

Expression and Purification of a Novel Computationally Designed Antigen for Simultaneously Detection of HTLV-1 and HBV Antibodies

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ABSTRACT

Computational tools are reliable alternatives to laborious work in chimeric protein design. In this study, a chimeric antigen was designed using computational techniques for simultaneous detection of anti-HTLV-I and anti-HBV in infected sera.

Databases were searched for amino acid sequences of HBV/HTLV-I diagnostic antigens. The immunodominant fragments were selected based on propensity scales. The diagnostic antigen was designed using these fragments. Secondary and tertiary structures were predicted and the B-cell epitopes were mapped on the surface of built model. The synthetic DNA coding antigen was sub-cloned into pGS21a expression vector.

SDS-PAGE analysis showed that glutathione fused antigen was highly expressed in *E. coli* BL21 (DE3) cells. The recombinant antigen was purified by nickel affinity chromatography. ELISA results showed that soluble antigen could specifically react with the HTLV-I and HBV infected sera.

This specific antigen could be used as suitable agent for antibody-antigen based screening tests and can help clinicians in order to perform quick and precise screening of the HBV and HTLV-I infections.

Keywords: Blood screening; Computational analysis; HBV; HTLV-I; Transfusion

INTRODUCTION

Individuals who have severe anemia, dangerous low platelets counts or have lost blood following accident or surgery need to receive blood. Donated blood can be life saving for these individuals.

Blood born viruses (BBVs) are group of viruses that are transmitted via blood samples from infected patients to healthy persons.¹ Human immunodeficiency virus (HIV) is classified as BBVs.² Blood samples must not be used to transfusion unless it is negative according to diagnostic tests of BBVs. If one or more of these virus screening tests were clearly reactive, the blood and any derived component cannot be used for transfusion. Hepatitis B virus (HBV) and human T lymphotropic virus type I (HTLV-I) are members of

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BBV group.² Infection with these viruses is relatively frequent in north eastern cities of Iran.³⁻⁶ People are infected with one or both viruses are not allowed to participate in transfusion. People suffering from chronic infection of HBV or HTLV-I are mostly asymptomatic and not aware of their infection.^{7,8} In blood screening, such carriers are detected using enzyme-linked immunosorbent assay (ELISA) method. In these methods, the structural proteins are used as capture antigen for detection of anti-HTLV-I and anti-HBV antibodies in serum samples. Presence of anti-HBV and anti-HTLV-I antibodies shows that person is infected by HBV or HTLV-I respectively.^{9,10}

The commercial HTLV-I ELISA tests use a combination of two recombinant proteins (gp21, r-gp46 or p19 peptide) as capture antigens. These assays assure detection of HTLV-I specific antibodies.¹¹ Also the commercial HBV core antibody tests use recombinant hepatitis B core protein (rHBc) as capture antigen to detect HBV anti-HBc specific antibodies. This antibody appears before hepatitis B surface antigen antibody (anti-HBs) or hepatitis B e antibody (anti-HBe) in circulation and could be used for early detection of HBV infected individuals.

We presume that a chimeric antigen can be designed for simultaneous detection of anti-HBV and anti-HTLV-I antibodies in serum of infected patients for screening purposes. Design of such antigen can reduce substantially screening costs. To examine this possibility, we decided to design a novel diagnostic antigen including immunodominant fragments of HBV and HTLV-I diagnostic antigen.

There are two general strategies to design the chimeric diagnostic antigens, rational design and directed evolution. In rational design, the researchers use detailed knowledge regarding protein structure and function to design and make desired changes. Design of chimeric protein for detection of *Acinetobacter baumannii* is one of the studies that used this strategy.¹²

We chose computational tools for rational design of antigen because these software accelerate the design of chimeric proteins by approximately 10-20 folds and can provide profound insight into the design of infectious diagnostic agents before any experimental analyses.¹³

MATERIALS AND METHODS

Chemicals, Enzymes and Strains

All reagents used in this study were analytical grade

and were purchased from Sigma Chemical (St. Louis, MO) unless otherwise indicated. Bacterial culture media were purchased from Merck (Germany), restriction enzymes from Takara (Japan), plasmid extraction and DNA extraction kits from Intron (Korea), Western blotting and Ni-NTA agarose resin from Qiagen (Valencia, CA). *Escherichia coli* DH5 α (Invitrogen, San Diego, CA) and BL21 (DE3) (Novagen, Madison, WI) strains were used for plasmid manipulation and overexpression experiments, respectively. The expression vector pGS21a was obtained from Genscript (USA).

