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Antigenic properties of dense granule antigen 12 protein using bioinformatics tools in order to improve vaccine design against *Toxoplasma gondii*

Purpose: *Toxoplasma gondii* is an opportunistic parasite infecting all warm-blooded animals including humans. The dense granule antigens (GRAs) play an important role in parasite survival and virulence and in forming the parasitophorous vacuole. Identification of protein characteristics increases our knowledge about them and leads to develop the vaccine and diagnostic studies.

Materials and Methods: This paper gave a comprehensive definition of the important aspects of GRA12 protein, including physico-chemical features, a transmembrane domain, subcellular position, secondary and tertiary structure, potential epitopes of B-cells and T-cells, and other important features of this protein using different and reliable bioinformatics methods to determine potential epitopes for designing of a high-efficient vaccine.

Results: The findings showed that GRA12 protein had 53 potential post-translational modification sites. Also, only one transmembrane domain was recognized for this protein. The secondary structure of GRA12 protein comprises 35.55% alpha-helix, 19.50% extended strand, and 44.95% random coil. Moreover, several potential B- and T-cell epitopes were identified for GRA12. Based on the results of the Ramachandran plot, 79.26% of amino acid residues were located in favored, 11.85% in allowed and 8.89% in outlier regions. Furthermore, the results of the antigenicity and allergenicity assessment noted that GRA12 is immunogenic and non-allergenic.

Conclusion: This research provided important basic and conceptual data on GRA12 to develop an effective vaccine against acute and chronic toxoplasmosis for further *in vivo* investigations. More studies are required on vaccine development using the GRA12 alone or combined with other antigens in the future.

Keywords: *Toxoplasma gondii*, Epitope mapping, Computational biology, Vaccines

Introduction

Toxoplasma gondii, an intracellular protozoan, is an opportunistic parasite infecting all warm-blooded animals including humans [1,2]. *Toxoplasma* infection is acquired by incidental consumption of oocysts shed from cats in contaminated water or vegetables, ingestion of tissue cysts presents in undercooked infected meat, congenitally from pregnant mothers to the fetus, and rarely by organ transplantation and blood transfusion [3]. *T. gondii* is categorized into three major strain types (type I, type II, and type III) that differ together in their epidemiological patterns, pathogenicity, and

virulence [4]. Most human infections caused by type II strains in Europe and North America. Interestingly, this genotype is also common in agricultural animals from these zones [5]. Although *T. gondii* often causes subclinical infection, it may cause neuropsychological manifestations including hydrocephalus, blindness, mental retardation, and encephalitis and severe complications of newborns with congenital infection and immunocompromised individuals [6]. Furthermore, *T. gondii* infection may lead to remarkable economic losses due to stillbirths, abortion, and neonatal deaths in livestock which is the main source of the infection to humans [7].

The common drugs which generally recommended for toxoplasmosis treatment, only affects on the tachyzoites multiplication during the initiation of infection, whereas they cannot eliminate the encysted parasites within infected hosts [8]. Therefore, the development of effective vaccines against this pathogen is an important goal because of the worldwide public health and economic losses in the livestock industry [9]. According to the results of various studies, the most promising vaccine candidates for the *T. gondii* include the dense granule (GRA), surface antigens, rhoptry, and microneme proteins [10]. *Toxoplasma* enters its host cells via an active motility-based invasion mechanism. Invasion and formation of parasitophorous vacuoles (PV) require the temporary secretion of specialized parasite proteins from Apicomplexa-specific organelles, namely the micronemes and the rhoptries [11]. Shortly after PV formation, the parasite secretes the contents of the dense granules into the nascent PV [12]. It has been reported that the GRA antigens play a critical role in parasite survival and virulence and in forming the parasitophorous vacuole [13]. These proteins are strongly produced by the parasite and represent circulating antigens during acute and chronic periods of infection, and are of primary importance to host immunity [14]. GRA12 is a key *Toxoplasma* virulence factor that resists host interferon-γ (IFN-γ) activated innate immunity [15]. Bioinformatics is one of the newest scientific fields involved in biological issues [16]. They have been extensively used to analyze gene and protein expression and predict structural, immunogenicity and general features of proteins. Study and comparison of the physical, chemical, and immunogenicity characteristics of proteins can increase our knowledge regarding them and help the researchers select the proper epitopes for vaccine investigations [17]. Bioinformatics had several advantages compared to traditional methods, including relatively low cost, low time requirements, high precision, accuracy, and so forth [16]. So, the recognition of protein epitopes characters using bioinformatics techniques will be beneficial for diagnostic purposes and development of the vaccine [18].

Therefore, the present study was targeted toward the analyzing of the characteristics of GRA12 utilizing bioinformatics tools to introduce potential epitopes for designing an appropriate vaccine against *T. gondii*.

Materials and Methods

Sequence availability
In the first stage of research, the complete amino acid sequence of GRA12 was obtained from a publicly available sequence database, the National Centre for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/protein/) at FASTA format for bioinformatics analysis.

Physical and chemical properties
Different physico-chemical parameters of the GRA12 protein, including number of amino acids, molecular weight (MW), theoretical isoelectric point (pI), aliphatic index, total number of positive and negative charged residues, estimated half-life in mammal's reticulocytes, yeast and *Escherichia coli*, instability index, extinction coefficients, and grand average of hydropathicity (GRAVY) were predicted using the Expasy ProtParam online server (https://web.expasy.org/protparam/).

Prediction of post-translational modification sites of GRA12
Phosphorylation and acylation sites of GRA12 protein were analyzed using NetPhos 3.1 server (http://www.cbs.dtu.dk/services/NetPhos/) and CSS-Palm service (http://csspalm.biocuckoo.org/online.php), respectively.

Prediction of transmembrane domains and subcellular localization of GRA12 protein
To predict the transmembrane structure and subcellular location of the GRA12 protein, we used TMHMM ver. 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/) and PSORT II prediction (http://psort.hgc.jp/form2.html) servers, respectively.

Secondary structures analysis and three-dimensional model constructed
Garnier-Osguthorpe-Robson (GOR) secondary structure prediction (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_gor4.html) and PSIPRED (http://bioinf.cs.ucl.

ac.uk/psipred) online services were utilized to predict the secondary structure of GRA12 protein. Next, DiNNNA online service (unified software for cysteine state and disulfide bond partner prediction) was used (http://clavius.bc.edu/ ~clotelab/DiANNA/). Also, we used SWISS-MODEL (a homology modeling technique; https://swissmodel.expasy.org/) to construct three-dimensional (3D) models of the GRA12 sequence.

Refinement and validation of the three-dimensional modeled structure

To improve the quality of template-based protein model prediction, the best model (produced by SWISS-MODEL) was selected and refined with GalaxyRefine at http://galaxy.seoklab.org/cgi-bin/submit.cgi?type=REFINE. The GalaxyRefine database first rebuilds side chains and conducts side chain repacking and next overall structure relaxation via molecular dynamic simulation. Validation of the 3D proposed model was evaluated by the Ramachandran plot using the SWISS-MODEL program at https://swissmodel.expasy.org/assess. A Ramachandran plot is a way to visualize energetically favored regions for backbone dihedral angles against amino acid residues in protein structure (https://swissmodel.expasy.org/assess/help). Also, the overall quality of the model was evaluated with ProSAweb at https://prosa.services.came.sbg.ac.at/prosa.php.

Prediction of B-cell epitopes

Several servers were applied to analyze the linear B-cell epitopes. At first, BCPREDS (B-cell epitope prediction server; http://ailab.ist.psu.edu/bcpred/predict.html) was used to predict the continuous B-cell epitopes by recruiting a compound of a subsequence kernel and a support vector machine (SVM) approach with an output reliability of 74.57% [19]. The program runs with the following characteristics: 20 amino acid epitope length, a specificity 75%, and the use overlap filter. Next, we employed a web-based algorithm Bcepred (B-cell epitope prediction) server to predict the linear B-cell epitopes, using physicochemical properties (http://crdd.osdd.net/raghava/bcepred/bcepred_submission.html). This server is able to predict highest precision of 58.70% at threshold 2.38 and lets users to predict B-cell epitopes via any of the physical and chemical properties (accessibility, hydrophilicity, polarity, flexibility/mobility, exposed surface, and turns; http://crdd.osdd.net/raghava/bcepred/). Default parameters have been used for estimation using the Bcepred database. In addition, we also used ABCpred (artificial neural network based B-cell epitope prediction) server to predict B-cell epitope(s) in an antigen sequence (http://crdd.osdd.net/raghava/abcpred/). Also Bepipred linear epitope, beta-turn, surface accessibility, flexibility, antigenicity, and hydrophilicity were employed using the IEDB (Immune Epitope Database) servers to which available online at http://tools.iedb.org/bcell/. Besides, discontinuous B cell epitopes were estimated using ElliPro (http://tools.iedb.org/ellipro/) from the 3D epitope structure protein data bank file. For the prediction, the default minimum score of 0.5 and the maximum distance (Angstrom) of 6 was applied.

