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Constructing Chimeric Antigen for Precise Screening of HTLV-I Infection

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ABSTRACT

Individual preparation of two human T-cell lymphotropic virus type I (HTLV-I) diagnostic GST fused peptides (MTA-1 and GD21) is time-consuming and expensive. The aim of this study was to design a novel single chimeric antigen (SCA) to obviate separate expression of proteins and reduce the cost of reagent preparation.

Structural protein fragments, including immunodominant B cell linear epitopes, were selected and different SCAs were designed. Tertiary structure, epitope exposure, solubility and stability were calculated for each SCA and compared with each other. The synthetic DNA encoding the interested SCA was sub-cloned into pET32a expression vector, expressed as a soluble form in *Escherichia coli* BL21 (DE3) cells and purified under native condition using affinity chromatography.

The SDS-PAGE results indicated that thioredoxin-fused SCA was successfully expressed as a soluble form in *E. coli* BL21 (DE3) cells. The results of ELISA confirmed that SCA reacted with anti-HTLV-I antibodies in a concentration-dependent manner.

Our results indicated that the designed SCA may be a good candidate for the screening of HTLV-I carriers with antigen–antibody-based tests.

Keywords: Antigenicity; Chimeric protein; ELISA; HTLV-I

INTRODUCTION

The human T-cell lymphotropic virus type I (HTLV-I) is a human retrovirus that causes a variety of human diseases, including adult T-cell leukaemia/lymphoma (ATLL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP).[1] It is estimated that HTLV-I has already infected around 10–20 million people worldwide.[2]

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This infection is endemic in some parts such as north-eastern Iran. In Mashhad (a city of Iran), it is estimated that 2%–3% of the general population is infected with HTLV-I.[3] HTLV-I carriers have no clinical manifestations and transmit the virus through blood transfusion, breast feeding and sexual contact with other healthy individuals.[4] The detection of asymptomatic carriers by screening methods such as enzyme-linked immunosorbent assay (ELISA) is a major part of HTLV-I prevention programs to prevent the introduction of new infection.[5,6] The presence of anti-HTLV-I antibodies in a person indicates that he/she may be infected with HTLV-I.[7]

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In HTLV screening using ELISAs, MTA-1, GD21 and K55 peptides are used for the detection of anti-HTLV-I/-II antibodies. GD21 interacts with anti-HTLV-I and anti-HTLV-II antibodies, whereas MTA-1 and K55 peptides are specific for HTLV-I and HTLV-II, respectively.[7,8] These peptides are expressed as a separate fusion protein in *Escherichia coli* cells and used in most of the commercial kits.[9]

Preparation of these peptides as three separate GST-fusion proteins is a time-consuming and expensive process. Furthermore, although two or more antigens can be used for diagnostic assay, in multi-antigen assays, the efficiency of detection could be affected by equivalent binding of antigens, competition for binding and spatial distribution of antigenic determinants in fusion proteins binding to solid surface.[10] Therefore, there is a tendency for use of chimeric antigens to avoid these problems and improve the sensitivity and specificity of the tests.

There are several studies that have used synthetic chimeric peptides derived immunodominant regions of HTLV-I structural proteins (amino acids 175 and 199 of gp46, amino acids 374–400 of gp21 and amino acids 100–129 of p19) to develop a diagnostic method for the detection of antibodies.[11-14] Some of these studies have suggested that the p19 peptide has maximum sensitivity and specificity in detecting HTLV-I antibodies and could detect all HTLV-I-infected sera if used as a chimer with one or two envelope-derived peptide.[11,15,16]

The aim of the present study was to design a novel single chimeric antigen (SCA) from HTLV-I diagnostic antigen fragments, including the immunodominant B-cell epitopes of gp46, gp21 and p19. We expected that incorporation of the p19 fragment in the SCA design would increase the sensitivity of HTLV-I detection.