Serum Samples

Serum samples used in this study were collected from patients with hepatitis B (anti-HBc positive) or HTLV-1 (anti-HTLV-1 positive) infection referring to Razavi hospital of Mashhad city in Iran. All serum samples were diagnosed as positive when tested with commercial immunodiagnostic ELISA tests (MP Diagnostics™ HTLV-I/II ELISA 4.0 and ORTHO HBc ELISA Test). Samples from healthy volunteers were used as controls and were test confirmed with the ELISA.

Sequence, Databases and Antigen Design

Presences of antibodies against structural proteins are major strategy for detection of HBV and HTLV-I infection carriers. The core antigen of hepatitis B virus (HBc) and surface (gp46) and core (p19) protein of HTLV-I were selected for current study. Related sequences were picked from Uniprot database available at <http://www.uniprot.org/>. Multiple sequences of each antigen were separately aligned by Muscle¹² at <http://www.ibi.vu.nl/programs/pralinewww/>. Needle program in http://www.ebi.ac.uk/Tools/psa/emboss_needle/ was used for pair wise comparison of sequences. Single amino acids substitutions were determined and most conserved residues for such positions were selected. Linear and conformational B-cell epitopes of HBV and HTLV-I diagnostic antigens were predicted using IMTECH at <http://www.imtech.res.in/raghava/btxpred/link.html> and ElliPro at http://tools.immuneepitope.org/tools/ElliPro/iedb_input. Also several supplementary criteria such as antigenicity, accessibility, hydrophobicity and beta-turn formation were used in epitope characterization. In each antigen, the fragments that involve high density of immune-

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dominant epitopes were selected. The fragments fused together by linker (sequence: GSGGSG) to find the best epitope, exposing chimeric antigen. This linker contains glycine and serine residues and is a flexible linker that could separate properly different domains in chimeric proteins.

Physico-Chemical Properties

For evaluation of chimeric diagnostic antigen (CDA) stability parameters such as half-life, instability index, aliphatic index and grand average hydropathicity (GRAVY) were computed by Expasy's ProtParam software available at: <http://web.expasy.org/protparam/>.

Folding Evaluation

Folding capacity of CDA was analyzed using FoldIndex available at:

<http://bip.weizmann.ac.il/fldbin/findex>. Secondary structure of CDA was predicted using Garnier-Osguthorpe-Robson (GOR) secondary structure prediction method. Tertiary structure of different constructs was predicted by various software such as Phyre¹³ at

<http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index>, and I-TASSER¹⁴ at:

<http://zhanglab.cmb.med.umich.edu/I-TASSER/>.

The generated models were evaluated using Ramachandran plot, energy frustration and Z-score calculation. For z-score calculation, *protein data bank* (PDB) files submitted to ProSA-web¹⁵ at:

<https://prosa.services.came.sbg.ac.at/prosa.php> to recognize the errors in generated models. Ramachandran plots of models structures were calculated at rampage at:

<http://mordred.bioc.cam.ac.uk/~rapper/rampage.php>.

Distribution of energy in models was analyzed by frustratometer at [http:// bioinf.qb.fcen.uba.ar/frustra/](http://bioinf.qb.fcen.uba.ar/frustra/).

