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Original Article

Computational comparison of two new fusion proteins for multiple sclerosis

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Abstract

Multiple sclerosis (MS), as one of the human autoimmune diseases, demyelinates the neurons of the central nervous system (CNS). Activation of the T cells which target the CNS antigens is the first autoimmune event in MS. Myelin oligodendrocyte glycoprotein (MOG) and myelin basic protein (MBP) are two proteins of the myelin sheath and have been shown to be among the high antigens contributing to the pathogenesis of MS. Production of the drugs with high specificity for the immune system diseases is a concern for various researchers. Therefore, tolerogenic vaccines are considered as a new strategy for the treatment of MS by presenting specific antigens. This study aimed to design and compare two fusion proteins by a combination of two neuroantigens linked to interleukin-16 (IL-16) (MOG-Linker-MBP-IL16 and MBP-Linker-MOG-IL16) as vaccines for MS. In this study, at first two models MOG (aa 11-30) linked to MBP (aa 13-32) was made by Modeler 9.10 and simulated for 20 ns via Gromacs 5.1.1 package. Then simulated antigen domains connected to the N-terminal domain of IL-16 and obtained structures simulated for 50 ns. The results revealed that both constructs had stable structures and the linker could keep two antigenic fragments separate enough, preventing undesired interactions. While MOG-Linker-MBP-IL16 showed better solubility, more accessible surface areas, more flexibility of its IL-16 domain, and better functionality of its IL-16 domain as well as more specific cleavage of its related epitopes after endocytosis lead to a better presentation of its antigenic property.

Keywords: Fusion protein; MBP; MOG; Molecular dynamics simulation; Multiple sclerosis; Vaccine.

INTRODUCTION

Multiple sclerosis (MS) is an inflammatory disease that affects the central nervous system (CNS) and usually starts between the ages of 20 and 40 years old (1). Multiple sclerosis causes disability by defects in sensory and motor functions of neurons (2). It is not clear what factors are causative in MS, but it is believed that environmental factors and their interactions with special genes are the sources of this disease (3). Some studies have revealed that MS is induced by autoreactive T cells (1,4,5). Therefore, it is considered as an autoimmune disease caused by $CD4^+$ Th1 and Th17 cells (2,3,5). The role of $CD4^+$ T cells in MS is supported by lots of samples with experimental autoimmune encephalomyelitis (EAE). It is also indirectly supported by the fact that major histocompatibility complex (MHC) or the certain MHC class II molecules show the strongest genetic risk factor, which is possibly due to their role in providing antigen molecules of myelin sheath proteins for $CD4^+$ T cells (1,2,5). It is assumed that activation of the inactivated T cells by CNS antigens, especially by MHC-antigen complex, is the first autoimmune reaction that occurs in MS pathogenesis (4). Subsequent immune responses to different proteins of the myelin sheath of CNS including myelin oligodendrocyte glycoprotein (MOG) and myelin basic protein (MBP) lead to some pathological damages (1,6). Myelin basic protein plays a major role in the structure and function of the myelin sheath (4,5). In addition, it is the first factor found in the homogeneous liquid of the brain and spinal cord as the cause of the EAE (4).

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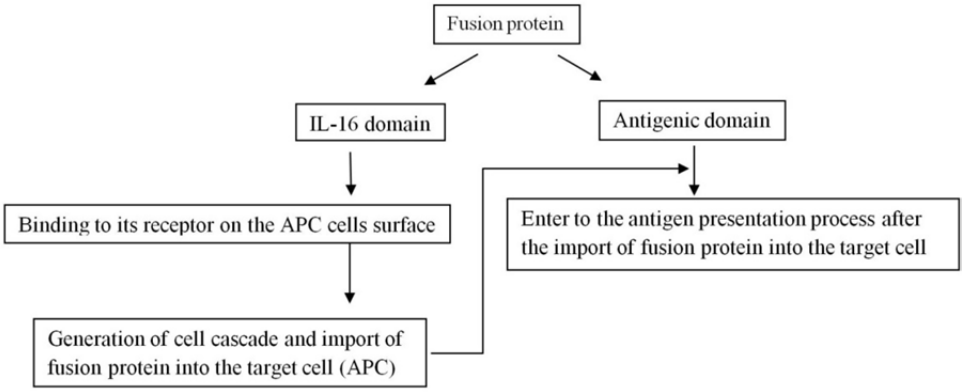

Fig. 1. A schematic image of the functional mechanism of neuroantigen-cytokine fusion proteins. IL, interleukin; APC, antigen-presenting cell.