Prediction of MHC-I and MHC-II binding epitopes

To analyze the half maximal inhibitory concentration (IC50) values of peptides that bind to the major histocompatibility complex (MHC) class I (http://tools.iedb.org/mhci/) and class II (http://tools.immuneepitope.org/mhcii) molecules, the IEDB online service was employed [20]. IEDB suggests making choices based on a percentile value of ≤1% for each (MHC allele, length) mixture to cover most of the immune responses [21]. Six alleles (H2-Db, H2-Dd, H2-Kb, H2-Kd, H2-Kk, and H2-Ld) were selected as MHC class I mouse molecules. The prediction for MHC-I was rendered according to the IEDB recommended method with the length of 10-mer. On the other hand, three alleles (H2-IAb, H2-IAd, and H2-IEd) were selected as mouse MHC class II molecules. The prediction method was done based on the IEDB recommended method and the length of predicted peptide was 15 amino acids with sort by percentile rank. Besides, to predict peptides binding to MHC-I and MHC-II molecules two online servers NetMHCcons 1.1 (http://www.cbs.dtu.dk/services/NetMHCcons/) and NetMHCIIpan 3.2 (http://www.cbs.dtu.dk/services/NetMHCIIpan/) were used respectively. NetMHCcons provide the user with the option of choosing the MHC molecule from a long list of alleles or alternatively upload the MHC protein sequence of interest. NetMHCIIpan predicts the three human MHC class II isotypes HLA-DR, HLA-DP, and HLA-DQ, as well as the mouse molecules (H-2) [22]. Estimation values are provided in the nano Molars (nM) IC50 values and rank percent. A peptide is known as a strong binder in NetMHCcons if the rank percent is below 0.5% or the binding affinity (IC50) is less than 50 nM. The peptide is also considered a poor binder if the percentage rank is less than 2% or the binding affinity (IC50) is less than 500 nM. Similar to the abovementioned, the length of predicted peptides

in NetMHCcons and NetMHCIIpan methods were 10 and 15 mer, respectively.

Prediction of cytotoxic T lymphocyte epitopes

Cytotoxic T lymphocyte (CTL) epitopes prediction was performed using CTLpred online server which available at http://www.imtech.res.in/raghava/ctlpred/index.html [23]. CTLpred is a method of predicting CTL epitopes that are critical in the development of vaccine design. The prediction was employed by the combined method. By default, the cutoff scores of the artificial neural network (ANN) and the SVM were set at 0.51 and 0.36, respectively. The cutoff value is used to distinguish the epitopes and non-epitopes. The accuracy of the combined prediction method was 75.8% (http://crdd.osdd.net/raghava/ctlpred/about.html).

Antigen probability, allergenicity, and solubility evaluation

The ANTIGENpro (http://scratch.proteomics.ics.uci.edu/) and VaxiJen ver. 2.0 (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) online servers were applied for estimated the antigenicity of the GRA12 protein. ANTIGENpro is an alignment-free, sequence-based, and pathogen-independent protein antigenicity predictor. It is the first predictor of the entire protein antigenicity trained using reactivity data obtained by analysis of protein microarray (http://scratch.proteomics.ics.uci.edu/explanation.html#ANTIGENpro). VaxiJen ver. 2.0 server is a new antigen prediction approach based on auto-cross covariance alteration of protein sequences into uniform vectors of principal amino acid properties. The accuracy of this server is varied from 70% to 89%, depending upon the target organisms (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen_help.html). The allergenicity of GRA12 protein was predicted by the AlgPred (http://www.imtech.res.in/raghava/algpred/) and AllergenFP ver. 1.0 (http://ddg-pharmfac.net/AllergenFP/) online servers. In AlgPred, a systematic effort has been made to combine different approaches to predict allergenic proteins with high precision. The hybrid approach (immunoglobulin E epitope+SVMc+ MAST+ARPs BLAST) was used with 85% accuracy at a threshold -0.4. Also, prediction of protein solubility upon overexpression was predicted using (http://scratch.proteomics.ics.uci.edu/) [24]. AllergenFP server identifies allergens based on their physicochemical properties with 88% accuracy.

Ethics approval

This study was approved by the Ethical Committee of Tarbiat Modares University (IR.MODARES.REC.1398.009).

Results

Gene information and general characteristics of GRA12

The amino acid sequence of GRA12 protein was obtained from the NCBI under accession no. ACH87600.1 in FASTA format. GRA12 protein contains 436 amino acid residues with the MW of 47.880 kDa, its theoretical pI is 9.28. The total number of negatively (Asp+Glu) and positively charged residues (Arg+Lys) were 36 and 44, respectively. The extinction coeffi-
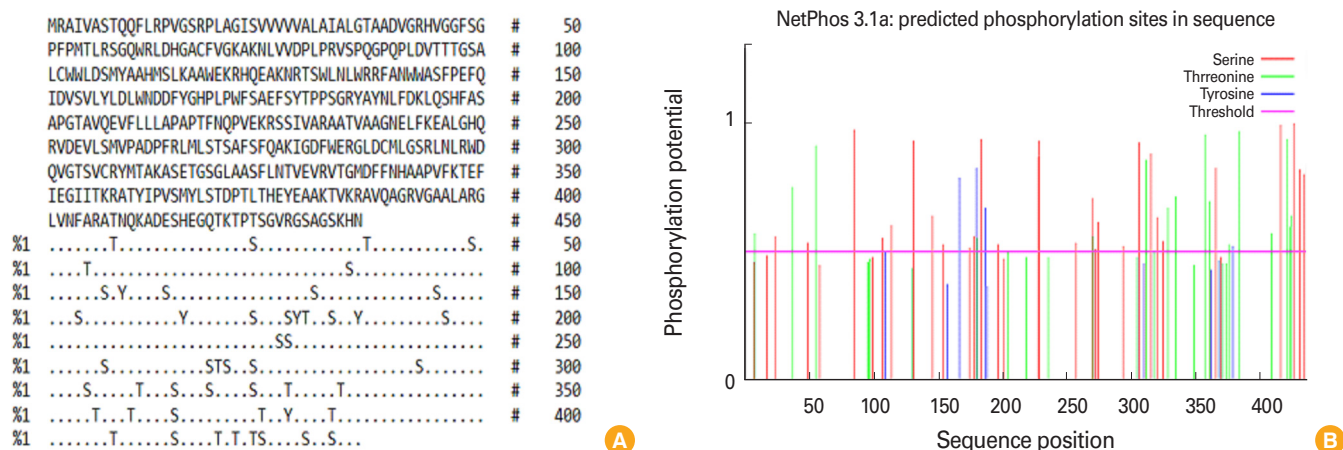
Fig. 1. Bioinformatics analysis of the phosphorylation sites of GRA12. (A) If the residue is predicted not to be phosphorylated, either because the score is below the threshold or because the residue is not serine, threonine, or tyrosine, that position is marked by a dot ('.'). Residues having a prediction score above the threshold are indicated by 'S', 'T', or 'Y', respectively (http://www.cbs.dtu.dk/services/NetPhos-2.0/output.php). (B) Predicted phosphorylation sites in GRA12 sequence. GRA, dense granule antigens.

cient was 81,150 M⁻¹cm⁻¹ at 280 nm in water. The predicted half-life was 30 hours in mammalian reticulocytes, >20 hours in yeast, and >10 hours in *E. coli*. The instability index for this protein was computed to be 37.06 and this classifies the protein as stable. Furthermore, the Aliphatic index was 78.81 and the GRAVY of this protein was -0.098. Additionally, the instability indexes of GRA12 were counted 37.06.

Prediction of post-translational modification sites of GRA12

To analyze the phosphorylation and acylation sites of GRA12 protein, NetPhos 3.1 and CSS-Palm servers service were used, respectively. The results of the analysis showed 49 phosphorylation sites (serine, 28; threonine, 16; tyrosine, 5) (Fig. 1A, B) and four acylation sites in the GRA12 protein. The acylation sites of the GRA12 sequence are presented in Table 1.