B-Cell Epitope Prediction

Linear B cell and discontinuous epitopes of designed antigen were predicted at <http://www.imtech.res.in/raghava/btxpred/link.html> and <http://www.imtech.res.in/raghava/cbtope/respectively>.¹⁶ Surface exposed B-cell epitopes having cutoff more than 0.7 were selected and further analyzed using VaxiJen¹⁷ (<http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html>) to check the antigenicity. Location of Conformational epitopes on protein surface was characterized by Episearch software at:

<http://curie.utmb.edu/episearch.html>.

Computational Analysis of Expression Capacity

Expression of protein is a complex process and depends on most various factors. First protein expression probability was predicted by ESPRESSO online tool available at

<http://mbs.cbrc.jp/ESPRESSO/Submission.php>. This

web tool is a sequence based predictor for estimating protein expression for different protein expression systems. Sub-cellular location of CDA was predicted by CELLO available at <http://cello.life.nctu.edu.tw/>.

This online tool predicts sub-cellular localization of the expressed protein to the cytoplasm, inner membrane, periplasm, outer membrane and extracellular space with accuracy of 89%. Toxicity of protein can significantly reduce protein expression level and in some cases pause it thoroughly. Also transmembrane protein insert to membrane during expression might lead to cell death and low expression. Probable biochemical functions (potential toxicity) and presence of transmembrane segments of CDA were predicted by ProFunc server (<http://www.ebi.ac.uk/thornton-srv/databases/profunc/index.html>) and SAPS (<http://www.ebi.ac.uk/Tools/seqstats/saps/>), respectively.

Protein solubility was predicted by different online tools such as protein solubility predictor¹⁸ at <http://www.biotech.ou.edu/>.

Construction of Expression Plasmid Encoding the CDA

The gene sequence coding CDA was codon optimized for expression in *E. coli* and was obtained from Genscript. Optimization of sequence dose not change amino acids coded by DNA, hence could not affect adversely on selected epitopes. The pUC57 vector containing the gene of interest flanked by *Bam*HI and *Eco*RI restriction sites was digested with corresponding enzyme according to standard protocols. The gene of interest was inserted into the multiple cloning site of pGS21a expression vector. The obtained expression vector (2µl) was transformed into *E. coli* strain DH5α chemically competent cells. Transformed bacteria were grown in Luria-Bertani (LB) media containing 100µg/ml ampicillin and the correctness of the sequence was verified by restriction digest and DNA sequencing. Subsequently isolated plasmid transformed into BL21 (DE3) chemically competent cells for protein expression.

Expression of CDA

E. coli BL21 harboring the pGS21a expression constructs were grown in LB medium (5ml) supplemented with 100µg/ml ampicillin and incubated at 37°C overnight with shaking at 250rpm. Fresh LB liquid medium (50ml) containing 100µg/ml ampicillin was inoculated by 5ml of pre-culture. This culture was incubated at 37°C and periodically the OD600nm of the culture was checked until the OD600nm reached to 0.5. Then culture was induced by 1mM IPTG and incubated at 25°C with shaking at 250rpm for additional 6h. Cells were harvested by centrifugation at 5000g at 4°C for 5 min. CDA expression was evaluated on 12% SDS-PAGE and visualized by Coomassie-blue staining.

Protein Solubility Determination

To examine solubility of CDA, *E. coli* expressing CDA were cultured in 50ml of LB (LB: pH 7.0) medium supplemented with ampicillin (100µg/ml). The cultures were grown at 37°C by shaking at ~250rpm. When the OD600nm of culture reached 0.5, CDA expression was induced with isopropyl-β-D-thiogalactoside (IPTG: 1mM) and the culture was further incubated for 10h at 25°C. The cells were harvested by centrifugation at 5000g at 4°C for 15min. The cell pellet was resuspended in 5ml of lysis buffer for native purification. Lysozyme was added at concentration of 1mg/ml and was further incubated on ice for 30min. The cells were disrupted by sonication for 10min with 20s intervals between pulses. The cell lysate was centrifuged at 10000xg at 4°C for 30min. This segregated the suspension into two phases where the supernatant contained the soluble CDA while the insoluble CDA was present in the pellet. These samples were analyzed on a 12% poly-acrylamide gel and amount of proteins were quantified by Bradford assay.