Myelin oligodendrocyte glycoprotein mostly exists on the outer surface of the oligodendrocyte membrane and has a direct access to antibodies and can be used as a target for both humoral and cellular immune responses in MS (2).

Because the tissue damage in MS is exclusively the result of a CNS autoimmune inflammation process, there is a huge tendency for the study of the immune system components, and the investigation of immune cells has been changing in the last decades (7). Nowadays, researchers are mostly looking for drugs with high activity on the immune system. Accordingly, treatments of MS have been changed to be immune-based therapies. To this aim, antigen-specific tolerance, which is an antigen-specific immunomodulation method, may be applicable for MS-related antigens to reduce the immune response to the MS antigens (5,8). Therefore, tolerogenic vaccines generally introduce a new class of vaccines designed for restoring the immunological tolerance and the immune homeostasis, and consequently, reversing the autoimmune disease. These vaccines induce a long-term, antigen-specific, and inhibitory memory that blocks pathogenic T cell responses, in which the effector T cells are inhibited by induced regulatory T cells (9). In a new approach to design a tolerogenic vaccine for MS, a natural cytokine with or without a linker is fused into a neuroantigen domain (10). In this regard, one of the cytokines with anti-inflammatory function is interleukin 16 (IL-16). According to the experimental studies, those vaccines, in which neuroantigen part is located at N-terminal, and functional part of IL-16 is located at C-terminal, have an effective tolerogenic function (11,12). The functional mechanism of these fusion proteins are illustrated in Fig. 1.

As there is an ongoing investigation into these vaccines, we decided to design new fusion vaccines by considering different arrangements of two neuroantigen epitopes (e.g., MOG (aa 11-30) and MBP (aa 13-32)) followed by IL-16 (12). Then, their properties were analyzed by employing the molecular dynamics (MD) simulation method.

MATERIALS AND METHODS

The amino acid sequences of MOG (aa 11-30) (PIRALVGDEVELPCRISPGK), MBP (aa 13-32) (KYLATASTMDHARHGFLPRH), and IL-16 were obtained from UniProt (MOG id: Q16653; MBP id: P02686; IL-16 id: Q14005, respectively). Homology modeling, which is considered as a comparative modeling method, is a technique that allows us to build a model of the 3D structure of a target protein using its amino acid sequence and one experimental 3D structure of a similar protein (template) (13,14). Protein data bank (PDB) templates for MOG and MBP protein were obtained from blastpserver (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=Blast Search&LINK_LOC=blasthome) with the highest percent of overlap with the target sequence. These templates were 1QCL for the modeling of MBP (aa 13-32), 1PY9 for MOG (aa 11-30), 2MRU for the enterokinase linker (GDDDDKG). Afterwards,

1000 model were made for each epitope using Modeller 9 software via PDB templates (15). Subsequently, Procheck software, (16) ERRAT (17), and verify3D (18) servers were used to select the best models. Moreover, MOG and MBP epitopes in fusion proteins were modified. According to the databases, in MBP (aa 13-32), amino acids in the positions of 19, 20, 25, and 31 include phosphoserine, phosphothreonine, and citrulline (in the last two positions), respectively. The charge of phosphoserine and phosphothreonine residues was considered to be -2e (19). In this work we used the functional C-terminal part of IL-16 (PDB code: 1I16) containing 130 amino acids. The 3D structure of ubiquitinated IL-16 was made via connecting the ubiquitin (PDB code: 2ZNV) to IL-16 via Discovery Studio 3.5 Client (http://accelrys.com/resource-center/ downloads/ updates/ discovery - studio/ dstudio35/latest.html). Ubiquitination of IL-16 was based on phosphosite databases (http:// www.phosphosite .org/ homeAction.action), and three ubiquitin were connected to lysines of 57, 103, and 122 of IL-16 (20). Free MOG and MBP epitopes, ubiquitinated IL-16 domain, and antigenic domain (MOG epitope + MBP epitope) were separately utilized for MD simulation by Gromacs 5.1.1 package under G43A1 force field, SPC216 water model and the time step of 1 fs, for 20 ns (21-23) with the same procedure as mentioned in the previous works (24,25).