MATERIALS AND METHODS

Chemicals, Enzymes and Strains

All the reagents used in this study were of analytical grade and were purchased from Sigma Chemical (St. Louis, MO) unless otherwise indicated. The bacterial culture media were purchased from Merck (Germany), restriction enzymes were obtained from Takara (Japan), plasmid extraction and DNA extraction kits were bought from Intron (Korea) and Western blotting and Ni-NTA agarose resin were purchased from Qiagen (Valencia, CA). *E. coli* DH5α (Invitrogen, San Diego, CA) and BL21 (DE3) (Novagen, Madison, WT) strains were used for plasmid manipulation and overexpression experiments, respectively. The protein molecular standard was obtained from Sinaclon (Iran).

Serum Samples

The serum samples used in this study were obtained from patients referred to Razavi hospital, Mashhad city, Iran. All the samples were diagnosed as positive when tested with commercial HTLV-I ELISA kits (MP Diagnostics™ HTLV-I/II ELISA 4.0). Samples from healthy volunteers were used as controls and analysed using ELISA.

Computational Antigen Design

Different sequences of gp46, gp21 and p19 antigens were selected from Uniprot database (http://www.uniprot.org/) and separately aligned by Muscle (http://www.ebi.ac.uk/Tools/msa/muscle/). As the amino acid sequences were identical, one sequence for each antigen (Access No. gp46 and gp21: P23064, p19: P03345) was selected for subsequent analysis. The antigenic fragments, including immunodominant peptides (150–289 from gp46, 320–460 from gp21 and 100–130 from p19) were selected and different SCAs (with different orders of fragments) were designed using an amino acid linker (5′-GSGGSG-3′). The molecular weight (MW), half-life, instability index (calculated using Protparam server, http://us.expasy.org/tools/protparam.html) and secondary and tertiary structures (predicted by I-TASSER at http://zhanglab.ccmb.med.umich.edu/I-TASSER/) were separately determined for each SCA. After energy minimisation, the Z-score (calculated by ProSA, https://prosa.services.came.sbg.ac.at/prosa.php) and Ramachandran plot (http://mordred.bioc.cam.ac.uk/~rapper/rampage.php) were determined for the evaluation of the generated models. The location and accessibility of the immunodominant peptides were evaluated using Episearch software. The solubility of SCAs was predicted by an online protein solubility predictor tool (http://www.biotech.ou.edu/).

Expression Optimisation

The amino acid sequence of the SCA was reverse-translated (http://www.bioinformatics.org/sms2/

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rev_trans.html) to nucleotide sequence, and the sequence was submitted to an online optimizer tool to optimise it according to the *E.coli* codon usage (http://genomes.urv.es/OPTIMIZER/). The internal ribosome-binding sites, which could significantly decrease the translation efficiency, were removed from the optimised sequence.

Construction of the Expression Plasmid

The DNA-encoding SCA was synthesised by Biomatik and sub-cloned in the plasmid pET32a using *BamHI/EcoRI* according to standard protocols. The accuracy of sub-cloning was confirmed by sequencing. In pET-32a, the gene of interest was expressed in fusion with thioredoxin (Trx) and contained an amino terminal of six histidine (His) tag. The pET32a-Trx-SCA was transformed into *E. coli* BL21 (DE3), the cells were grown in LB agar medium containing 100μg/ml ampicillin and some of the surviving colonies were selected for further analysis.

Small-Scale Expression

E. coli BL21 (DE3) harbouring the pET32a expression constructs were grown in Luria-Bertani (LB) medium (5ml) supplemented with 100μg/ml ampicillin at 37°C overnight under constant shaking at 250rpm. Fresh LB liquid medium (50ml) containing 100μg/ml ampicillin was added to 5ml of the pre-culture and incubated at 37°C. The optical density (OD_{600nm}) of the culture was periodically checked until the value reached 0.6. Subsequently, the culture was induced using 1mM IPTG and grown at 25°C for 8h, and the cells were harvested by centrifugation at 5000 ×g at 4°C for 15min. The cell pellets were resuspended in phosphate-buffered saline (PBS) and stored at −70°C for further analysis.

