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# In Silico Structural Prediction and Production of a Chimeric Recombinant Dickkopf-1 (DKK-1) Antigen

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**ABSTRACT**

Dickkopf (DKK) family of proteins are known as antagonists for the Wnt-β-catenin signaling pathway. It is suggested that the Dickkopf-1 (DKK-1) has a role in several diseases such as hepatocellular carcinomas, hepatoblastomas, Wilms' tumors, lung cancer and Myeloma bone disease.

The aim of the present study was to produce a chimeric-recombinant DKK-1 protein in order to induce immune response against the antigen.

The recombinant Dickkopf-1 (rDKK-1) protein was designed using bioinformatics analysis. The standard methods were used for cloning, expression and purification. The structure of recombinant protein was analyzed by spectroscopy methods. Enzyme-linked immunosorbent assay (ELISA) and Western blotting were performed to confirm the recombinant protein using a commercial anti-DKK-1 (whole protein) polyclonal antibody. The immunogenicity of the recombinant DKK-1 was assessed by immunizing, intraperitoneally, BALB/C mice four times with the 31-kDa and 45-kDa purified rDKK-1 cloned in pET28a and pET32a vectors respectively. The antibody titer was measured in due course of time.

Stronger immunogenic parts of the protein were selected based on in-silico predictions and recombinant protein was successfully designed. The chimeric gene was sub-cloned, expressed, purified and refolded. The purified protein was confirmed by Western blotting and ELISA. The three dimensional structural was confirmed by CD spectrum and predicted structures by bioinformatics tools, revealed the stability of helix structures. rDKK-1 protein was capable of inducing immune response with high titer antibody and excessive humoral immune response. No significant difference was observed between immunization by 31-kDa and 45-kDa antigen.

**Keywords:** Antibody titer; Chimeric antigen; Dickkopf-1; In silico prediction; Refolding

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## INTRODUCTION

Dickkopf (DKK) is the family of proteins including DKK-1, DKK-2, DKK-3 and DKK-4 are known as



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antagonists for the Wnt/β-catenin signaling pathway.[1]The Wnt/β-catenin pathway is critical for cell fate determination during gastrulation in embryogenesis and tissue renewal in adults. Dysregulation of this pathway is tightly associated with cancer and bone anomalies.[2] X-ray crystal structure of the DKK-1 C-terminal domain bound to LRP6 has shown that a single molecule of DKK-1 can inhibit several Wnts.[3] DKK-1 is a secreted protein with two cysteine rich regions that binds to low-density lipoprotein receptor-related protein (LRP5/6), leading to inhibition of the Wnt pathway. Wnt signaling is under precise regulation by a delicate balance of extracellular agonists and antagonists.[4,5]

Curiously, previous reports indicated that both down- and up-regulation of DKK-1 expression level is linked to various malignancies.[6-10] Moreover, several studies revealed that the DKK-1 level is significantly increased in the sera of lung cancer patients and myeloma bone disease.[11,12] These fluctuations in DKK-1 expression levels, in various cancers, represent this antigens an ideal target for early cancer detection or non-invasive screening. Hence, the antigen may be used to develop antibodies (polyclonal and monoclonal) for the purpose of diagnostic tools designing. Sequence alignment of DKK-1 protein from human and mice showed that there was a high similarity between them. In order to induce immune system, it would be required to make several desirable changes through the recently available bioinformatics tools. Prediction of structural properties and solubility of heterologous proteins using bioinformatics analysis can improve experimental results. Furthermore, although *Escherichia coli* was recognized as an easy to handle and cost-effective platform for production of recombinant proteins in high yield,[13] production of eukaryotic proteins such as DKK-1usinga prokaryotic expression system is encountered with several drawbacks including codon usage bias and lack of eukaryotic post-translational modification, which entails in silico studies prior to starting experiments. In this study, we used different bioinformatics and in-silico prediction tools for the investigation of epitope mapping, prediction of tertiary structure and structure comparison of the rDKK-1 protein with the experimentally determined 3-dimensional structure presented in the RCSB PDB website.

## MATERIALS AND METHODS

### Bioinformatics Analysis

### Sequences and Databases

The respective gene and protein sequences were selected based on information obtained from the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov) and relevant published articles.[14,15] Protein tertiary structures were acquired from Protein Data Bank (PDB) (http://www.rcsb.org/pdb/home/home.do). Post translation modifications, such as glycosylation and disulfide bonds were analyzed using data presented in the UniProt website (www.uniprot.org). In order to expose immunogenic epitopes, the sequences were fused to each other by Gly-Ser linkers (Figure 1). The physico-chemical parameters such as molecular weight were computed using the ProtParam tool on ExPASy server (http://us.expasy.org/tools/protparam.html).