Protein Purification

The supernatant of the cell lysate was transferred into 1 x 5cm column packed with 1ml Ni⁺⁺-NTA resin. The resin was washed with 3 column volumes of binding buffer, following with 5 volumes of washing buffer. The adsorbed CDA 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 chromatographic steps. The purified CDA was analyzed with 12% SDS-PAGE.

ELISA

Binding characteristics of purified CDAs were evaluated using indirect ELISA technique in 96 well micro-titer plates. Wells were coated with the purified CDA (0-15µg/ml) at 4°C. After an overnight incubation, the coated wells were washed with PBS buffer and blocked by 1% bovine serum albumin (BSA) for 2h at 37°C. Then the wells were washed with PBS buffer and 50µl of pulled 10 infected sera (containing anti-HBV or anti-HTLV-I antibodies) was added per well and they were incubated at 37°C for 1h. Subsequently the wells were washed 3 times with PBS-T buffer (5min each), and incubated with peroxidase conjugated anti-human IgG at 37°C for 1h. After washing, 50µl of 3,3',5,5'-Tetramethylbenzidine (TMB) was added into wells and incubated at 37°C for 15min. The enzymatic reaction was stopped by using H₂SO₄ (0.1N: 50µl) and absorbance was read at 450nm using a microtiter plate reader. The wells coated with BSA were used as negative control. All ELISA analysis was done as triplicate.

RESULTS

Computational Design of Diagnostic Antigen

Based on primary sequence analysis, appropriate fragments (64 to 144 of HBC, 57 to 112 of gp46 and 90 to 130 of p19) that served our purpose were selected and used for chimeric antigen named as CDA. A graphical display of CDA is shown in Figure 1.

Folding Capacity

FoldIndex results (Figure 2) indicated that the C-terminal region of CDA form disordered structures after folding process. The disordered regions could act as initial core for protein aggregation which may results in inclusion body formation.



Figure 1. Graphical display of CDA. The length of fragment and position of linkers are graphically illustrated.

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No template with similar fold to CDA was found in PDB library in LOMETS. Hence ab-initio was found to be the best way for prediction of 3D structure. Secondary and tertiary structures of this construct have been shown in Figures 3 and 4). Tertiary structure model was analyzed for model quality by Z-score calculation. The z-score of the built model was in the range of native proteins with similar size (Figure 5). The confidence score (C-score) of I-TASSER model was -2 (typical range -5 to 2) that signify model has high

confidence. Ramachandran plot of CDA has been shown in Figure 6. In I-TASSER generated model 63% and 11% of total residues are located in favored and allowed region, respectively. Approximately, 26% of residues are located in outlier region. Also, Energy frustration analyses of I-TASSER model (Figure 7) indicated that more residues located on low energy positions. The highest degree of frustration is at position of 20 to 25 at N-terminal of CDA.

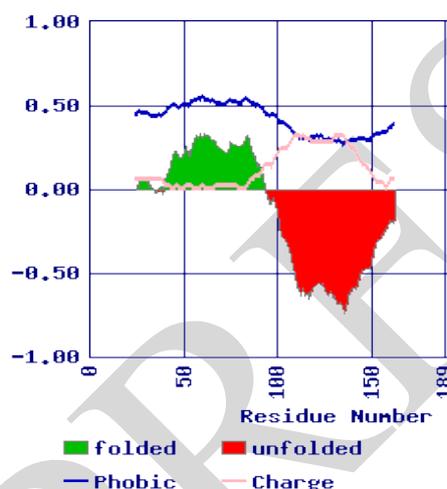


Figure 2. Disordered region prediction. The threshold value is shown as level line.

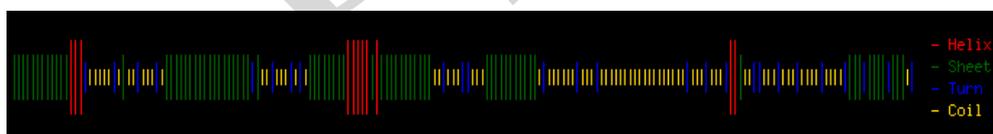


Figure 3. The secondary structure of CDA. Extended strand: purple, Coil: red, Helix: blue.