Expression Confirmation by SDS-PAGE and Immunoblot Analysis

The cell suspensions were mixed with 2× sample buffer and heated for 15min at 100°C. To confirm the expression of the recombinant protein, SDS-PAGE was employed. The SDS-PAGE was performed using acrylamide: bisacrylamide at a ratio of 30:0.8, with acrylamide at a concentration of 12% and 4% w/v for running gel and stacking gel, respectively. The non-transformed and non-induced BL21 cells were used as negative control. Protein MW markers were used to determine the MW of the proteins.

For Western blot analysis, the proteins were transferred to nitrocellulose membrane, following separation on SDS-PAGE, and blocked with 5% non-fat dried milk powder in PBS–0.5% Tween-20. Subsequently, antibodies were added and specific binding was revealed with the anti-His tag antibodies in Western blotting detection system.

Protein Solubility Determination

To check the solubility of the antigen under conditions for expression, the *E. coli* pellets obtained from 50ml of culture following IPTG induction were resuspended in PBS and subjected to sonication on ice. After sonication, the cell lysate was centrifuged at 12,000 rpm for 20 min at 4°C. The clear supernatant and pellets were analysed using SDS-PAGE in 12% polyacrylamide gels.

Protein Purification

The cell lysate supernatant was transferred into a 1×5-cm column packed with 1.5 ml of Ni^+-nitrilotriacetic acid (NTA) resin. The resin was washed with three-column volumes of binding buffer, followed by five volumes of washing buffer. The adsorbed antigen was eluted from the resin using an imidazole gradient (0–500mM in 10-column volumes of elution buffers). A flow rate of 0.5 ml/min was used in all the chromatographic steps, and SDS-PAGE was performed to identify the purity of the purified antigen.

ELISA

The recombinant antigen was diluted in PBS and concentrations of 0, 1.25, 2.5, 5, 10 and 20μg/ml were adsorbed onto the odd wells of the ELISA microplates. In the even wells, Trx diluted in PBS was added as the negative control. The plates were incubated overnight at 37°C and then blocked by 1% bovine serum albumin (BSA) for 2h at 37°C. Ten pooled infected sera (50 μl) were added to each well and incubated for 1h at 37°C. After washing for six times, peroxidase-labelled anti-human antibody (diluted in PBS) was added to the wells and the plates were incubated for 1h at 37°C and then washed four times. The reaction was developed by adding 50μl of 3, 3′, 5, 5′-tetramethylbenzidine (TMB) substrate solution to each well. After 15min, the enzymatic reaction was stopped using 0.1N H_2SO_4 and the plates were read at 450nm. Furthermore, we evaluated 10 HTLV-I-infected serum samples that gave positive results with commercial tests. All the analyses

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were performed in duplicates.

RESULTS

Antigen Design

The immunodominant fragments of gp46, gp21 and p19 (residues 150–289 from gp46, residues 320–460 from gp21 and residues 100–130 from p19) were selected and fused together using (GSGGSG) linker sequences. A graphical display of the construct designed by DOG1.0 software is shown in Figure 1.

This construct had more stability and epitope accessibility, when compared with other possible constructs with selected fragments (data not shown). The average molecular weight of the construct was calculated to be 49kDa. The half-life of SCA in E.coli cell was more than 10 hours. Secondary structure of SCA was predicted using GOR method (Figure 2). The complex threading method was exploited to produce 3D models of the chimeric protein (Figure 3). Localisation of immunodominant fragment on protein structure was showed in Figure 4.

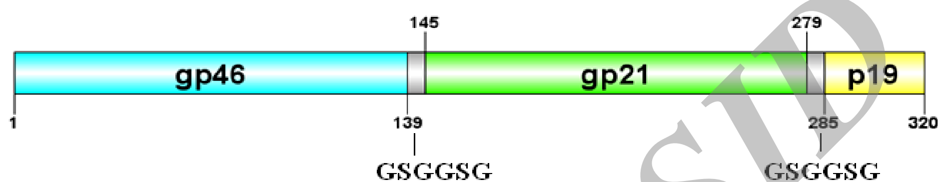

Figure 1. Graphical display of the construct consisting of gp46, gp21 and p19 fused together via linkers (shown in black) expressed in *E. coli*. The number denotes the amino acid position and grey interval denotes the amino acid linker position.