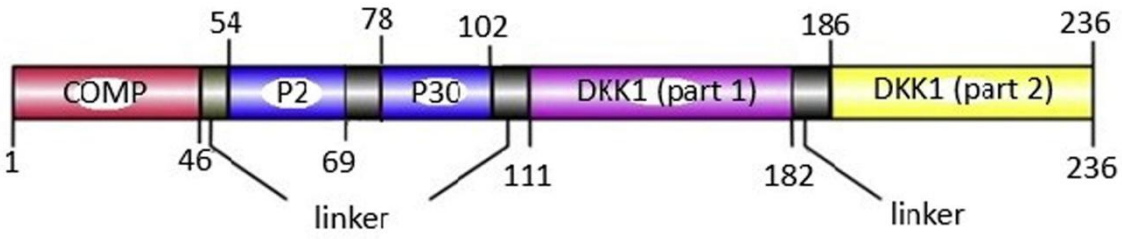


**Figure 1. Schematic representation of chimeric recombinant DKK-1 construct consisting of cartilage oligomeric matrix protein (COMP), tetanus toxin epitopes and two truncated parts of the DKK-1 protein, fused together using appropriate linkers for expression in *E. coli*.**

**B-cell Epitope Mapping**

For the prediction of antigen immunogenicity, full-length DKK-1 protein sequence was subjected to web-based B-cell epitope prediction algorithms, ABCpred (www.imtech.res.in/raghava/abcpred) and BcePred (http://www.imtech.res.in/raghava/bcepred/analysis), to predict continuous linear B-cell epitopes. The most immunogenic segments were selected.

**Secondary and Tertiary Structures and Protein Solubility Prediction**

The secondary structure prediction was performed by SOPMA and PSIPRED servers using consensus prediction from multiple sequence alignments. Three dimensional (3D) model of the designed construct was evaluated by the Iterative Threading ASSEmbly Refinement (I-TASSER) and MODELLER homology-based online servers. The YASARA molecular graphics program was used to visualize the modeled 3D structures. Stereochemistry quality of the resulting protein structures was validated by RAMPAGE Ramachandran plot. Finally, protein solubility was evaluated using recombinant protein solubility prediction tools PROSO (http://mips.helmholtz-muenchen.de/proso/proso.seam) and SOLpro (http://biotech.ou.edu).

**Experimental Analysis**

**Cloning and Expression of the Chimeric Protein**

The codon optimized nucleotide sequence encoding rDKK-1 was synthesized for expression in prokaryotic systems by GeneArt (Invitrogen, USA). Synthesized sequences were sub-cloned into pET28a (+) and pET32a (+) vectors using *EcoRI* and *HindIII* restriction enzymes. Colony PCR and double digestion was exploited to screen colonies. Finally, *Ecoli* Rosetta-Gami 2 (DE3) bacteria were transformed with the pET-rDKK-1 construct.

Single clones of Rosetta-Gami 2 (DE3) harboring recombinant plasmid pET-28a/rDKK-1 or pET-32a/rDKK-1 vectors were grown in 5 mLof Luria-Bertani (LB) medium containing 50 µg/mL antibiotic at 37°C, shaking at 150 rpm overnight. Prior to induction by IPTG (isopropyl-b-thiogalactopyranoside, Sigma, USA), 10 mL of LB medium was inoculated with 100 µL of overnight culture and incubated at 37°C until $OD_{600}$ reached 0.6.

Optimized condition for the expression of recombinant DKK-1 was determined using induction with different IPTG concentrations (0, 0.25, 0.5, 0.75 and 1 mM), incubation at different temperatures (4, 18, 30 and 37°C) and varying the induction time (1, 2, 3, 4, 5 and 18h). Briefly, induced cells were harvested by centrifugation, re-suspended in lysis buffer and treated by ultrasound sonication. Non-induced cells were harvested as negative control.[16] Expression and solubility of rDKK-1 was analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% gel, as described previously.[17,18]

**Purification and Refolding of rDKK-1**

Following incubation at 18°C for 18h, cells were harvested, dissolved in lysis buffer and kept at room temperature for 30 min, then treated with an ultrasound sonication at 1 min×2 cycles. The mixture was centrifuged at 8,000g for 10 min and the pellet was re-suspended in denaturing buffer (100 mM NaH2PO4, 10 mM Tris-HCl, 8 M urea, pH 8), followed by shaking at 150 rpm for 1.5h at 37°C. The suspension was centrifuged at 14,000 rpm for 1 h at 4°C.[19] Purification of rDKK-1 was performed through two different methods; in the first method, the supernatant was applied onto a Ni-NTA column resin (QIAGEN, Germany). After washing the column with 10 ml of washing buffer (100 mM NaH2PO4, 10 mM Tris-HCl, 8 M urea, pH 6.3), protein refolding was performed on-column using a urea gradient. The purified protein was eluted by 5 ml elution buffer (50 mM NaH2PO4, 300 mM NaCl, 250 mM imidazole, pH 8), and washed with PBS (phosphate-buffered saline) on a 10 kDa Amicon ultra centrifugal filter (Merck, USA).[20]

In a second method, the supernatant was diffused through a 50 kDa filter, and the filtrate was further centrifuged on a 10 kDa filter. In order to refold the protein, the supernatant was diluted4 times by adding drop by drop of PBS buffer. The mixture was then washed three times with PBS and the refolding solution was concentrated on a 10 kDa filter by centrifugation at 3500g for 20 min. Concentration of the purified protein was estimated by Bradford assay.[21]

**Confirmation of the Purified rDKK-1 Protein by Western blot Analysis and ELISA**

The purified rDKK-1 protein was confirmed using commercial anti-DKK-1 polyclonal antibody (Elabscience, USA) by Western blot analysis and enzyme-linked immunosorbent assay (ELISA). For Western blotting, rDKK-1 protein expressed in pET32a was subjected to 11% SDS–PAGE. Further steps was

performed according to the standard procedures. Commercial active human DKK-1 protein (Abcam, UK), BSA (Bovine serum albumin), and recombinant DKK-1 purified through two distinct methods were coated (1 µg per well) onto the wells of polystyrene microtiter plates (Jet Biofil, China), and the absorbance was measured at 450 nm.