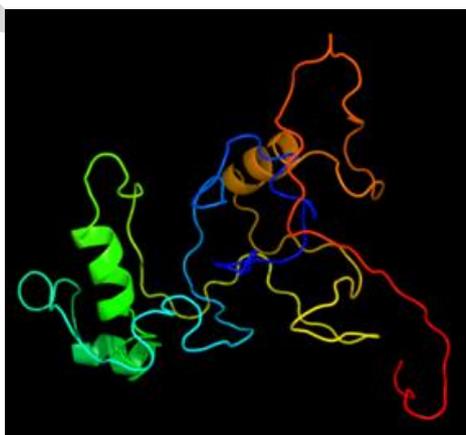


Figure 4. The tertiary structure of CDA. The 3D structure predicted by I-TASSER software. The structure is shown in ribbon model.

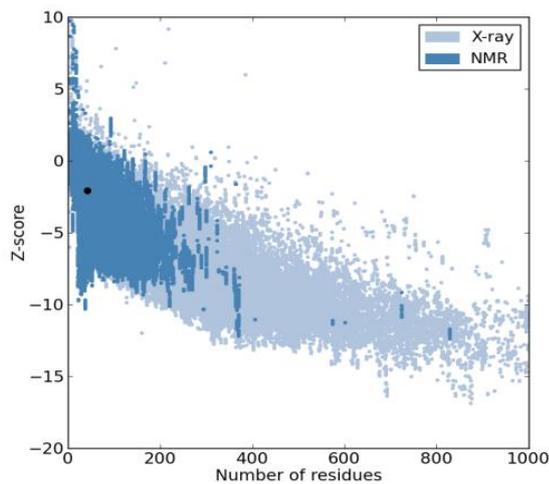


Figure 5. Z-score plot for tertiary structure of CDA. The Z-score was used for model quality. Its value is displayed in a plot that contained the z-score of all experimentally determined protein chains in current PDB. In this plot, groups of structures from different source are distinguished by different colors. It can be used to check whether the z-score of the input structure is within the range of source typically found for native proteins of similar size. The value of Z-score is highlighted as a black colored dot. The value is in the range of native conformations.

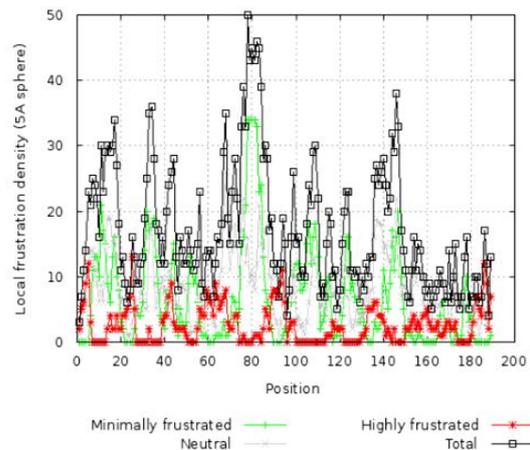


Figure 7. Energy frustration of protein structure. In this figure, the minimally frustrated interactions are shown in green lines, and highly frustrated contacts in red lines. Each dot represents pair interaction between amino acids numbered in the axis. The highest energy distribution was at residues of 20-25.

B-Cell Epitopes

The CDA was evaluated by various computational immune-informatics tools. Based on Vaxigen result, the CDA has enough antigenicity for stimulating of immune system and could be a viral antigen. More than 20 linear epitopes with scores higher than 0.6 were predicted for primary sequence of CDA (Table 1). These epitopes mostly belong to hepatitis B core and HTLV-I surface proteins.

Expression Analysis

The results of expression analysis indicated that CDA could overexpress in *E. coli* cells. The sequence of protein has no cluster of particular amino acid, charge or repeat in sequence. Function analysis has indicated no toxic function or transmembrane segment. The CDA has 97% chance to be insoluble when over expressed in *E. coli* BL21 (DE3).