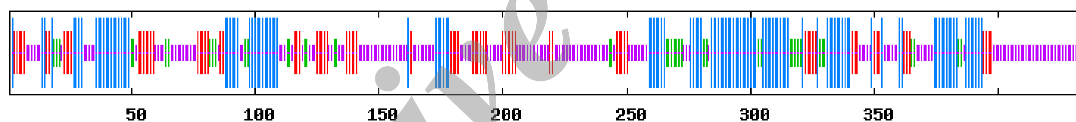

Figure 2. The secondary structure of SCA: alpha helix: blue, extended strand (red), beta turn (green), coiled coil (violet).

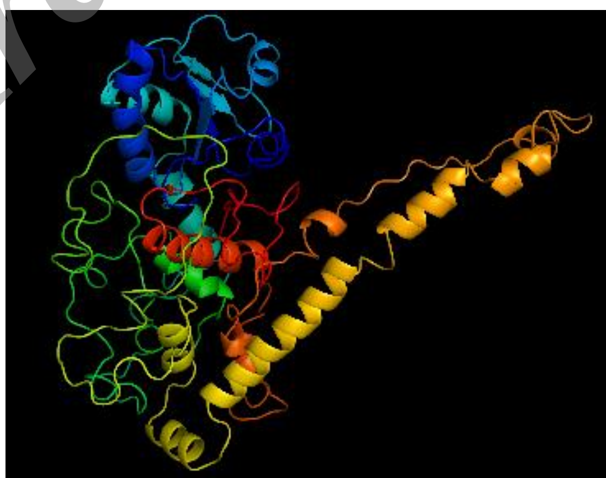

Figure 3. Modelled structure of the chimeric protein predicted by I-TASSER. The structure was represented in ribbon

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model.