The obtained final structures of antigenic domains and ubiquitinated IL-16 were connected to each other and two fusion proteins were constructed in this way: construct (1), MOG (aa 11-30) + enterokinase linker + MBP (aa 13-32) + IL-16; and construct (2), MBP (aa 13-32) + enterokinase linker + MOG (aa 11-30) + IL-16. Then, the fusion proteins were simulated for 50 ns with the same conditions.

After MD simulation, thermodynamics, and structural parameters of free epitope domains (MOG (aa 11-30) and MBP (aa 13-32)), antigenic domains, IL-16 domain, and two constructs were calculated via analysis commands of Gromacs package during simulation. In addition, ten PBD structures were extracted from the last 10 ns of MD simulation of fusion proteins. Then, PQR files were obtained from pdb2pqr server (http://nbcr-222.ucsd.edu/pdb2pqr_2.1.1 (26). Solvation free energies were calculated via apbs1.5 software (https://sourceforge.net/ projects/apbs/) by defaults parameters (27). Isoelectric point (pI), aliphatic index (28), and instability index (29) of two fusion proteins were performed via Expasy's Prot Param Server (http://web.expasy.org/protparam/) (30). Instability Index is the weighted sum of dipeptides that occur more frequently in unstable proteins when compared to stable proteins and offers an estimate of *in vitro* protein stability. Proteins with instability index of less than 40 are predicted to be stable proteins (31). Also, for checking and approving the structure, and function of fusion proteins, Pep Cleave-cd4 server (http:// peptibase.cs.biu.ac.il/PepCleave_cd4/) was used. This server predicts excision position of an antigen for creating a proper epitope for $CD4^+$ T cells (32). Aggrescan3D server (http://biocomp.chem.uw.edu.pl/A3D/) was also used to assess and compare the solubility of the constructs, and aggregation propensity in protein structures as well as the rational design of protein solubility. One of the information obtained from this server was the average score for each protein construct, either a positive or a negative value; the negative values indicated solubility status of the construct (33).

RESULTS

Structural analysis of fusion proteins

In this study, we used computational methods to understand the properties and structure of the designed recombinant vaccines (constructs). The results of fragments (epitopes and antigenic domains) of constructs are not mentioned for brevity. The results of modeling conducted by Procheck software, Verify3D, and ERRAT servers, and theoretical pI, instability index, aliphatic index of final structure of constructs 1 and 2 obtained from Expasy's Prot Param Server and the average of solvation free energies obtained from apbs software and also the results of MD simulation analysis are summarized in Table 1.

Table 1. The results from structural analysis performed by servers and MD[1] simulation during the last 15 ns of MD simulation.

Analysis		Protein						
		Construct 1			Construct 2			Free IL[2]-16
Theoretical pI[3]		5.79			5.79			-
Instability index		39.50			38.63			-
Aliphatic index		89.44			89.44			-
Aggrescan 3D score		-1.0005			-1.044			-
ERRAT (Overall quality factor)		50			42.3			-
Verify3D (percent of the amino acids that have 3D-1D score > = 0.2)		54.4%			52.20%			-
Procheck	Most favored	74.2%			74.2%			-
	Additional allowed	21.2%			21.9%			-
	Generously allowed	4%			4%			-
	Disallowed	0.7%			0%			-
RMSD[4] (nm)		1.3 ± 0.07			1.1 ± 0.04			-
Rg[5] (nm)		2.59 ± 0.01			2.46 ± 0.02			-
Number of hydrogen bonds	Intermolecular (protein-sol)	811 ± 21			797 ± 18			-
	Intramolecular (protein-protein)	294 ± 10			295 ± 9			-
Solvent accessible surface area (nm^2)		MOG[6] 25.3	MBP[7] 24.6	IL16 173.4 ± 3.0	MOG 18.6	MBP 22.3	IL16 165.7 ± 2.4	177.7 ± 2.5
The number of contacts between Ag[8]-IL16 less than 0.6 nm		138.5 ± 9.3			220.6 ± 10.3			-
COM[9] (nm)	MOG-MBP	1.2 ± 0.04			1.6 ± 0.04			-
	Ag-IL-16	3.3 ± 0.07			3.2 ± 0.03			-
Solvation free energy (Kj/mol)		-24606.6 ± 697			-23428.1 ± 556			-

[1] MD, molecular dynamic; [2] IL, interleukin; [3] pI, isoelectric point; [4] RMSD, root mean square deviation; [5] Rg, radius of gyration; [6] MOG, myelin oligodendrocyte glycoprotein; [7] MBP, myelin basic protein; [8] Ag, antigen; [9] COM, center of mass.