Prediction of transmembrane domains and subcellular localization of GRA12 protein

The results of the TMHMM server ver. 2.0 showed that the GRA12 sequence has only one transmembrane domain, which is shown in Fig. 2. Moreover, the prediction of subcellular localization of GRA12 by using PSORT II was as follows: 39.1%

mitochondrial, 21.7% cytoplasmic, 13.0% Golgi, 4.3% vacuolar, 4.3% nuclear, and 17.4% endoplasmic reticulum.

Secondary structures analysis and three-dimensional model constructed

Prediction of secondary and 3D structures of a protein has a significant effect on the biological function of it. The secondary structure of GRA12 protein was obtained by GOR IV and PSIPRED online services. The data extracted from the GOR IV server demonstrated that the proportions of the random coil, alpha-helix, and extended strand in the GRA12 sequence were 44.95% (196/436), 35.55% (155/436), and 19.50% (85/436), respectively (Figs. 3, 4). The total number of cysteines in the

Table 1. The acylation sites of GRA12 sequence

ID	Position	Peptide	Score
ACH87600.1 GRA12 [*T. GONDII*]	68	WRLDHGACFVGKAKN	0.652
ACH87600.1 GRA12 [*T. GONDII*]	102	TTTGSALCWWLDSMY	0.007
ACH87600.1 GRA12 [*T. GONDII*]	289	FWERGLDCMLGSRLN	3.702
ACH87600.1 GRA12 [*T. GONDII*]	307	DQVGTSVCRYMTAKA	4.859

GRA, dense granule antigens.
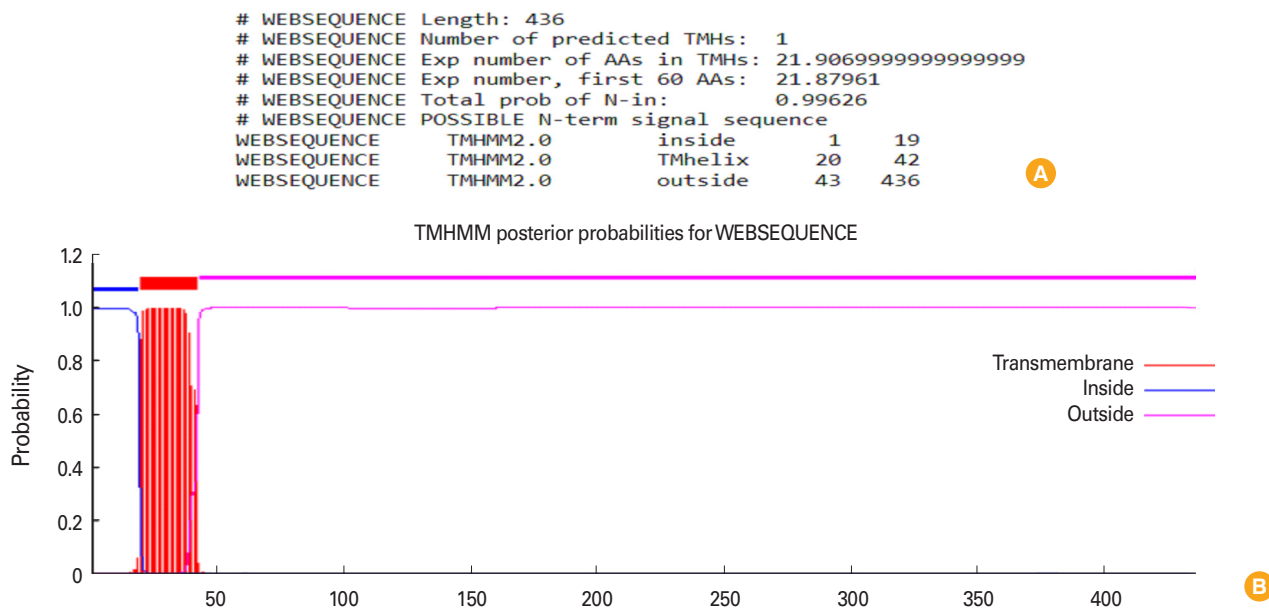
Fig. 2. Prediction of transmembrane helices in GRA12 proteins. (A) Some statistics and a list of the location of the predicted transmembrane helices and the predicted location of the intervening loop regions. (B) Analysis of the transmembrane domain of GRA12. Number of predicted TMHs, the number of predicted transmembrane helices; exp number of AAs in TMHs, the expected number of amino acids in transmembrane helices (if this number is larger than 18, it is very likely to be a transmembrane protein [*OR* have a signal peptide]); exp number–first 60 AAs, the expected number of amino acids in transmembrane helices in the first 60 amino acids of the protein (if this number more than a few, you should be warned that a predicted transmembrane helix in the N-term could be a signal peptide); total prob. of N-in, the total probability that the N-term is on the cytoplasmic side of the membrane; possible N-term signal sequence, a warning that is produced when "exp number–first 60 AAs" is larger than 10 (http://www.cbs.dtu.dk/services/TMHMM-2.0/TMHMM2.0.guide.html#output); GRA, dense granule antigens.

GRA12 sequence using DiNNNA software was 4. More details are shown in Table 2. To predict and construct the 3D structure of GRA12 protein, the online service of SWISS-MODEL was employed. Results from SWISS-MODEL for GRA12 showed that three templates were found to match the target sequence. So, the model with the best sequence identity and coverage was chosen. The output of SWISS-MODEL such as the 3D model predicted for GRA12, protein global quality estimate,
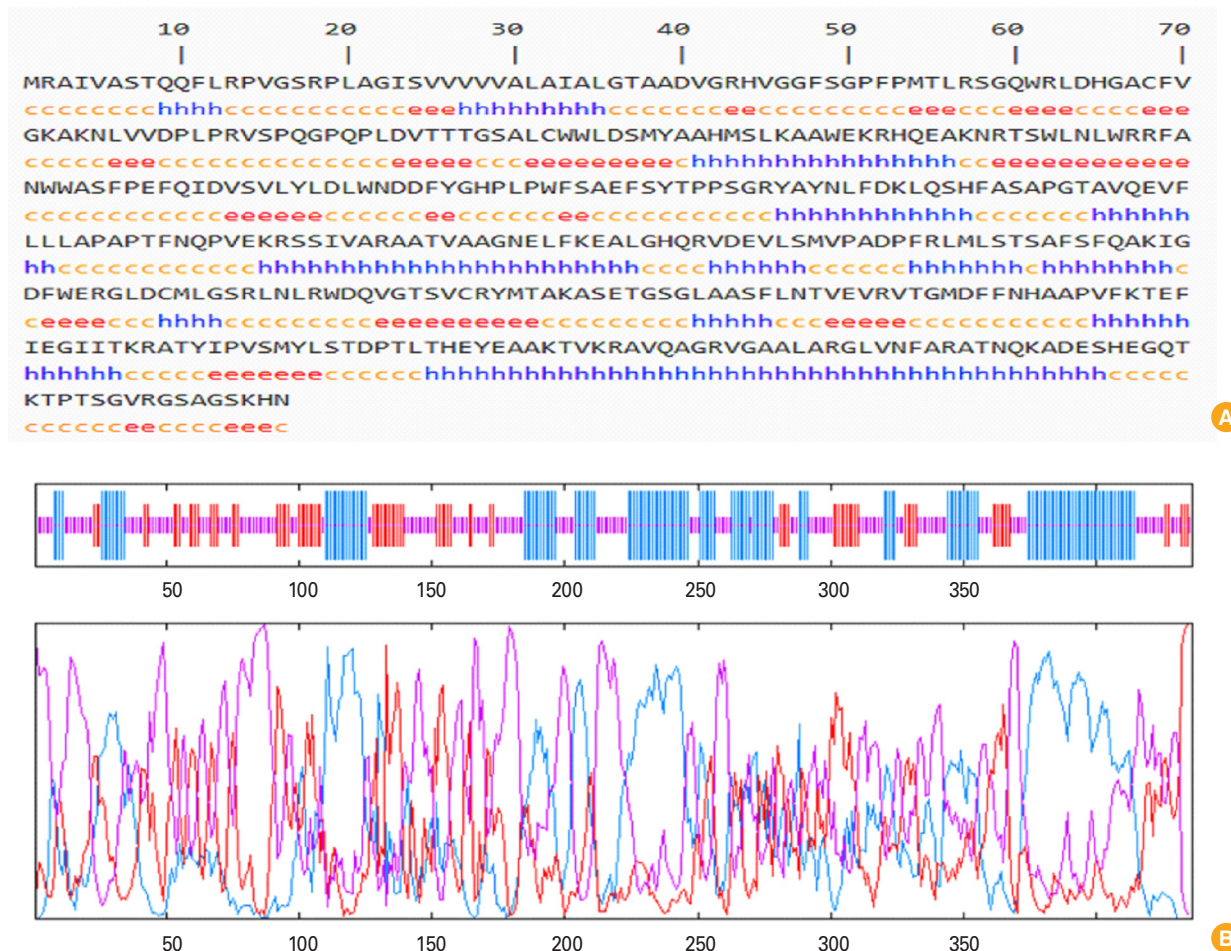
Fig. 3. Analysis of secondary structure of GRA12 using GOR IV. (A) Predicted secondary structure. (B) Graphical frame of secondary structure prediction of GRA12 protein. GRA, dense granule antigens; h, helix; e, extended strand; c, coil.