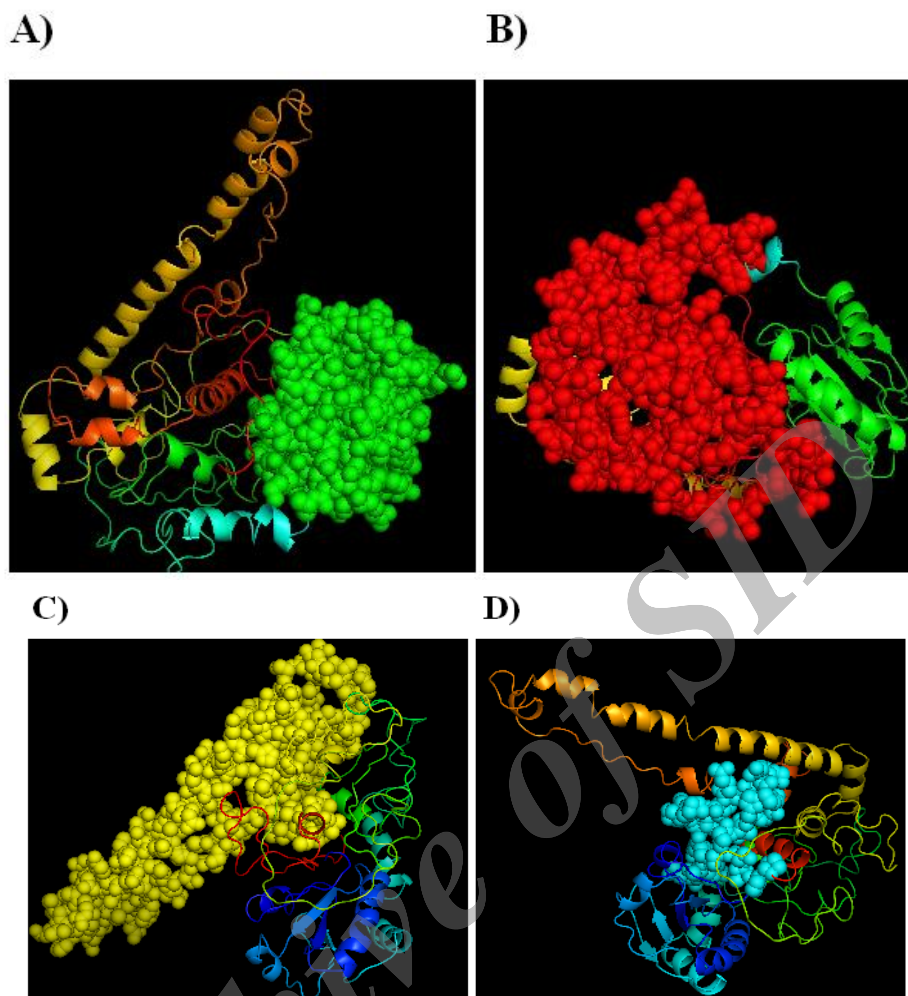

Figure 4. Localisation of immunodominant fragments on the protein structure. A) Trx tag (green), B) gp46 fragment (red), C) **gp21 fragment (yellow) and D) p19 fragment (cyan).**

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The generated models were checked using ProSA (Figure 5) to find the potential errors in the 3D models. The ProSA server calculated a score (Z-score) for each input model; if the score was out of the native protein score range, then the structure probably contained errors. The Z-score indicated the energy separation of the native and average of the misfolds in the units of standard deviation. The Z-score of the generated model was at the borderline of that of the native proteins.

Furthermore, the quality of the model was investigated using Ramachandran plot calculation (Figure 6). For a good quality model, the plot is expected to have more than 90% residues in the most favoured region. The Ramachandran plot analysis of the model revealed that 94% of the amino acids were located in the favoured and allowed regions, indicating that the predicted model has good quality. Moreover, according to solubility prediction, the SCA has poor solubility and forms inclusion bodies.

Computational Expression Optimisation

The synthetic chimeric genes were analysed by rare codon analyses tool. The codon optimisation index (CAI) on the native chimeric gene was increased from 0.65 to 0.89 after optimisation. The distribution of the frequently used codons in the native gene was 41%, which was significantly improved to 62% in the optimised gene sequence. The overall GC content, which indicates transcription and translation efficiency, was improved from 50.79% to 55.96% after codon optimisation. Furthermore, there were three negative regulatory cis-elements in the native gene sequence,

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which were removed after optimisation.

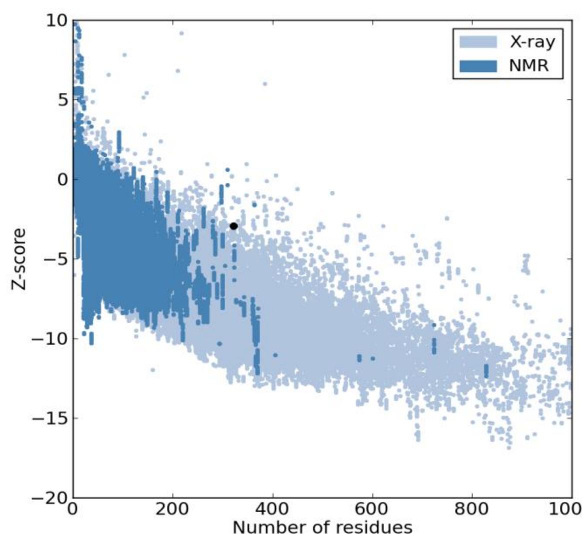

Figure 5. ProSA-web Z-score plot of the chimeric protein. The Z-score was used for overall model quality evaluation. Its value is displayed in a plot containing the Z-score of all experimentally determined protein chains in the current PDB. In this plot, the groups of structures from different sources are distinguished by different colours, which can be used to check whether the Z-score of the input structure is within the range of the source typically found for the native proteins of similar size. The value of Z-score is highlighted as a black dot and is in the range of native conformations.