**Circular Dichroism Spectropolarimetry**

The far-UV circular dichroism (CD) measurements were recorded using J-810 circular dichroism spectropolarimeter (JASCO, Japan) with a 1-mm path length at room temperature. Protein concentration was0.2 mg/mL in the PBS buffer pH 7.2. The represented spectra are the results of averaging at least three accumulations.

**Antigenicity Testing**

All animal experiments were conducted in strict accordance with approved conditions and standard guidelines by the International Animal Studies Committee. All procedures were performed according to the approved guidelines by the ethics committee of the Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran (approval ID: IR.TMU.REC.1394.7). To examine immunogenicity of recombinant DKK-1, mice (n=15) were divided into two groups (test and control). In the test groups, the purified rDKK-1 from both vectors pET28a and pET32a were intraperitoneally injected in two groups (n=5) of six-week-old female BALB/c mice (Pasture institute, Iran). During initial injection, 50 µg of rDKK-1 was emulsified in Freund's complete adjuvant (Sigma, USA) and boosters injections were performed four times by a minimum interval of two weeks with 20µg of rDKK-1 emulsified with incomplete Freund's adjuvant. The mice (n=5) receiving PBS were used as control. The animals were bled (40 microlitter from each mouse) at five days after the immunizing injections (First, second ,third and fourth) and sera were collected for further analysis. The rDKK-1-specific antibody responses were analyzed by indirect ELISA briefly as follow. Wells of microtiter plates were coated with an optimal concentration of rDKK-1 expressed recombinant protein in pET28a (1 µg per well) and incubated at 4°C overnight. BSA solution was coated as control. The plates were washed with PBST (PBS with 0.05% Tween-20) and blocked with 5% skim milk at 37°C for 60 min. After washing, 100 µL of serum at serial dilutions (from 1:500 to 1:64000 in order to find the best reacting dilution of antibody with the antigen) was added and incubated for 60 min. at 37°C. After washing steps, plates were incubated with rabbit anti-mouse immunoglobulin G horseradish peroxidase (Sigma, USA) (1:5000 in PBST) at 37°C for 60 min. further steps were performed according to the indirect ELISA protocol.

## RESULTS

**Bioinformatics Analysis**

Respective sequences for construct design were selected from computerized data banks and previously published studies.[14,15] The linear B-cell epitope regions were predicted using BcePred and ABCpred software that utilized different physico-chemical scales and recurrent neural network for prediction. Theoretical biochemical parameters of rDKK-1 were computed by ProtParam software and showed that the molecular weight of this protein would be 31 kDa and 45 kDa when expressed in pET-28a and pET-32a, respectively. The 109 aa thioredoxin fusion tag (Trx-tag) from pET-32 vector is responsible for the extra 14kDa molecular weight.[15]

**Prediction of Secondary and Tertiary Structures**

SOPMA, an improved software in protein secondary structure prediction, showed the sequence length of 266 amino acids for rDKK-1, where 76 amino acids (29%) were included in structure of alpha helices, 43 amino acids (16.17%) were in extended strand (Ee) and 118 amino acids (40.36%) in random coil (Cc) (Figure 2). The results showed no sign of $3_{10}$ helix (Gg), Pi helix (Ii), beta bridge (Bb) or bend region (Ss) structures.

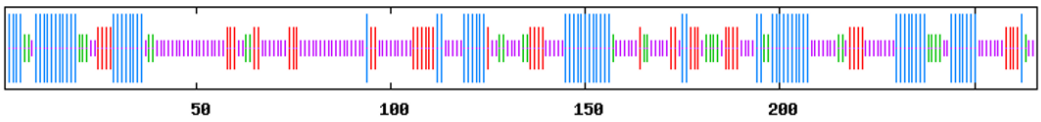


**Figure 2. Secondary structure prediction of Dickkopf-1 (DKK-1) by self-optimized prediction method (SOPMA). Graphical results for the secondary structure prediction of rDKK-1. Helix: blue; extended strand: purple; coil: red.**