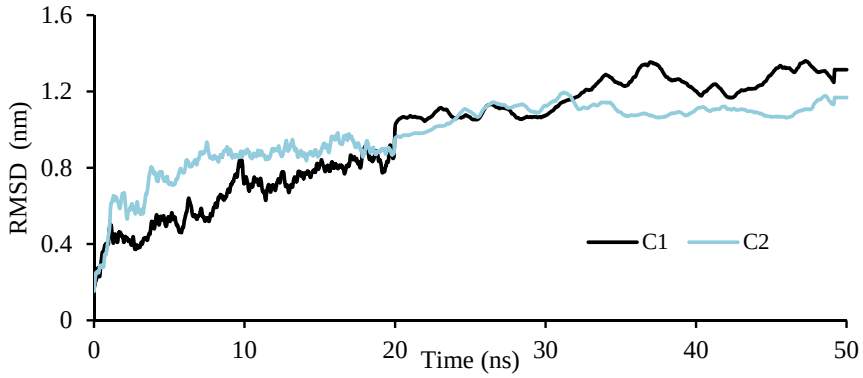

Fig. 2. Root mean square deviation (RMSD) plots for construct 1 (C1) and construct 2 (C2). The average of backbone RMSD of construct 1 and construct 2 are 1.3 ± 0.07 and 1.1 ± 0.04 (nm) respectively.

Figure 2 shows root mean square deviation (RMSD) plots for constructs 1 and 2 during 50 ns MD simulation. Root mean square deviation of both constructs reached a plateau after about 35 ns in MD simulation run. Then all analyses were conducted in the last 15 ns of MD simulation. In this study, we considered both MOG and MPB atoms as antigenic domains.

Figure 3 shows the distance between center of mass of two antigenic domains and IL-16 domain in two constructs and the number of contacts less than 0.6 nm between them during 50 ns MD simulation.

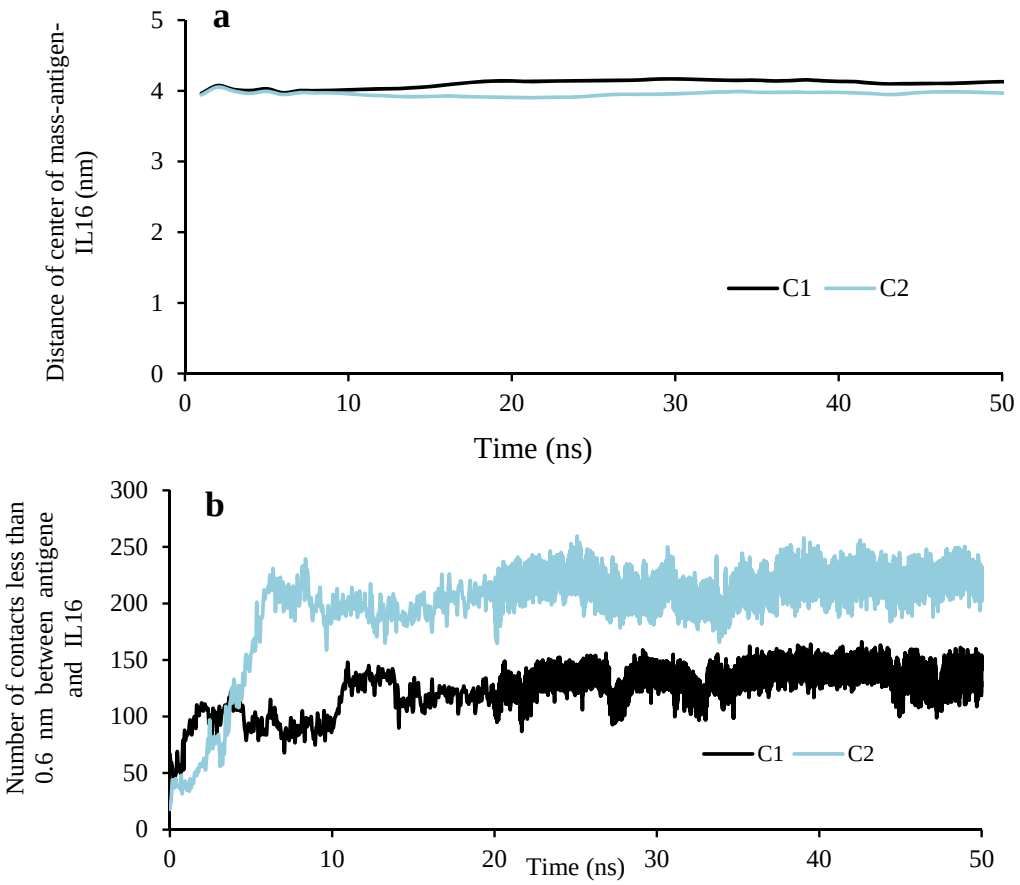

Fig. 3. (a) The distance between center of mass two of antigenic domain and IL-16 domain in two constructs and (b) the number of contacts less than 0.6 nm between them during 50 ns molecular dynamic simulation.