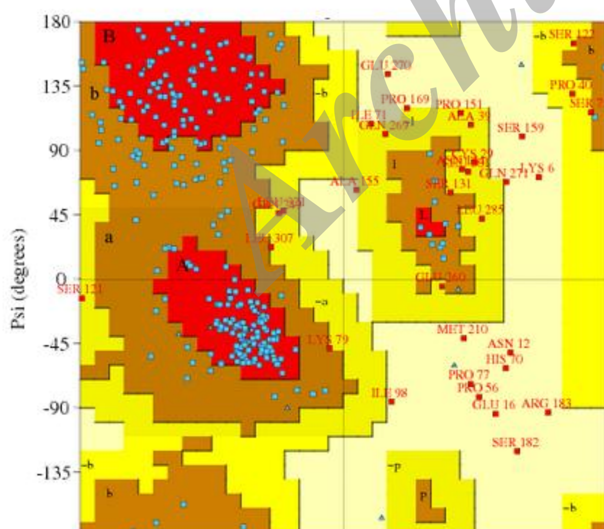

Figure 6. Validation of the protein structure using Ramachandran plot. The Ramachandran plot revealed that 63% of the amino acid residues were in the favoured regions (A, B and L) and 26.8% of the residues were in the allowed regions (a, b, l and p).

Construction of the Expression Plasmid

As the Trx tag enhances protein solubility, it is used for the expression of proteins with poor solubility. The synthetic DNA was sub-cloned into the pET32a expression vector that incorporates Trx at the N-terminus of the protein. The recombinant plasmid was transformed into the chemically competent *E. coli* BL21 (DE3) cells and subsequently confirmed by sequencing.

Small-scale Expression

The pET32a-SCA was predicted by Expasy online server to encode the recombinant fusion protein with a MW of approximately 49kD (Trx-His-SCA). Small-scale cultures were induced by adding IPTG at a final concentration of 1mM and incubating for 8h at 25°C to identify the expression capacity by SDS-PAGE analysis. Trx-His-SCA was expressed with the expected MW in the transformed *E. coli* BL21 (DE3) cells. The results of the SDS-PAGE analysis revealed that this protein constituted a relatively large fraction of the total proteins of the host cell, when compared with the negative controls without recombinant plasmid or IPTG induction (Figure 7).

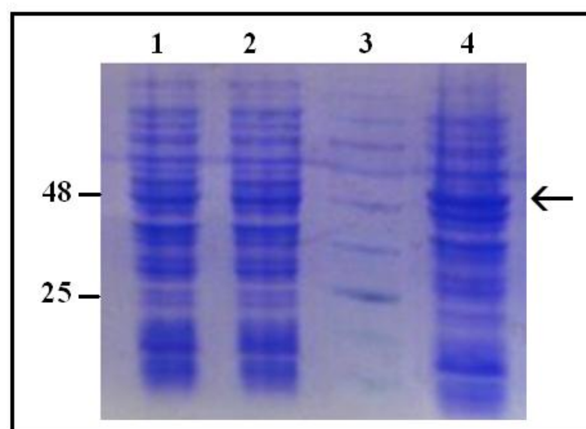

Figure 7. Preliminary expression analysis of the His-tagged fusion protein. Non-transformed bacteria and non-induced cells were used as negative control. The expression of the protein was analysed using 12% SDS-PAGE. Lane 1: Non-transformed *E. coli* BL21 (DE3); Lane 2: Non-induced *Ecoli* BL21 (DE3); Lane 3: Protein

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marker and Lane 4: Induced *E. coli* BL21. Arrow indicates the position of the expressed protein.

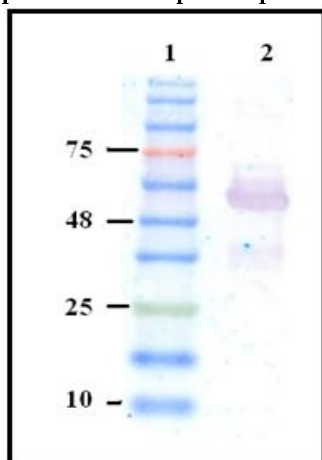

Figure 8. Western blot analysis of the expressed protein. Mouse monoclonal anti-poly-His antibody served as the primary antibody. Lane 1: Protein marker and Lane 2: Expressed His-tagged protein.