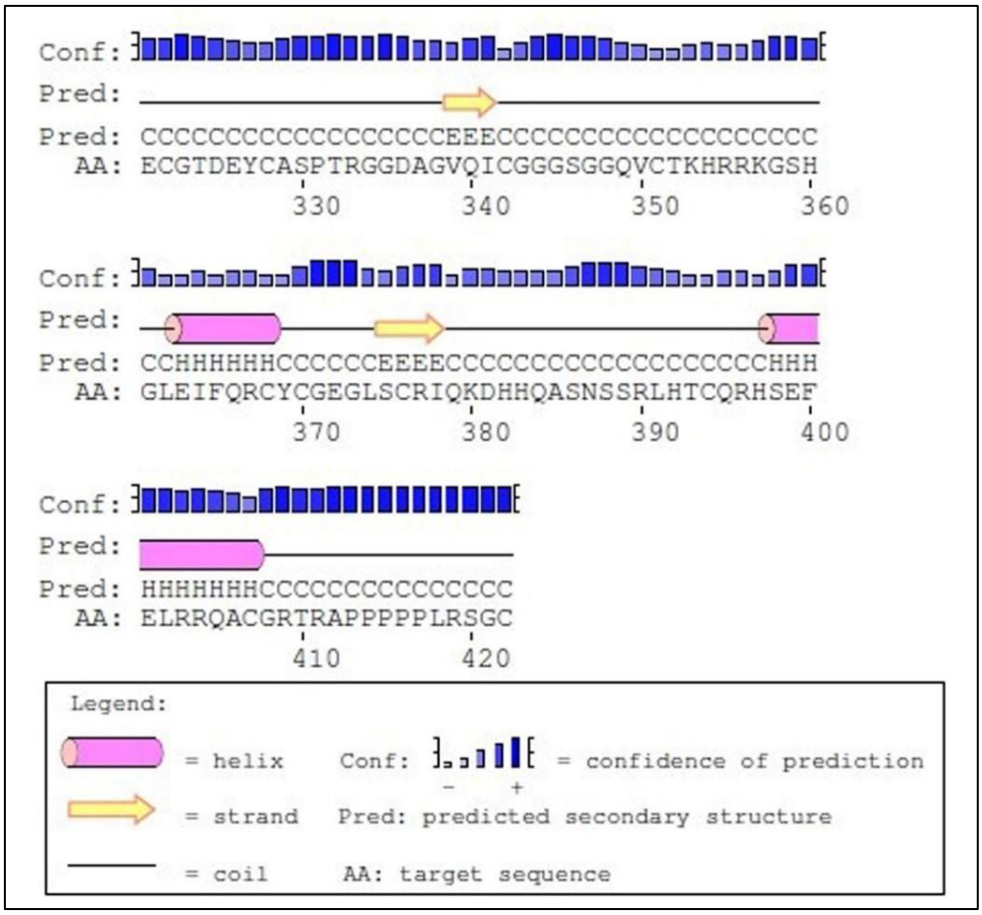


**Figure 2. Secondary structure analysis of rDKK-1, expressed in pET-32a/rDKK-1 by the PSI- blast based secondary structure prediction (PSIPRED) online tool**

The structural comparison between rDKK-1 and the determined structure of DKK-1 in PDB site using PSIPRED online tool indicated no exact similarity locations of helices and sheets between the two models. As an example, just the strand located at Leu, Ser, Cys, Arg, Ile and Gln residues in PDB model is present between Leu 375 and Gln 378 in rDKK-1(Figure 3).

The quality of model was verified by RAMPAGE via assessment of the Ramachandran plot and 93.1% of residues were predicted to place in the favored region.

As illustrated in Figure 4, a tertiary structure of rDKK-1 was predicted by several software. The results obtained from MODELLER showed higher similarity to folded form of DKK-1 protein presented in protein data bank than that of I-TASSER.

**Protein Folding and Solubility**

Solubility and folding of rDKK-1 heterologous protein was evaluated and compared to DKK-1 by PROSO (http://biotech.ou.edu/) and Fold Index (bip.weizmznn.ac.il/fldbin/findex). Data suggested that the solubility and folding were improved in rDKK-1 as a result of COMP domain addition. In PROSO, the input protein sequences are categorized into two classes; yes (soluble) or no (insoluble). The probability of belonging to a class is also provided as a number between 0.5 and 1. The result was no (0.925) for DKK-1 and yes (0.577) for rDKK-1. Data analysis provided by FoldIndex signified that the chimeric state directly improved the folding properties of the predicted model (Figure 5). PROSO renders a statistical model to predict protein solubility based on overexpression of the target protein in *Escherichia coli*. This software estimated that DKK-1 and rDKK-1 have a zero percent chance of solubility, when overexpressed in *E. coli*.