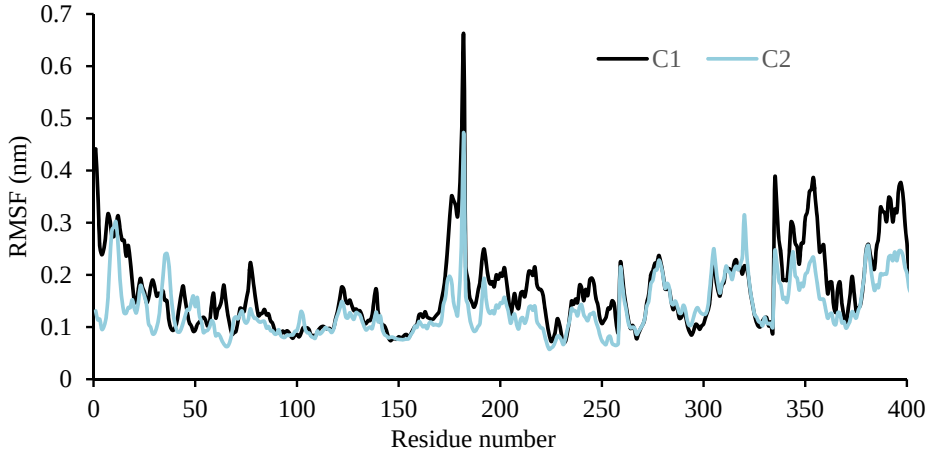

Fig. 4. The root mean square fluctuation (RMSF) plot of constructs 1 and 2 during the last 20 ns of molecular dynamics simulation.

Figure 4 shows the root mean square fluctuation (RMSF) plot of compounds 1 and 2 during the last 20 ns of MD simulation. We can see that RMSF compound 1 is greater than compound 2.

Table 2 shows the area under the RMSF plot and F_{overal} for free IL-16, free MOG and free MBP epitopes, IL-16 domain in constructs 1 and 2, MOG and MBP fragments in constructs 1 and 2 during the last 15 ns of 50 ns MD simulation.

Table 2. The area under the RMSF[1] plot for free IL[2]-16, MOG[3] and MBP[4] epitopes in the last 5 ns, and IL-16 domain, MOG and MBP epitopes in constructs 1 and 2 in the last 15 ns of the time simulation.

Protein		Area under MSF plot	F_{overal}
Free interleukin-16		63.1	0.188
Free antigen (MOG + MBP epitopes)		6.8	0.392
	Full protein	69.3	0.187
Construct 1	IL-16 domain	59.5	0.184
	Antigen (MOG + MBP) fragment	8	0.399
	Full protein	55.8	0.146
Construct 2	IL-16 domain	48.1	0.144
	Antigen (MOG + MBP) fragment	6.2	0.331

[1] RMSF, root mean square fluctuation; [2] IL, interleukin; [3] MOG, myelin oligodendrocyte glycoprotein; [4] MBP, myelin basic protein.

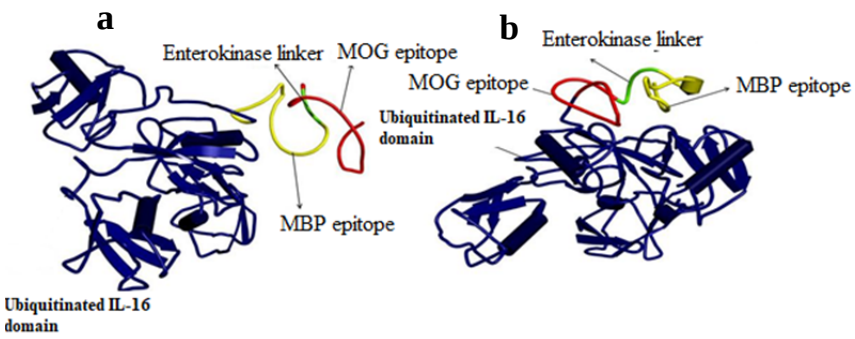

Fig. 5. The final structure of (a) construct 1 and (b) construct 2 after 50 ns molecular dynamics simulation.