Solubility Test

To determine the distribution of the over-expressed protein in the soluble and insoluble fractions, the supernatant and pellets were analysed by SDS-PAGE after sonication. The results showed that most of the expressed recombinant protein was present in the soluble fraction (Figure 9).

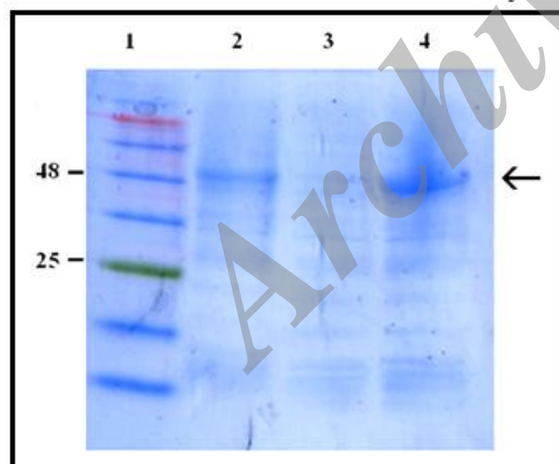

Figure 9. Solubility test of the Trx-His-SCA. The samples were submitted to 12% SDS-PAGE and stained with Coomassie blue G-250. The samples were directly resuspended in SDS-loading buffer and boiled for 10min. Lane 1: Protein MW marker; Lane 2: *E. coli* BL21 (DE3) induced with 1mM IPTG, Lane 3: Pellets of *E. coli* BL21 (DE3) induced with 1mM IPTG and Lane 4: Supernatant of *E. coli* BL21 (DE3) induced with 1mM IPTG. Arrow indicates the position of the expressed SCA.

Purification

As the target protein was expressed as a soluble form, the purification step was performed under native condition at room temperature. The His-tagged protein was immobilised on resin and the unbound proteins were washed. Afterwards, the antigen was recovered using elution buffer and immediately dialysed overnight against PBS at 4°C. The purified protein was subjected to SDS-PAGE analysis and the results indicated that the protein purity was more than 90% (Figure 10).

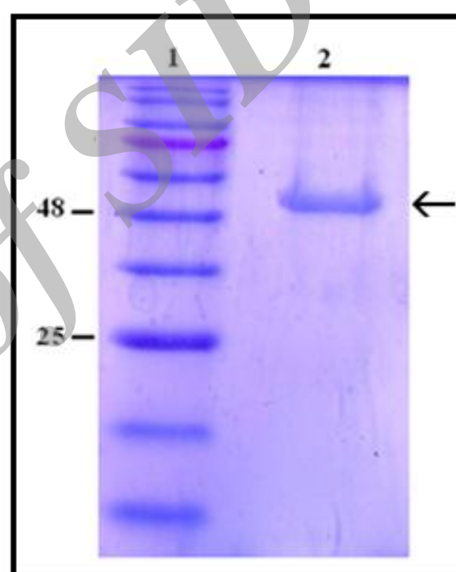

Figure 10. SDS-PAGE analysis after purification with Ni-NTA agarose column. Lane 1: Protein marker and Lane 2: Purified protein. Arrow indicates the position of the expressed SCA.

Indirect ELISA

ELISA was performed for assessing the reactivity of the recombinant antigen (Figure 11). The results indicated that the purified antigen reacted in a concentration-dependent manner towards the pooled positive samples, suggesting that the epitopes were accessible to the antibodies and were functional. The results of the analysis of 10 infected and 10 healthy sera showed that the recombinant proteins carrying HTLV-1 could react with anti-HTLV-1 antibodies with no false-positive or false-negative results.