Expression of the CDA in *E. coli* strain BL21 (DE3)

The N-terminally GST tagged CDA were expressed in *E. coli* BL21 (DE3). SDS-PAGE analysis of the cell lysate gave rise to prominent protein of about 48 kDa which corresponds well with calculated molecular weight.

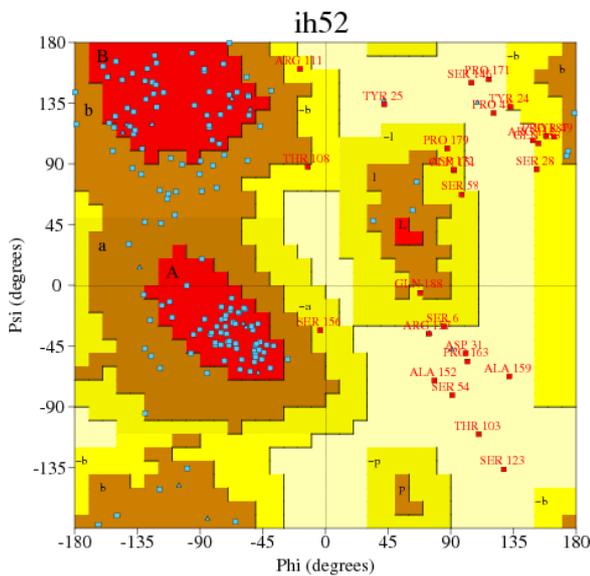


Figure 6. Ramchandran plot for validation of protein structure. The Ramchandran plot revealed that 74% of amino acid residues from modeled structure were incorporated in the favored regions (A, B and L) of the plot. 12% of the residues were in allowed regions (a, b and l) of the plot.

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Table 1. Linear B-cell epitopes predicted for CDA using IMTECH online tool.

Rank	Sequence	Start position	Score
1	GVWIRTPPAYRPPNAP	84	0.96
2	HATYSLYLFPHWIKKP	2	0.93
3	PHWIKKPNRNGGGYYS	11	0.93
4	SSPTHDPDSDPQIPP	164	0.92
5	STLPETTVVRRRGRSP	102	0.92
6	DSDPQIPPPYVEPTAP	172	0.90
7	PPAYRPPNAPILSTLP	90	0.87
8	GGSGQAQIPSRPAPP	147	0.87
9	TVLEYLVSFGVWIRTP	75	0.85
10	SPRRRTSPRRRRSQS	116	0.84
11	CQSWTCPYTGAVSSPY	42	0.83
12	GYYSASYSDPCSLKCP	23	0.83
13	RRRSQSPRRRRSQSRE	126	0.83
14	TGAVSSPYSGGSGWFH	50	0.80
15	PCSLKCPYLGCQSWTC	32	0.80
16	PYSGGSGWFHISCLTF	56	0.77
17	TVVRRRGRSPRRRTPS	108	0.77
18	GWFHISCLTFGRETVL	62	0.72
19	PNAPILSTLPETTVVR	96	0.64

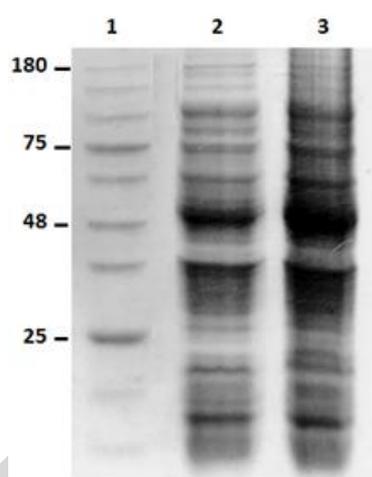


Figure 8. Expression analysis of the His-tagged fusion CDA. Non-induced cells were used as negative control. Expression of the protein was analyzed by 12% SDS-PAGE. Protein marker (lane 1), non-induced (lane 2) and induced bacteria (lane 3).