Table 2. Disulfide bonds prediction

|	Distance	Bond	Score
Cysteine sequence position			
68–102	34	LDHGACFVGKA–TGSALCWWLDS	0.01058
68–289	221	LDHGACFVGKA–ERGLDCMLGSR	0.01039
8–307	239	LDHGACFVGKA–VGTSVCRYMTA	0.01037
102–289	187	TGSALCWWLDS–ERGLDCMLGSR	0.01097
2–307	205	TGSALCWWLDS–VGTSVCRYMTA	0.01039
289–307	18	ERGLDCMLGSR–VGTSVCRYMTA	0.01043
Weighted matching predicted bonds			
68–307		LDHGACFVGKA–VGTSVCRYMTA	
102–289		TGSALCWWLDS–ERGLDCMLGSR	
Predicted connectivity			
1–4, 2–3			
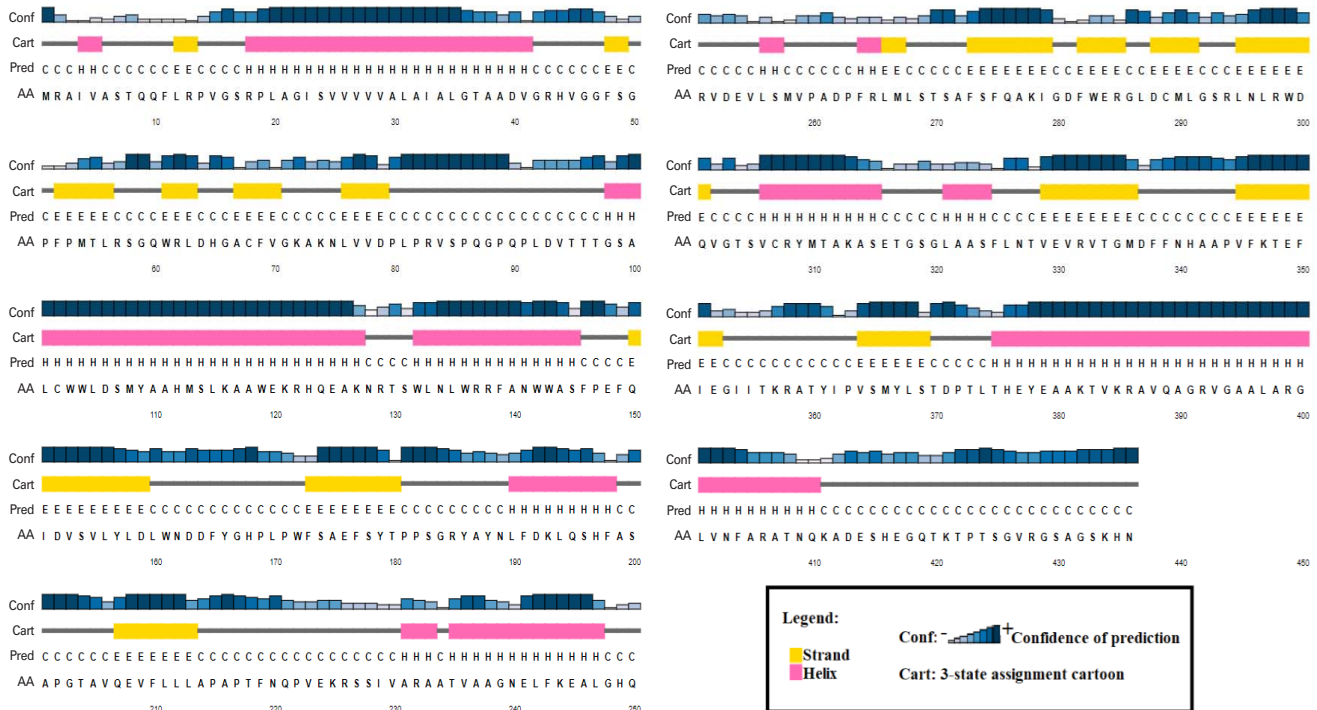
Fig. 4. Graphical result from secondary structure prediction of vaccine using PSIPRED.

sequence identity and coverage, model-template alignment, and the local quality estimate was rendered in Fig. 5.

Refinement and validation of the three-dimensional structures

The z-score which demonstrates overall model quality was -0.61, and a high number of residues have been found in the favored region. The quality of the 3D structure was improved (z-score of -0.98) after refinement (Fig. 6A, B). Protein validation before refinement indicated that in the initial model, 79.26% of residues were located in favored, 11.85% in allowed and 8.89% in outlier regions. After refinement of the 3D model, the results were changed as follows: 91.85% of residues in favored regions, 6.67% in allowed regions, and 1.48% in outlier regions of Ramachandran plot (Fig. 6C, D).

Prediction of B-cell epitopes

Prediction of an epitope can give researchers considerable information to recognize immunogenic peptides and the design of novel potential vaccines. The BCPREDS was employed to prediction of B-cell epitope. Therefore, nine predicted epitopes with a high score were presented in Table 3. The higher threshold score shows a greater binding affinity/specificity. Also, the output of the Bcepred server to predict the linear B-cell epitopes, utilizing physico-chemical characteristics (po-

Table 3. BCPred predictions of linear B-cell epitopes from GRA12 protein

Position	Epitope	Score
80	PLPRVSPQGPQPLDVTTTGS	0.999
411	KADESHEGQTKTPTSGVRGS	0.977
174	SAEFSYTPPSGRYAYNLFDK	0.952
41	VGRHVGGFSGPFPMTLRSGQ	0.948
213	LAPAPTFNQPVEKRSSIVAR	0.941
3	AIVASTQQFLRPVGSRPLAG	0.862
301	QVGTSVCRYMTAKASETGSG	0.834
118	AWEKRHQEAKNRTSWLNLWR	0.802
352	EGIITKRATYIPVSMYLSTD	0.759

GRA, dense granule antigens.