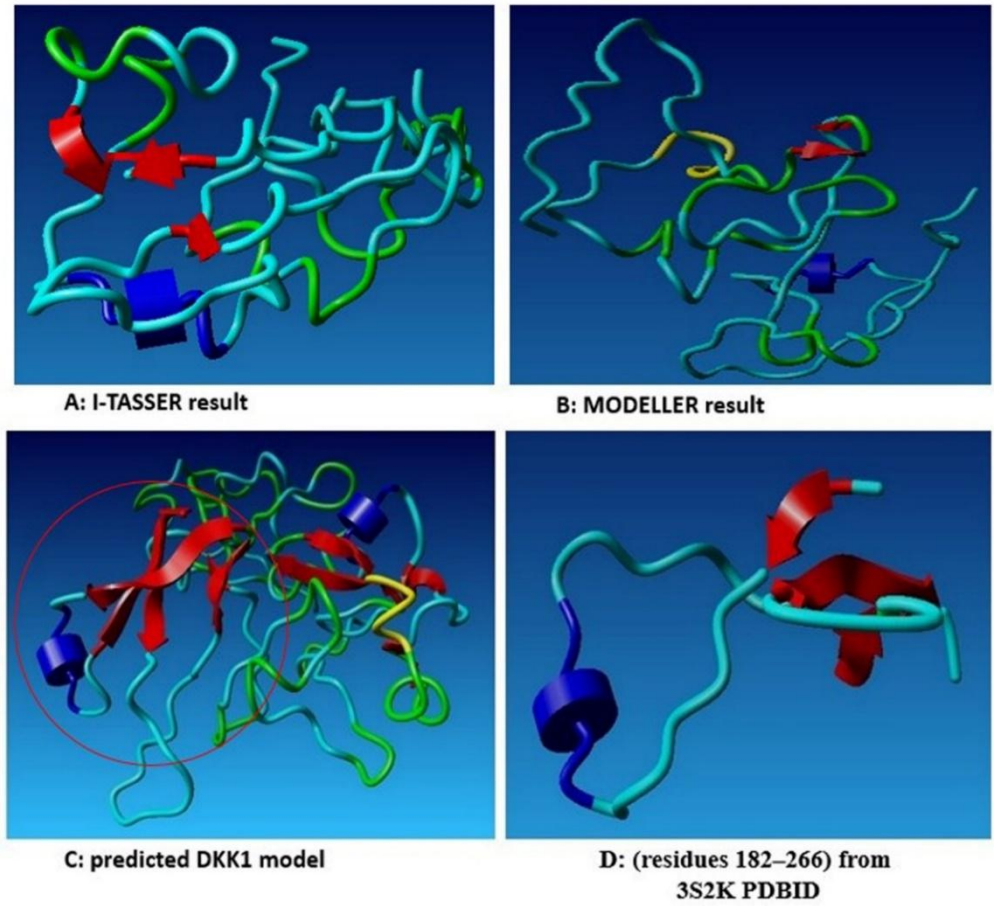


**Figure 3. Three dimensional structure prediction. A: recombinant Dickkopf1 (rDKK-1) predicted by Iterative Threading ASSEmbly Refinement (I-TASSER); B: rDKK-1 predicted by MODELLER homology-based online servers; C: predicted Dickkopf1 (DKK-1) model; D: defined structure of residues 182-266 from 3S2K protein data bank identification code (PDBID). The results were viewed by the yet another scientific artificial reality application (YASARA) software. Red: sheets; blue: helix.**

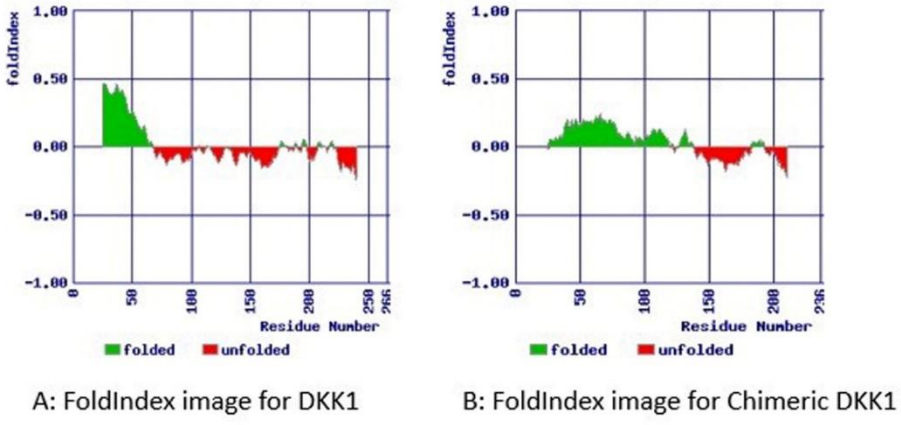


**Figure 4. FoldInedx analysis: improved protein folding in the chimeric protein by adding COMP domain. A: FoldInedx analysis for complete sequence of DKK-1 protein; B: FoldInedx analysis for chimeric sequence of recombinant DKK-1 protein.**

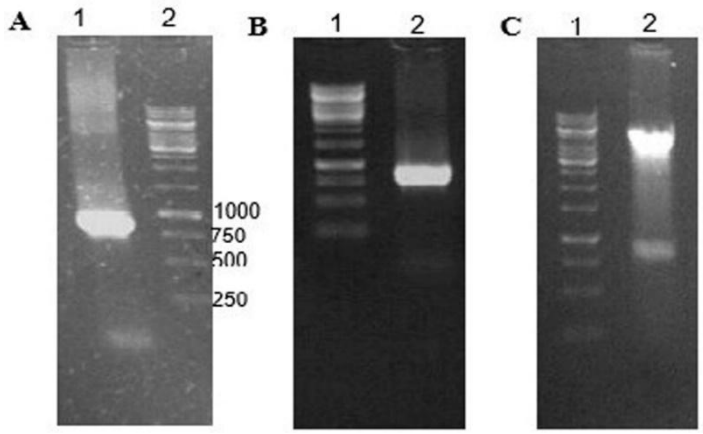


**Figure 5. Analysis of PCR products and cloning confirmation following agarose gel electrophoresis. A) Lane 1, PCR products of rDKK-1; Lane 2, DNA marker (Fermentas). B) Lane 2, colony PCR of rDKK-1 in pET-28a/rDKK-1. C) Lane 2, the recombinant plasmid digested by *Hind*III and *Eco*RI.**

**Cloning, Expression and Purification of Recombinant DKK-1**

Following codon optimization, chimeric DKK-1 gene was amplified by PCR (Figure 6A) and then cloned into pET-28a and pET-32a expression vectors. The gene insertion was confirmed by colony PCR (Figure 6B) and restriction enzyme digestion (Figure 6C).