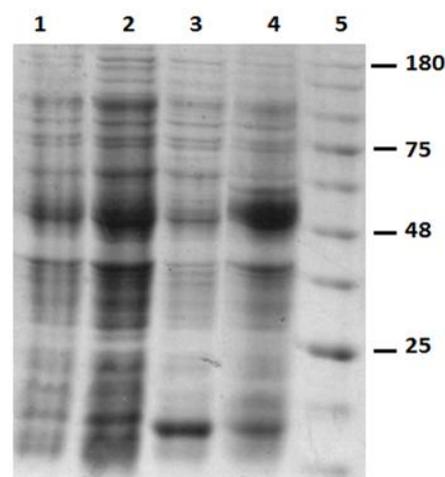


Figure 9. Solubility testing of CDA analyzed by 12% SDS-PAGE. Non-induced control (lane 1), induced bacteria (lane 2), supernatant (lane 3), pellets (lane 4) and protein marker (lane 5).

Solubility Analysis of CDA

To determine the distribution of expressed protein in soluble and insoluble fractions, all supernatant and pellet of cell lysates were analyzed after sonication by SDS-PAGE. The results revealed that the recombinant protein was expressed as insoluble at 25°C with solubility of about 7.5%.

Purification of CDA with Immobilized Metal Affinity Chromatography

His-tagged CDA was purified using the Mclab purification system and purity obtained was about 95% or higher. The majority of contaminant proteins were removed by washing of the protein bound column with 10 column volumes of binding buffer containing 20mM

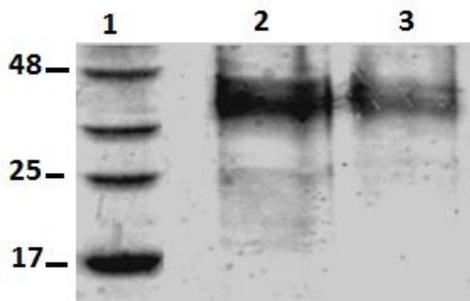


Figure 10. SDS-PAGE analyses after purification with Ni-NTA agarose column. Protein marker (lane 1), purified protein (lane 2 and 3).

midazole and washing buffer supplemented with 50mM imidazole. The purified CDA were analyzed on 12% SDS-PAGE. The prominent band corresponding to CDA was observed on the SDS-PAGE (Figure 10).

Antigenicity of CDA

The purified CDAs were detected using human anti-HBV and anti-HTLV-I sera in ELISA. The results showed that CDAs reacted well with known positive samples. This indicates that epitopes were displayed and were functional. The signals increased as more CDAs were coated on the wells (Figure 11). Also we examined the serum of 10 patients infected with HBV and HTLV-I using ELISA prepared with CDA. Result

of this evaluation indicated that the antibodies were detected successfully by CDA in infected serums.

DISCUSSION

The main strategy in this study was to design a chimeric antigen (named CDA) carrying various epitopes for simultaneous detection of HBV and HTLV-1 antibodies. Theoretically, CDA contains three immunodominant fragments that could be synthesized as unique construct in *E. coli* cells. Development of simultaneous screening can significantly reduce screening costs.

The HbC was selected for detection of HBV antibodies because anti-HbC appears before other HBV antibodies in circulation and are not found in vaccinated individuals.¹⁹ The surface and core proteins were selected for HTLV-I detection because in some of HTLV-I infected patients, antibodies against the envelope proteins (g46 and g21) or p19 are not present. In such patients exclusively envelope or core antibodies may be present. Screening test will be positive when such samples will be assessed by envelope and core proteins simultaneously.²⁰ Hence, different fragments from envelope and surface proteins were selected for CDA design.