larity, exposed surface, hydrophilicity, turns, flexibility/mobility exposed surface, and accessibility) are indicated in Table 4. Such parameters are very important for the antigenic properties of the GRA12 protein. Also, the ABCpred server was used to predict B cell epitopes of GRA12 which the results are listed in Table 5 according to their scores (just the epitopes over scores of 0.75 are listed in Table 5). The higher peptide score indicative the higher possibility to be as an epitope. All the peptides displayed in the table are above the selected threshold value. This server predicted 21 epitopes over
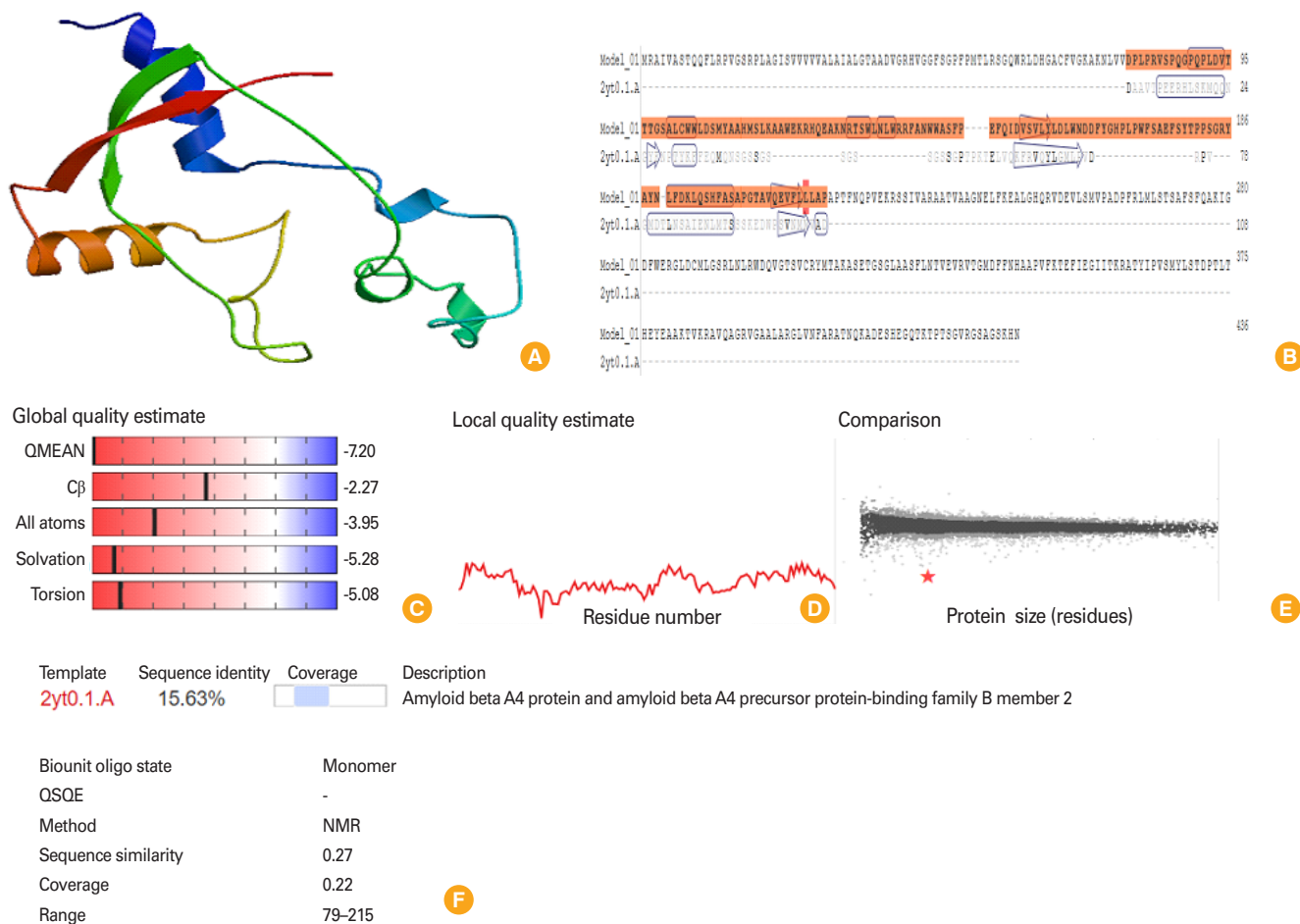
Fig. 5. Analysis of 3D structure constructed for GRA12 protein using SWISS-MODEL. (A) 3D structure prediction of GRA12 protein. (B) Model-template alignment. (C) Global quality estimate; QMEAN, a composite estimator, contains four individual terms all atoms, solvation, torsion, and Cβ atoms. Positive values demonstrate that the model scores higher than experimental structures on average. Negative numbers demonstrate that the model scores lower than experimental structures on average. The QMEAN Z-score itself is shown on top. (D) Local quality estimate; typically, it is expected that residues with a rating under 0.6 will be of low quality. (E) Comparison with non-redundant set of protein data bank structures; protein length (number of residues) is showed in x-axis and the normalized QMEAN score is showed in y-axis. The actual design is shown as a red star. (F) Sequence coverage and identity. 3D, three-dimensional; GRA, dense granule antigens.

Table 4. Predicted of linear B-cell epitopes, using physico-chemical characteristics using Bcepred online server

Prediction parameter	Epitope sequence
Flexibility	QFLRPVG; AAWEKRHQEAKNR; FSYTPPS; FNQPVEKRS; MTAKASETG; ATNQKADESHEGQTKTPTSGVRGSAG
Hydrophilicity	DVTTTGS; EKRHQEAKNRTS; GHQRVDE; TAKASETGSG; ARATNQKADESHEGQTKTPTSG; RGSAGSKHN
Accessibility	STQQFLRPVGSRP; LRSGQWRL; PRVSPQGPQPLD; KAAWEKRHQEAKNRTSWLN; EFSYTPPSGRYAYN; FNQPVEKRSS; GHQRVDEV; RLNLRWD; RYMTAKASETGS; ITKRATYIP; DPTLTHEYEAAKTVKRAV; ARATNQKADESHEGQTKTPTSG
Turns	DFFNHAA
Exposed surface	KAAWEKRHQEAKNRT; NQPVEKRS; TNQKADES; SKHN
Polarity	KAAWEKRHQEAKNRTS; NQPVEKRSSIV; GNELFKEALGHQRVDEV; EGIITKR; TLTHEYEAAKTVKRAV; QKADESHEGQTKT
Antigenic propensity	QQFLRPV; GISVVVVAL; KNLVVDPLPRVSPQ; FQIDVSVLYLDLW; FYGHPLP; VQEVFLLL; QRVDEVLSMVP; QVGTSVCRY; FLNTVEVRVTG; YIPVSMYLS

0.75 scores on our sequence, which the highest score was for linear epitope HFASAPGTAVQEVFLLLAPA (0.91). The average score (threshold) estimate for the GRA12 protein by IEDB prediction is as follows: Bepipred linear epitope prediction
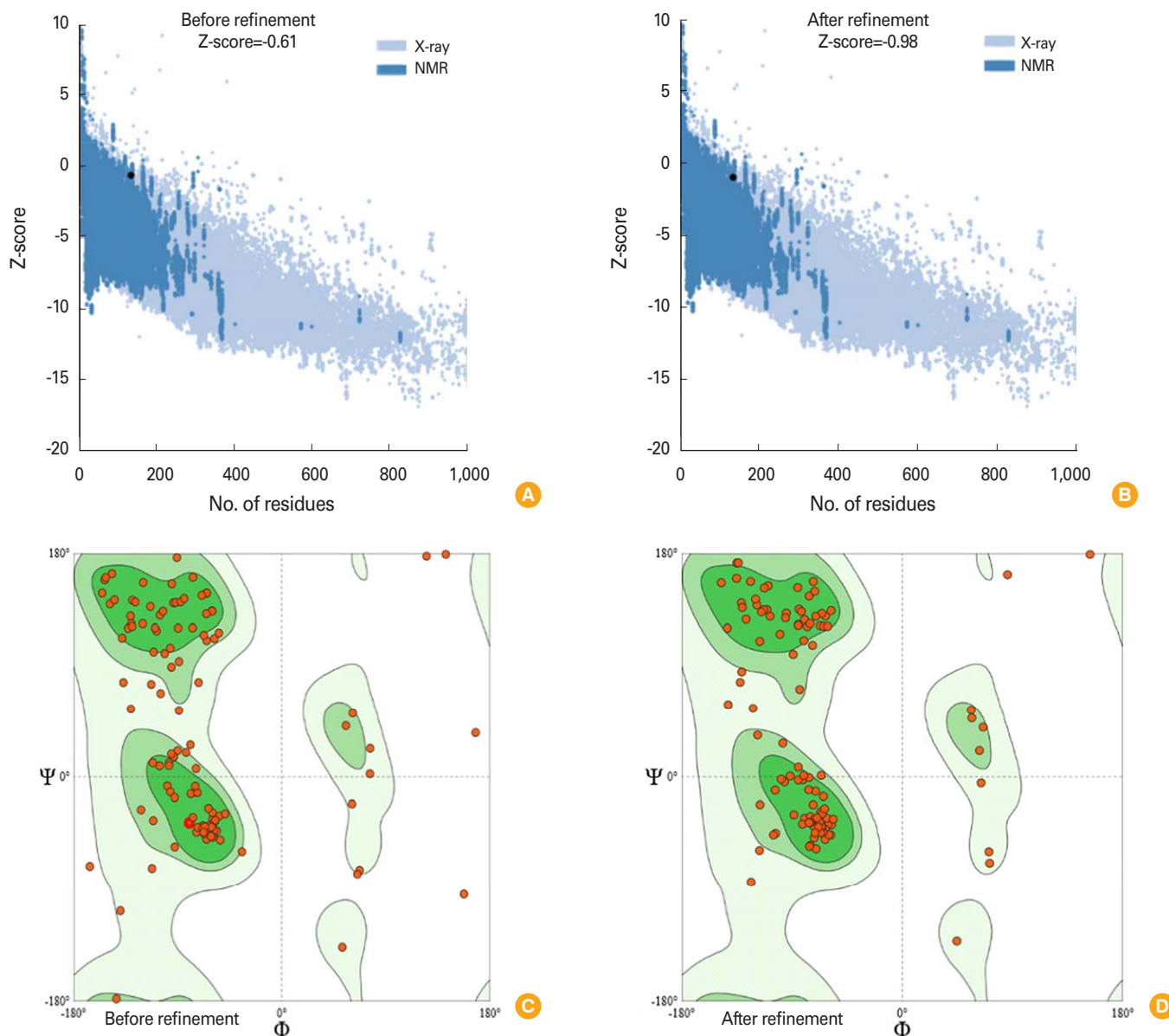
Fig. 6. Validation of 3D model of GRA12 protein using Ramachandran plot. (A, B) The z-score plot for 3D structure of predicted vaccine before and after refinement with ProSA-web server. The z-score of the initial model was -0.61 and after refinement processes was -0.98. (C, D) The analysis of Ramachandran plot of initial model showed 79.26%, 11.85%, and 8.89% of residues were located in favored, allowed, and outlier regions, respectively. The results after refinement were changed as follow: 91.85%, 6.67%, and 1.48% of residues were located in favored, allowed, and outlier regions, respectively. 3D, three-dimensional; GRA, dense granule antigens.