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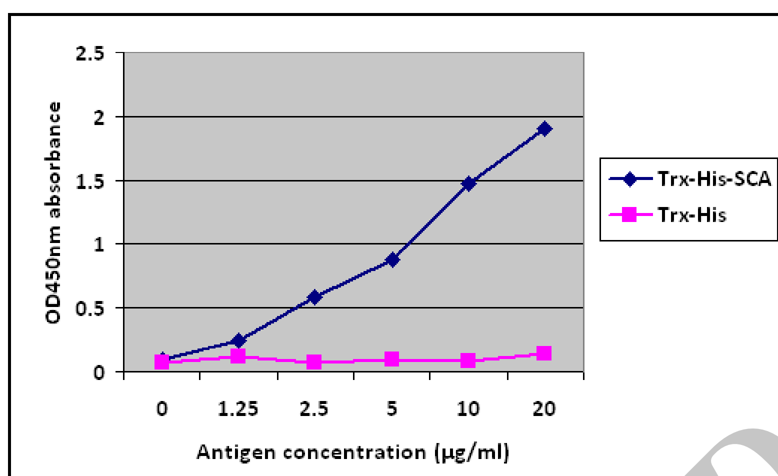

Figure 11. Binding activity of SCA to HTLV-I-infected sera. ELISA assay demonstrated a binding curve at various concentrations of fusion SCA to serum antibodies. Trx-His protein was used as the negative control under the same condition.

DISCUSSION

The aim of the present study was to design a chimeric protein for precise screening of HTLV-I infection. This study was conducted in three main stages: 1) antigen selection, 2) SCA design and screening and 3) small-scale preparation and evaluation of SCA.

In a previous study, Yamada et al. reported that the peptide corresponding to 100–130 of the p19 protein encoded by the *gag* gene presented the highest sensitivity and specificity in HTLV-I screening. However, they suggested that 1 of the 20,000 serum specimens was not positive for the p19 peptide. This specimen did not have an antibody for this peptide, but was positive when tested with peptides derived from gp46 and gp21 envelope proteins.[17] Based on these findings, we decided to use the immunodominant peptides of gp46, g21 and p19 for chimeric protein design.

The fragments were linked together using an amino acid linker (GSGGSG) in different orders. This linker contained glycine and serine residues that are flexible and allow the adjacent fragments to move freely relative to one another. The flexibility of the linker improved antibody–antigen interaction, orientation of epitopes, separation of fragments and structure conservation.[18]

It is suggested that codon optimisation may increase the expression level of chimeric constructs.[19,20] The viral protein sequences often contain codons that are rarely used in prokaryotic cells. It is difficult to overproduce proteins from sequences containing rare codons because the tRNAs that encode these codons are rapidly depleted during overexpression. To maximise the level of expression, we optimised the gene of interest by substituting the rare codons with more preferred ones. After optimisation, the designed antigen constituted 20% of the total cellular protein.

Inclusion bodies are protein aggregations with no biological activities. Denaturation or refolding of inclusion bodies is a time-consuming process and is difficult to accomplish for large-scale preparation.[21] Low-temperature induction, low inducer concentration and expression with fusion partner are used for the expression of proteins with poor solubility in a soluble form. As Trx is a highly stable and soluble protein, it enhances the stability and solubility of a target protein to which it is fused.[22] We used pet32a for the expression of SCA, which incorporated Trx tag in the N-terminus of the SCA. By using this fusion system, the SCA was expressed as a soluble form.

There are many controversial data regarding whether the immunoreactivity of solubility tag with control serum results in false-positive results.[23-26] Therefore, in the present study, Trx-SCA was used as the capture antigen and Trx-His tag was employed as the control in ELISA. Laboratory tests with the pooled

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sera from the infected patients indicated that the prepared protein Trx-SCA could react with anti-HTLV-I antibodies present in the pooled sera (fixed level) in a concentration-dependent manner. Furthermore, we also tested the 10 infected sera with commercial test kit and the designed antigen. By using our developed ELISA test, a sensitivity of 100% and specificity of 100% were achieved relative to HTLV-I infection test. These results indicated that the antigen prepared in this study could successfully detect HTLV-I-infected sera. To our knowledge, the present study is the first to successfully produce sufficient amount of HTLV-I diagnostic protein using computational tools to initiate novel assay development. However, our study has a main limitation. We did not examine specificity and sensitivity of this protein by large number of infected sera and healthy sera as negative control.

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