The cells carrying the expression construct were induced by IPTG and expression of rDKK-1 was verified by the presence of a protein band of around 31 kDa for pET-28a/rDKK-1 and 45kDa for pET-32a/rDKK-1 vectors in SDS-PAGE analysis, which also indicated overexpression of the heterologous protein by both constructs, under optimized conditions (Figure 7). The highest level of rDKK-1 expression was achieved when 0.5 mM IPTG was applied for induction at 18°C for 18h. The recombinant DKK-1 was entirely expressed in the form of insoluble inclusion bodies in the *E. coli* cytosol. Inclusion bodies were purified and solubilized in a denaturation buffer containing 8 M urea. Purification using Amicon ultra centrifugal filters yielded better results compared to the nickel column. Approximately 4 mg of rDKK-1 was obtained from 1 liter of bacterial culture in the filtration approach and 0.3 mg per liter by Ni-NTA column.

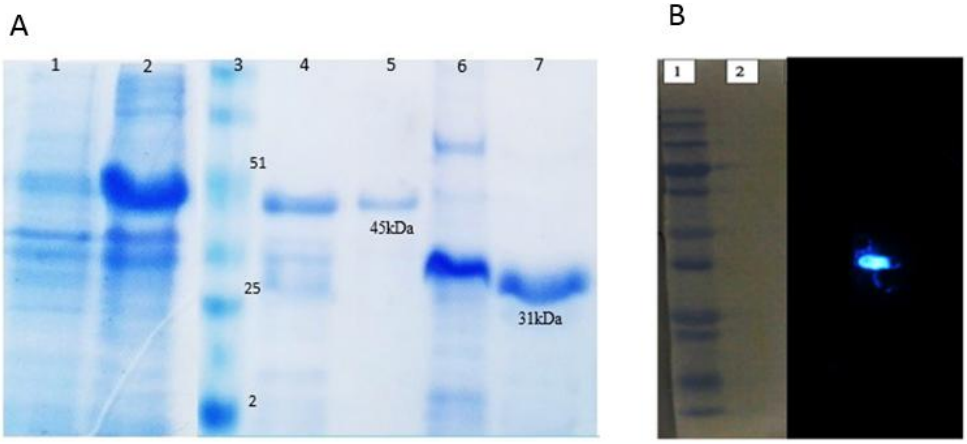


**Figure 6. A: SDS-PAGE analysis of rDKK-1 expression and purification. 1, Rosetta gami-2 (DE3) crude lysate transformed with pET-32a/rDKK-1 without induction; 2, Rosetta gami-2 (DE3) crude lysate transformed with pET-32a/rDKK-1 after induction; 3, protein marker (Fermentas sm0431); 4.PET-32a/rDKK-1 after sonication and centrifugation; 5, pET-32a/rDKK-1 after purifying via filtration; 6, pET-28a/rDKK-1 after sonication and centrifugation; 7, pET-28a/rDKK-1 after purifying via filtration. B: Confirmation of expressed rDKK-1 protein in pET32a vector by Western blotting. 1, pre-stained protein marker; 2, expressed rDKK-1 in pET32a band.**

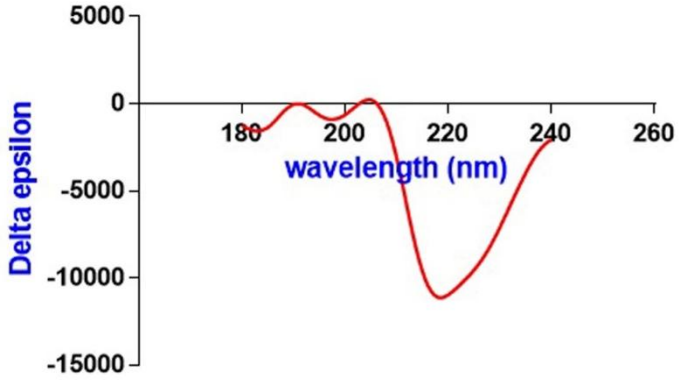


**Figure 7. Circular dichroism (CD) spectra of rDKK-1 at room temperature**

### Circular Dichroism (CD)

In order to analyze the content of secondary structure the far-UV CD spectra of rDKK-1 expressed in pET-32a/rDKK-1 was measured at room temperature (pH 7.2). Based on CD results, 27.8% helix, 39.6% random coils, 32.6% turn and 0.0% beta structure was reported for rDKK-1 (Figure 8).

### ELISA

Purified rDKK-1 through column or filtration approaches was confirmed by ELISA using anti-rDKK-1 polyclonal antibody and the results signified a better protein purification and the protein refolded via the filtration method (Figure9).