0.058, beta-turn 0.964, surface accessibility 1.000, flexibility 0.988, antigenicity 1.034, and hydrophilicity 1.108. The results of IEDB are shown as graphs (Fig. 7). Also, in the 3D model of the ElliPro, we found five discontinuous B-cell epitopes (Fig. 8).

Prediction of MHC-I and MHC-II binding epitopes

The IEDB online tool was recruited to estimate the IC50 values for peptide binding to the MHC class I and class II mole-

cules of GRA12. The T-cell epitopes on GRA12 were identified by bioinformatics analysis to have the ability to bind strongly to MHC class I and class II molecules. Three peptides with high affinity to MHC molecules were selected for each allele. The minimum percentile ranks for each MHC allele of GRA12 are shown in Tables 6 and 7. Also, info from NetMHCcons and NetMHCIIpan are described in Tables 8 and 9, respectively. The information portrayed in the table involving the

Table 5. B-cell epitope predicted from full-length protein using AB-Cpred server

Rank	Sequence	Start position	Score
1	HFASAPGTAVQEVFLLLAPA	197	0.91
2	SGQWRLDHGACFVGKAKNLV	58	0.90
2	NLFDKLQSHFASAPGTAVQE	189	0.90
3	TGMDFFNHAAPVFKTEFIEG	334	0.89
4	TSAFSFQAKIGDFWERGLDC	270	0.86
5	TAVQEVFLLLAPAPTFNQPV	204	0.84
5	LKAAWEKRHQEAKNRTSWLN	115	0.84
6	GSALCWWLDSMYAAHMSLKA	98	0.83
7	LPRVSPQGPQPLDVTTTGSA	81	0.82
8	APTFNQPVEKRSSIVARAAT	216	0.80
8	LDSMYAAHMSLKAAWEKRHQ	105	0.80
9	ARATNQKADESHEGQTKTPT	405	0.79
9	GTSVCRYMTAKASETGSGLA	303	0.79
9	SRPLAGISVVVVVALAIALG	17	0.79
9	MRAIVASTQQFLRPVGSRPL	1	0.79
10	DFYGHPLPWFSAEFSYTPPS	164	0.78
10	VLYLDLWNDDFYGHPLPWFS	155	0.78
11	VGGFSGPFPMTLRSGQWRLD	45	0.77
12	LAASFLNTVEVRVTGMDFFN	321	0.76
12	NWWASFPEFQIDVSVLYLDL	141	0.76
13	EYEAAKTVKRAVQAGRVGAA	377	0.75

Table 6. Prediction of MHC-I binding epitopes

Allele	Start-stop	Peptide sequence	Percentile rank[a]
H-2-Kb	270–279	TSAFSFQAKI	0.66
	365–374	SMYLSTDPTL	1.00
	359–368	ATYIPVSMYL	1.1
H-2-Db	99–108	SALCWWLDSM	0.4
	365–374	SMYLSTDPTL	0.42
	149–158	FQIDVSVLYL	0.51
H-2-Kd	308–317	RYMTAKASET	0.79
	197–206	HFASAPGTAV	2.2
	360–369	TYIPVSMYLS	2.85
H-2-Kk	375–384	THEYEAAKTV	0.94
	346–355	FKTEFIEGII	4.75
	243–252	FKEALGHQRV	5.0
H-2-Dd	342–351	AAPVFKTEFI	1.05
	133–142	LNLWRRFANW	1.2
	185–194	RYAYNLFDKL	2.0
H-2-Ld	168–177	DIPAAALRFF	3.0
	181–190	PPSGRYAYNL	3.1
	170–179	LPWFSAEFSY	3.4

MHC, major histocompatibility complex.
[a]Percentile rank=IC50 value; low percentile rank=high level binding.

Table 7. Prediction of MHC-II binding epitopes

Allele	Start-stop	Peptide sequence	Percentile rank[a]
H2-IAd	224–238	EKRSSIVARAATVAA	0.11
	106–120	DSMYAAHMSLKAAWE	0.12
	105–119	LDSMYAAHMSLKAAW	0.12
H2-IAb	193–207	KLQSHFASAPGTAVQ	0.24
	192–206	DKLQSHFASAPGTAV	0.27
	194–208	LQSHFASAPGTAVQE	0.27
H2-IEd	129–143	RTSWLNLWRRFANWW	1.49
	128–142	NRTSWLNLWRRFANW	1.85
	127–141	KNRTSWLNLWRRFAN	2.10

MHC, major histocompatibility complex.
[a]Percentile rank=IC50 value; low percentile rank=high level binding.

Table 8. Prediction of MHC-I binding epitopes on GRA12 using Net-MHCcons

Allele	Peptide sequence	IC50 (nM)	% rank	Binding level
H-2-Kb	SMYLSTDPTL	211.83	0.80	WB
	ATYIPVSMYL	263.01	1.00	WB
H-2-Db	AAPVFKTEFI	449.33	0.40	SB
	SALCWWLDSM	595.31	0.40	SB
	SMYLSTDPTL	763.52	0.50	SB
H-2-Kd	RYMTAKASET	350.34	0.50	SB
	RYAYNLFDKL	442.10	0.50	SB
	HFASAPGTAV	1,384.40	1.5	WB
H-2-Kk	QEAKNRTSWL	245.15	1.0	WB
	MDFFNHAAPV	659.75	2.0	WB
H-2-Dd	AAPVFKTEFI	5,441.03	0.80	WB
	TGSGLAASFL	9,550.53	2.0	WB
	TSWLNLWRRF	10,027.04	2.0	WB
H-2-Ld	HPLPWFSAEF	77.86	0.07	SB
	SPQGPQPLDV	1,133.26	0.50	SB
	RPLAGISVVV	1,222.43	0.80	WB

MHC, major histocompatibility complex; GRA, dense granule antigens; WB, weak binders; SB, strong binders.

used alleles, the predicted peptide, the Affinity/IC50 (nM), the percent rank and the binding level (strong or weak). Ultimately, the findings showed that that some GRA12 protein epitopes can strongly bind to MHC-I and MHC-II molecules.

Prediction of cytotoxic T lymphocyte epitopes
The ten high-ranking epitopes were selected based on their scores by CTLpred. More details are inserted in Table 10.

