This recombinant protein has a high sensitivity for detecting anti-HTLV-I/-II antibodies and by further validation could be used for HTLV-I/-II screening.

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