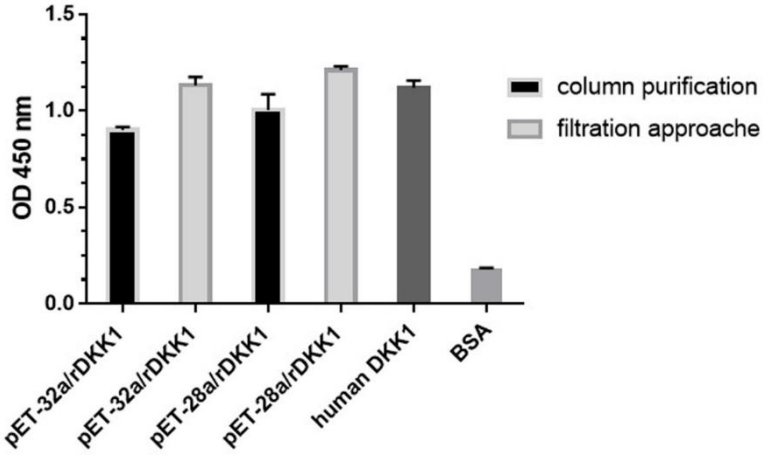


**Figure 8. Evaluation of purified rDKK-1 by indirect ELISA on rDKK-1 expressed in pET28a (+) and pET32a (+) vectors (1 µg/well), or purified by column- and filtration-based approaches. BSA was used as control. Commercial antibody was prepared as recommended, at 1:100 serial dilution. Each bar represents the mean±SD.**

### Serum Antibody Titers

Anti-rDKK-1antibody titers in the sera of BALB/c were evaluated by ELISA using purified rDKK-1 (expressed in pET28a vector) as an antigen. The antibody level was significantly increased after three boosters immunization with the recombinant protein produced in this study compared to the control group (even in 1:64000 dilution).The sera from immunized mice with recombinant proteins expressed in both vectors (pET28a and pET32a) and after three and four boosters showed almost the same antibody titer. On average, immunized mice exhibited 2.8-fold higher antibody level in comparison with the control group (Figure10). These results indicated that rDKK-1 imparts high immunogenicity.

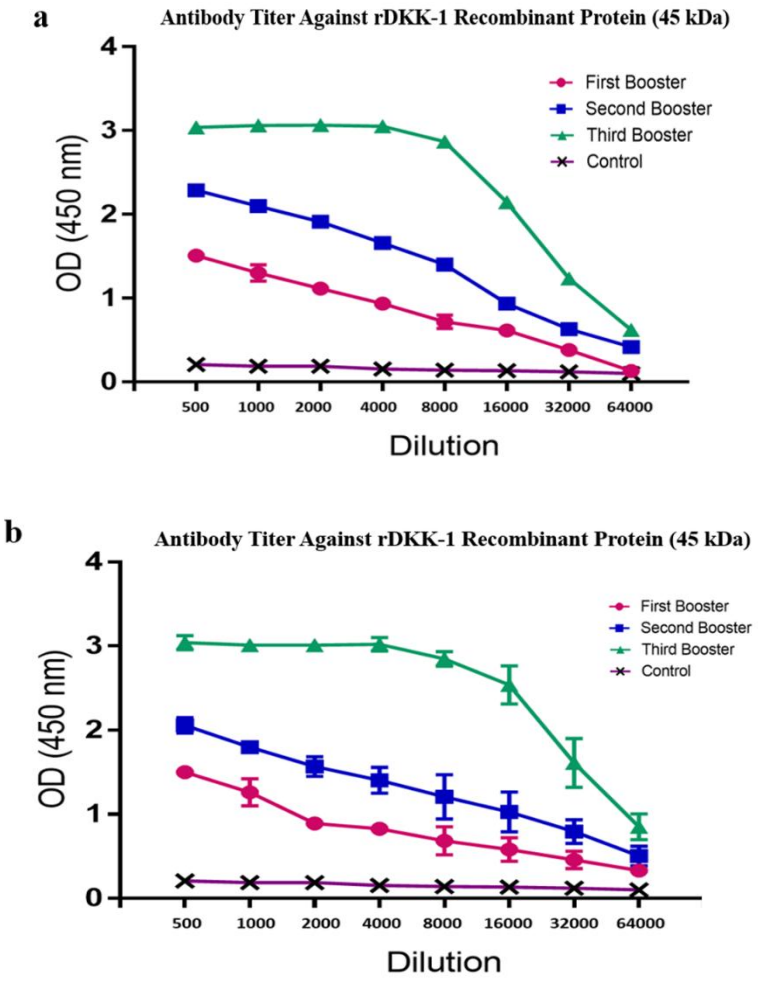


**Figure 10. Anti-rDKK-1 antibodies in the sera of immunized mice. Immunization was performed by the intra-peritoneal injection of the recombinant DKK-1. (a and b) represented mice immunized with expressed rDKK-1 proteins in pET28a (+) and pET32a (+) vectors respectively.**

## DISCUSSION

Apart from its role as negative regulator of the canonical Wnt signaling pathway, many studies suggest DKK-1as a potential tumor marker for cancer detection and prognosis.[8,10,22,23] Normally, DKK-1 expression is limited to a very low level in eukaryotic systems,[24] rendering recombinant technology the favorable approach to produce this antigen. Due to its efficiency and low production costs, *E. coli* is the most frequently employed expression host for the production of exogenous recombinant protein antigens.[25]

Bioinformatics approaches can lead to significant reduction in time, expense and failure in experimental attempts. Although bioinformatics predictions may not always be in concordance with experimental results which is accounted as the main limitations. However with the advancement of software and continues information regarding the relation between the structure and function of protein they could play an important role in vaccine design, development of protein suitable for antigen preparation used in immunoassay, structural studies, drug-protein and protein-protein interaction analysis.[26,27]

Herein, we have attempted to produce immunogenic rDKK-1 antigen along with anti-rDKK-1 polyclonal antibody reactive towards the native DKK-1 protein which may be used to develop highly sensitive and specific immunoassay. Linear B-cell epitopes of whole DKK-1 protein were predicted by the BcePred and

ABCpred servers and epitopes with high scores were selected, as the first step in antigen designing.