**Antigen probability, allergenicity, and solubility evaluation**
Predicted probability of antigenicity for GRA12 protein was 0.8095 and 0.4927 by ANTIGENpro and VaxiJen ver. 2.0 (Thres-
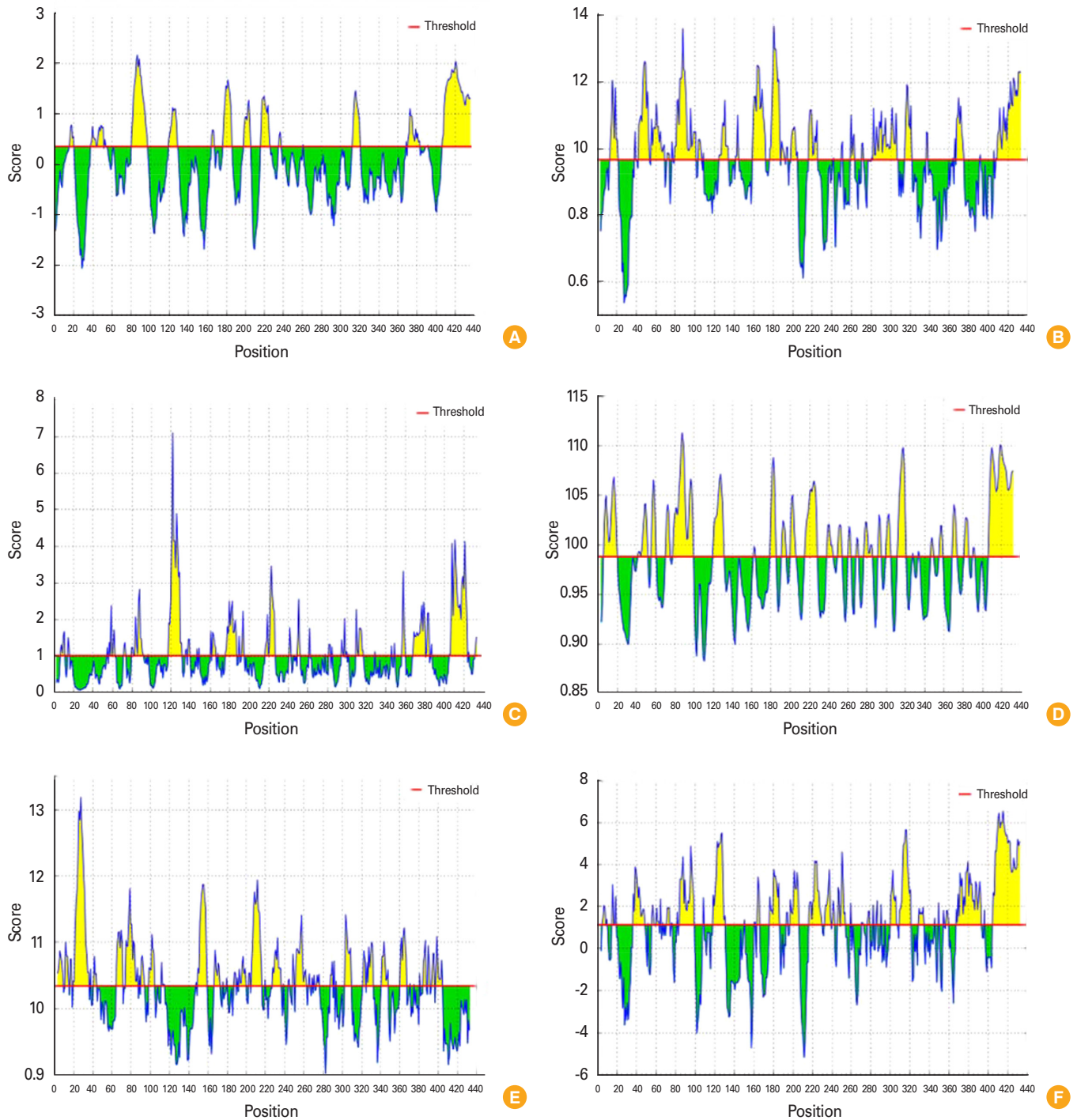
Fig. 7. The output of IEDB (Immune Epitope Database) online server. (A) Bepipred linear epitope prediction; (B) beta-turn; (C) surface accessibility; (D) flexibility; (E) antigenicity; (F) hydrophilicity. The x-axes represent the residue positions in the sequence, while the y-axes represent for each residue the correspondent score; The straight red line demonstrates the threshold or the average score; yellow colors representing that the residue might have a higher probability to be a part of the epitope; and green colors representing the unfavorable regions relevant to the properties of interest.

hold for this model was 0.5), respectively. The protein allergenicity was assessed by a hybrid approach at AlgPred and AllergenFP servers, which revealed that the GRA12 is a non-

allergen. The predicted solubility upon overexpression in *E. coli* was estimated 0.7737 by the SOLpro server.
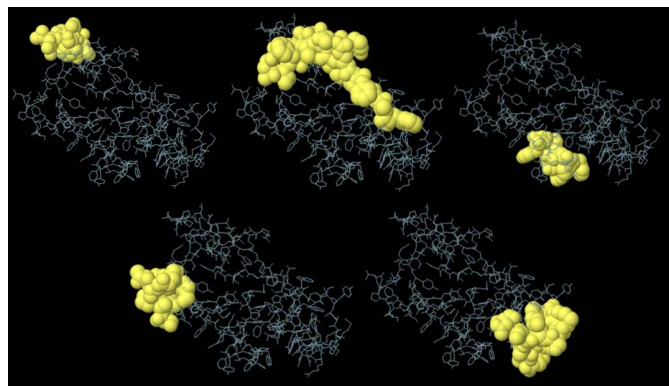
Fig. 8. Predicted discontinuous B-cell epitopes by ElliPro tool.

Table 9. Prediction of MHC-II binding epitopes on GRA12 using Net-MHCIIpan

Allele	Peptide sequence	IC50 (nM)	% rank	Binding level
H2-IAd	KRSSIVARAATVAAG	49.89	0.07	SB
	EKRSSIVARAATVAA	51.67	0.08	SB
	VEKRSSIVARAATVA	57.17	0.12	SB
	RSSIVARAATVAAGN	59.23	0.15	SB
	PVEKRSSIVARAATV	73.48	0.30	SB
	SSIVARAATVAAGNE	77.44	0.40	SB
	SIVARAATVAAGNEL	90.54	0.50	SB
	LDSMYAAHMSLKAAW	94.01	0.60	SB
	QPVEKRSSIVARAAT	102.22	0.70	SB
	WLDSMYAAHMSLKAA	105.52	0.70	SB
H2-IAb	KLQSHFASAPGTAVQ	71.29	0.04	SB
	LQSHFASAPGTAVQE	73.65	0.05	SB
	QSHFASAPGTAVQEV	76.94	0.05	SB
	DKLQSHFASAPGTAV	87.44	0.07	SB
	SHFASAPGTAVQEVF	95.58	0.08	SB
	HFASAPGTAVQEVFL	148.05	0.25	SB
	MDFFNHAAPVFKTEF	211.98	0.50	SB
	KRSSIVARAATVAAG	224.28	0.60	SB
	GMDFFNHAAPVFKTE	227.40	0.60	SB
	RSSIVARAATVAAGN	240.74	0.70	SB
H2-IEd	LDSMYAAHMSLKAAW	1,439.43	3.0	WB
	SMYAAHMSLKAAWEK	1,516.20	3.5	WB
	ADPFRLMLSTSAFSF	1,563.33	3.5	WB
	DPFRLMLSTSAFSFQ	1,617.21	4.0	WB
	DSMYAAHMSLKAAWE	1,674.37	4.0	WB
	WLDSMYAAHMSLKAA	1,689.09	4.0	WB
	PFRLMLSTSAFSFQA	1,713.29	4.0	WB
	KNRTSWLNLWRRFAN	1,711.61	4.0	WB
	RTSWLNLWRRFANWW	1,755.40	4.5	WB
	NRTSWLNLWRRFANW	1,738.46	4.5	WB

MHC, major histocompatibility complex; GRA, dense granule antigens; SB, strong binders; WB, weak binders.

Table 10. Predicted CTL epitopes of GRA12 using CTLpred

Peptide rank	Start position	Sequence	Score (ANN/SVM)
1	225	KRSSIVARA	0.90/1.3315333
2	23	ISVVVVAL	0.82/1.2331814
3	325	FLNTVEVRV	0.56/1.3002949
4	360	TYIPVSMYL	0.47/1.2816798
5	48	FSGPFPMTL	0.67/1.0597115
6	37	TAADVGRHV	0.83/0.87444574
7	62	RLDHGACFV	0.00/1.4831245
8	218	TFNQPVEKR	0.97/0.50220728
9	305	SVCRYMTAK	0.28/1.1701155
10	270	TSAFSFQAK	0.76/0.66275871

CTL, cytotoxic T lymphocyte; GRA, dense granule antigens; ANN, artificial neural network; SVM, support vector machine.

Discussion

Toxoplasma gondii is an opportunistic parasite infecting all warm-blooded animals including humans. Although *T. gondii* often causes subclinical infection, it may cause neuropsychological manifestations including hydrocephalus, blindness, mental retardation, and encephalitis and severe complications in congenitally infected newborns and immunocompromised individuals. Furthermore, *T. gondii* infection may lead to remarkable economic losses [1,6]. Therefore, vaccination is considered to be the only rational and useful choice to combat toxoplasmosis. For this aim, the first step in the successful production of the vaccine is the recognition of potential highly immunoprotective antigen(s) of the parasite [25].

Dense granule secretion starts when the parasite reaches the host cell, but it occurs mainly at the end of the invasion process, showing that the GRA proteins are likely involved in the development and proliferation of parasites [26]. These proteins are strongly produced by the parasite and represent circulating antigens during acute and chronic periods of infection, and are of primary importance to host immunity [14]. One of the most essential steps to design an efficient protein-based vaccine is use bioinformatics methods to evaluate the characteristics of the antigen. Bioinformatics methods play an undeniable role in the progress of vaccines to predict protein structures, functions, and other biological features [27]. One of the main purposes of bioinformatics is to increase our knowledge of biological processes and improve the health level using new vaccine design [28]. Given the importance of toxoplasmosis in human health and also recent developments

in designing DNA vaccine based on GRA proteins against this disease, we carried out a study to analyze and compare the structural, physical, immunogenicity and other features of GRA12 proteins using bioinformatics tools and websites to design proper vaccine against *T. gondii*.