Linker plays an important role in displaying the epitopes on surface of fusion proteins. Glycine and

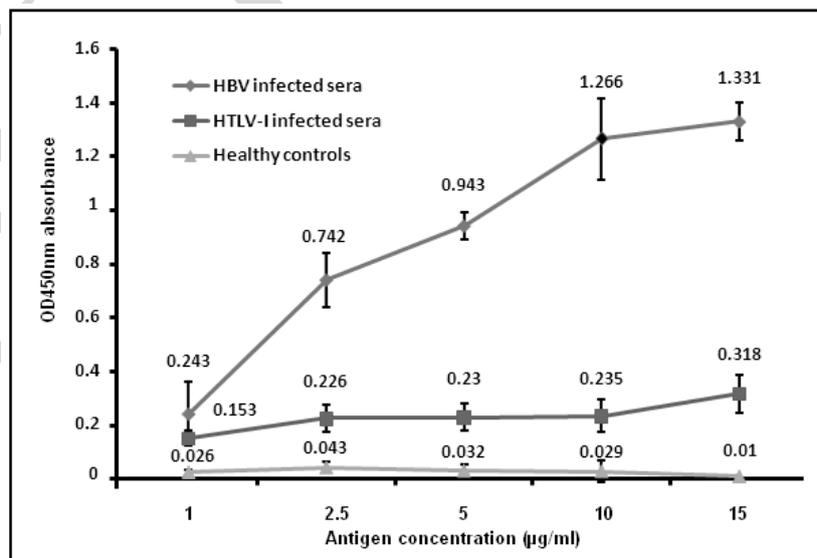


Figure 11. Binding activity of CDA to pooled infected sera. ELISA assay demonstrated a binding curve at various concentrations of CDA towards fixed concentration of serum antibodies. BSA was used as negative control under the same condition.

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serine residues form flexible linkers that could properly separate different domains in chimeric proteins.²¹ In the current study, GSGGSG was introduced between fragments for efficient separation. Also, linkers containing glycine and serine residues have sufficient flexibility that may enhance protein expression level.²²

FoldIndex and protein solubility prediction results indicated that CDA was expressed as inclusion bodies and has 0.0 percent chance for solubility in *E. coli* cells. These predictions were in consistency with previous studies. Envelope proteins of HTLV-I such as gp46 and also core antigen of hepatitis are aggregation prone and form inclusion bodies in *E. coli* cells.^{23,24} Inclusion bodies are mis-folded protein aggregates that have no biological activities. Refolding of inclusion bodies is time consuming and is difficult to be optimized in large scale.²⁵ Therefore, various strategies were selected for improving of protein solubility such as lower induction temperature (25C),²⁶ low concentration of IPTG (0.4mM IPTG)²⁷ and fusion with GST.²⁸ Glutathione tag could significantly improve solubility of proteins. With adaptation of these measures, only 9.5% of protein was expressed as soluble.

The computational epitope mapping results were validated by performance of ELISA. Our results indicated that this protein was specifically reactive towards anti-HBV and anti-HTLV-1. These results are in consistency with Olfa et al. In that study the OmcB protein of *Chlamydia tracomatis* was in silico analyzed in order to find the specific and immunodominant regions for development of sero-diagnosis method for *C. tracomatis* infection. Their results indicated that computational epitope mapping may be a useful candidate for diagnostic antigen design.^{29, 30}

However there are indications suggesting that the computational studies have not enough accuracy for prediction of linear epitopes. Van Regenmortel and Pellequer have compared the prediction efficacy of 22 different scales, taking into account both the correct and incorrect predictions, and showed that the prediction accuracy was not more than 50–60 %.³¹ The results of this study indicated that such constructs may be useful candidates for development of detection methods for simultaneous diagnosis of both infections. However, our study has some limitations. We examined only 10 infected sera for both viruses, therefore it is suggested that such antigens should be evaluated with large number of infected sera to

compare the immune-reactivity of such antigens with experimentally designed antigens.

In conclusion, this study demonstrated that computational software could be successfully applied for design of proteins. Also results have indicated that the use of recombinant proteins is effective in screening of HBV/HTLV-1 infected patients from uninfected individuals. These types of assays with such antigens have performance comparable to assays used for blood screening. Such antigens may be used for standard screening immunoassays and to reduce screening costs substantially.

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