The PII and P30 epitopes of tetanus toxin are reported to be beneficial to overcome humoral tolerance. Moreover, it has been proposed that inclusion of a COMP domain can improve protein solubility.[28] In view of these facts, our final chimeric protein construct consisted of DKK-1 immunogenic segments, COMP domain and the tetanus toxin epitopes.

The results of circular dichroism on rDKK-1 secondary structure corroborated with that predicted by SOPMA, and the same percentage of helix structure was reported. Although, no beta-sheet structure was detected according to CD results, which may be due to helix structure being more robust than beta strand, with higher stability during the purification procedure.[29] Secondary structural prediction by SIPRED had negligible similarity with determined structure of DKK-1 in 3S2K PDBID.

To investigate tertiary structure of the modeled protein, the structure of segments was obtained from the PDB website. The results obtained from MODELLER and I-TASSER server illustrated structural similarity of residues 182-266 from 3S2K PDBID.

The secondary structure of predicted models (alpha-helices and beta sheets) were more concordant with original DKK-1 structure in the case of predictions made by the MODELLER software. Our final chimeric protein showed desirable structural properties based on Ramachandran plot predictions.

In order to have the highest yield of protein expression, codon optimization was performed according to the *E. coli* codon usage. Thereafter, the DNA sequence of rDKK-1 protein was synthesized and cloned into the expression vectors.[30-32]Since DKK-1 contains several disulfide bonds, we primarily cloned the rDKK-1 gene into the pET22b (+) expression vector to allow periplasmic expression. However, this vector failed to expressed the recombinant protein (data not shown), which compelled us to pursue the cytoplasmic-refolding approach and sub-clone the heterologous gene into pET28a (+) and pET32a (+) expression vectors. The Rosetta-Gami 2 (DE3) and SHuffle strains of *E. coli* were chosen for protein expression to allow correct disulfide bond formation.[33] These *E. coli* strains provide a less reducing environment in their cytoplasm, thereby facilitating disulfide bond formation. Significantly high level ofrDKK-1 was expressed by the Rosetta-Gami 2 strain.

Despite our attempts to produce soluble rDKK-1 through the fusion of COMP domain, using various expression vectors and bacterial hosts and optimization of IPTG concentration, induction time and temperature, rDKK-1 was mostly expressed in the form of inclusion bodies. This may be regarded as the most probable limitations involved in bioinformatics prediction of protein structure in silico. Although, several studies suggest that adding COMP domain can improve recombinant protein solubility, rDKK-1 was predicted to be expressed in an insoluble form by PROSO, which may be the consequence of free cysteine residues and inability of the prokaryotic expression system to form correct disulfide bonds in the cytoplasm.[30,34] Formation of inclusion bodies and poor protein folding can be caused by several factors, including high local concentration of the recombinant protein, lack of post-translational modification, reducing environment of the *E. coli* cytoplasm and improper interactions with chaperones and other enzymes.[35]

In this study apart from in silico evaluations of the number of sequences, the expressed protein was purified and refolded following two different approaches. The first approach was the conventional Ni-NTA column and protein refolded using gradient concentration of urea. In the second method, which was developed in our lab, Amicon centrifugal filtered protein solutions were refolded by pulsatile dilution that allows refolding of recombinant proteins at high protein concentrations. Although the method is time consuming however it was found to be easy as well as highly efficient in case of insoluble recombinant proteins. Moreover, the filtration approach led to a higher concentration ratio and the yield of the rDKK-1 was ~10-fold higher than that of the customary Ni-column-based method. Our results corroborate previous studies and confirmed that dilution could be useful in the refolding of the cysteine-rich recombinant proteins from inclusion bodies.[15,36]

The reactivity of purified rDKK-1 was confirmed by Western blot analysis and ELISA test using commercial anti- DKK-1 polyclonal antibody. However, there are some limitation in this study such as the computer predication or not always sufficient to conclude the experimental result in vivo. Although our results presented here our conclusive and remarkably accurate.

In conclusion, we proposed that in-silico study may end up to a better protein design used in further studies. We also suggest that the refolding process may lead to a higher yield of recombinant protein, when operated onto centrifugal filters rather than Ni-NTA column. A comparison between ELISA results from pET28a (+)/rDKK-1 and pET32a (+)/rDKK-1 antigen indicated higher immunoreactivity of the protein expressed by pET28a (+)/rDKK-1, which may be explicable by the fused thioredoxin tag in pET32a (+)/rDKK-1. In addition, based on our results, the recombinant proteins (pET28a (+)/rDKK-1 and pET32a (+)/rDKK-1) elicited a strong immune response in mice model. Detection of anti-rDKK-1 antibody with OD value of 2.5 following the third booster and OD value at 1:64000 dilution indicated that this truncated protein is a good immunogen. The produced antibody can be used in the ELISA assay for DKK-1 titration in serum of cancer patients for detection and prognosis of cancer in further experiment.[5,6]

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