The amino acid sequence of GRA12 protein contains 436 residues and the MW of the GRA12 was predicted to be 47.880 kDa, which represents a good antigenic nature (it has been said the antigens with MW of <5–10 kDa are considered as a weak immunogenic) [29]. Also, we calculated the aliphatic index and GRAVY of the GRA12 sequence, which were 78.81 and -0.098, respectively. Briefly, the high aliphatic index shows that the target protein is more stable in a wide spectrum of temperatures. Also, GRAVY's negative value reflects protein hydrophilicity, which could have a better interaction with the surrounding molecules of water. Additionally, the instability indexes of GRA12 were counted 37.06. The index of instability provides an assessment of a protein's stability in a test tube. The GRA12 sequence was classified as a stable protein because the value smaller than 40 is predicted as stable. In the current study, only one transmembrane domain was predicted for GRA12 sequence which the details are shown in Fig. 2. It is well known that post-translational modifications (PTMs) play a key role in cellular control mechanisms [30]. Also, the recognition of phosphorylation sites on proteins is a great tool to analyze signaling networks and functional correlations between signaling proteins [31]. For this purpose, we used two online servers to predict the phosphorylation and acylation sites of GRA12 protein. The results revealed that GRA12 protein contains 53 potential PTM sites (49 phosphorylation and four acylation sites) on the sequence, suggesting that these sites may affect protein functions and activity. In order to predict the structural elements of GRA12 protein, the PSIPRED and GOR IV methods were employed. The outcomes of the secondary structure of GRA12 demonstrated that this protein comprises 35.55% alpha-helix, 19.50% extended strand, and 44.95% random coil. It is obvious that beta-turn and alpha-helix located in the inner parts of the protein, with high hydrogen-bond energy, can preserve a protein's structure and thus induce a strong interaction with antibodies [17]. In addition, we predicted four disulfide bonds in the GRA12 sequence using the DiNNNA server. Disulfide bonds also known as disulfide bridges play a key role in the structure and function of a protein [32]. Sequences of disulfide bonds similar to or less than five are easier to predict than those with more than five bonds. Also, precise disulfide bond prediction can diminish

the conformational space to boost the 3D structure modeling of proteins and protein folding [33]. It is well known that protein's main biological function relies on its spatial structure. As obvious, the prediction of tertiary structures is the ultimate aim of determining a function of the protein. Hence, it is highly important to understand the protein structures and to realize the relations between both structures and functions [25]. So, given the importance of tertiary structure in the biological function of proteins, we constructed the 3D structure of GRA12 using the SWISS-MODEL server. In order to validate the produced 3D model by SWISS-MODEL, the generation of the Ramachandran plot using the SWISS-MODEL program method was recruited. For this purpose, we selected the best model by SWISS-MODEL, and then refinement was performed using GalaxyRefine. According to the output of Ramachandran plot, protein validation before refinement indicated that in the initial model, 79.26% of residues were located in favored regions, 11.85% in allowed regions, and 8.89% in outlier regions. After refinement of the 3D model, the results were changed as follows: 91.85% of residues in favored regions, 6.67% in allowed regions, and 1.48% in outlier regions of the Ramachandran plot. A powerful humoral and cell mediated immunity are promoted during the course of *T. gondii* infection [34]. So that, the production of specific-immunoglobulin G antibodies prevented and restricted the attachment of parasites to respective host cell receptors. In addition, it can help immune cells, such as macrophages to easily eliminate *T. gondii* and prevent infection from reactivating [34]. On the other hand, the production of IFN-γ from T cells plays a main role in the restriction of acute and chronic infection. This important cytokine prevents the bradyzoites reactivation within tissue cysts of the host. the protection from *T. gondii* infection is mediated through the direction of CD4+ and CD8+ T cells; nonetheless, it is obvious that the function of CD8+ T cells and IFN-γ is more important to reduce infection [35]. Epitope, a part of an antigen, is identified by B-cells, T-cells, and molecules of the host immune system. Only a few amino acid residues including an epitope (instead of the entire protein) are enough to induce protective responses; thus, prediction and recognition of this substantial segment of amino acid residues can be a key to understand the pathogenesis and immune mechanisms of a pathogen and above all in development of epitope-based vaccines and immunodiagnostic test [36]. Epitope prediction methods are mostly based on several features (not one) of the proteins because researchers believe that only by evaluating one character we cannot access adequate and ac-

curate epitope information [37]. Hydrophilicity, beta-turns, secondary structure, and surface accessibility are the important features of the amino acids that can present significant and useable data of epitopes for biological studies including DNA vaccine [38]. Therefore, a peptide with excellent of the above-mentioned markers can easily interact with an antibody and generally act as an epitope. So, in the current study, several online services were applied to predict the epitopes of B-cell and T-cell. The findings of linear B-cell epitopes revealed that GRA12 protein has positive epitopes and acceptable indexes using BCPREDS, Bcepred, Bepipred, and ABCpred online servers. For example, our results indicated that nine potential antigen epitopes were predicted in GRA12 by BCPREDS. Also, Bcepred's prediction accuracy for models based on different properties ranges from 52.92% to 57.53% and this server permits users to predict epitopes of B-cell using any of the physico-chemical properties (accessibility, hydrophilicity, polarity, flexibility/mobility, exposed surface, and turns) [39]. In addition, the online server of ABCpred predicts B-cell epitopes in an antigen sequence using ANN. It is the first server developed using fixed-length patterns based on recurring neural network (machine-based technique). With an accuracy of 65.93%, this server can predict epitopes using recurrent neural network. It is important to mention that the prediction of discontinuous epitopes is crucial for the interaction between antibodies and antigens [40]. So, the ElliPro tool was used to predict discontinuous B-cell epitopes. We found 5 conformational B-cell epitopes in the 3D model of the GRA12 protein.

MHC molecules present T-cell epitopes to T-cells. Binding peptides to the MHC is a crucial step in the T-cell antigen presentation process and as well as a significant factor in the choice of potential epitopes. We used two online databases to analyze the IC50 values of peptides that bind to the MHC class I and class II molecules for GRA12. Based on obtaining results, the T-cell epitopes on GRA12 were found to have the ability to bind strongly to MHC class I and class II molecules. The results of these databases showed that both tools identified approximately some specific peptides and the results were almost similar. It is worth mentioning that the lower percentile ranks (or IC50 values) indicate the higher-level affinity, which represents a better T-cell epitope and vice versa. We predicted the CTL epitopes using CTLpred server and then the ten high ranked epitopes were selected for GRA12 protein. CTLpred is a method for CTL epitopes prediction that is critical in the design of a vaccine. This approach is based on elegant machine learning techniques such as ANN and SVM.

Since all MHC binders may not act as T-cell epitopes; hence, a highly accurate prediction method for CTL epitopes is needed. The use of ANN and SVM is explored to solve the problem. Regarding the above two approaches, the CTLpred server uses consensus and combined prediction. Compared to individual methods (like ANN and SVM), consensus and combined prediction methods are more specific and sensitive [23]. It should be remembered that the detection of allergenic proteins is becoming very important due to the use of modified proteins in diets (genetically modified foods), biopharmaceuticals, therapeutics, etc. (http://crdd.osdd.net/raghava/algpred/). As a result, derived results from antigenicity (ANTIGENpro and VaxiJen servers) and allergenicity (AlgPred and AllergenFP servers) assessment highlighted GRA12 protein is immunogenic and non-allergen.

Conclusion

One of the first steps to design an optimal vaccine is to recognize potential antigens that can provide strong protective responses. So, a precise and comprehensive antigen analysis via bioinformatics methods is essential. This paper gave a comprehensive definition of the important aspects of GRA12 protein, including physico-chemical features, a transmembrane domain, subcellular position, secondary and tertiary structure, B-cell and T-cell potential epitopes, and other important characteristics of this protein using different and reliable bioinformatics methods. Good antigenicity, hydrophilicity, surface accessibility, and flexibility indexes were observed for GRA12. Also, the findings of epitope prediction using various bioinformatics online servers showed that GRA12 protein had several excellent B-cells and T-cells epitopes, indicating that it would become an excellent vaccine against *T. gondii*. This research provided important basic and conceptual data on GRA12 protein in order to develop an effective vaccine against acute and chronic toxoplasmosis for further *in vivo* investigations.

Recommendations

More studies are required on vaccine development *in vivo* using the GRA12 alone or combined with other antigens in the future.

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