Functional analysis of fusion proteins

These tolerogenic vaccines present the neuroantigen (NAg) to particular types of antigen-presenting cells (APCs) by the cytokine receptors on the same APCs. After they bind to their receptor on the surface of $CD4^+$ T cells, they create a molecular cascade that modulates APC function in order to generate the inhibitory or tolerogenic APC activities and simultaneously present the NAg to MHC class II Ag-processing pathways for antigen presentation by the same APCs (12). Thus, after the simulation, in order to predict the performance and effectiveness of simulated fusion proteins, PepCleave cd4 server was used. The results of this server showed that the epitope fragments in the protein construct 1 was cleaved with score 0.87 for MBP (aa 13-32) epitope and with score 0.4 for MOG (aa 11-30) epitope. The score of the server was 0.73 for MBP (aa 13-32) epitope, and 0.4 for MOG (aa 11-30) epitope in construct 2.

Panels a and b in Fig. 5 show the final structures of simulated models of both constructs 1 and 2 and different fragments of their structures after 50 ns MD simulation.

DISCUSSION

Besides their immunogenic ability, neuroantigen vaccines, which are designed for MS, should be able to inactivate the autoreactive $CD4^+$ T cells. Due to the difficulty of the experimental studies related to these vaccines, computational studies seem to be useful and reasonable for the primary examinations of these molecules because they are almost quick and inexpensive.

The results of Procheck software, Verify3D, and ERRAT server showed that the models were reliable and stable (Table 1). The two fusion proteins had an identical pI, meaning that replacement of epitopes does not affect pI of proteins. The results of the analysis of the servers confirmed the proper structure and function of fusion proteins. The primary structure analysis of proteins obtained from Expasy's Prot Param server also showed the structural stability of both constructs because of the instability index of less than 40. In addition, aliphatic index of both constructs showed they were stable. Aggrescan3D server results indicated that both constructs had negative average scores, so both protein

structures were soluble (Table 1). In sum, these results showed the selected models were suitable and proper as the starting structures for MD simulation.

The results of RMSD showed that both constructs gained a stable structure, and systems reached equilibrium during simulation. Therefore, the simulation time would be enough. When the changes of RMSD of these two constructs were compared, we could determine that RMSD of construct 2 was 0.2 nm smaller and had less structural flexibility than that of construct 1 (Table 1).

The small change in radius of gyration (Rg) of proteins (about 0.01 nm) during simulation indicated the stable tertiary structure of them. The Rg plots also display that construct 2 had more compact structure. The extent of decrease was 0.13 nm; hence, construct 2 had less flexibility than construct 1 did. In other words, construct 1 exhibited more Rg than construct 2, less compact structure, more flexibility and more binding power to the receptor, because conformational change or flexibility in the protein, results in a series of rearrangements that lead to a complex with tighter binding (34). Consequently, the results of this analysis are consistent with RMSD analysis.

The small changes of hydrogen bonds in the number of inter-molecular hydrogen bonds among proteins and water, and intra-molecular hydrogen bonds of proteins indicated that all proteins reached the stable and soluble structure due to the high number of their intra-molecular and inter-molecular hydrogen bonds in the simulation. These results indicates more inter-molecular hydrogen bonds between water and protein in construct 1, thus, solubility of construct 1 was more than that of construct 2.

The solvent accessible surfaces of IL-16 in construct 1 and free IL-16 were almost similar. Therefore, this construct could be bonded to the IL-16 receptor without the interference of its antigenic domain, but surface of IL-16 in construct 2 was less than that in free IL-16. Then, construct 2 was probably impaired in its function for binding to its receptor on antigen-presenting cells (APC). Apparently, IL-16 in construct 2 was covered with other parts of fusion protein. In addition, the analysis of epitopes' surface indicated the superiority of construct 1 over construct 2 because solvent accessible surface area was greater in construct 1 than that in construct 2. As a result, construct 1 had more antigen presentation ability. This analysis also confirmed the results of the other analyses of this study (Table 1).

The results of the distance between antigenic domain and IL-16 domain (Table 1 and Fig. 3a) showed that this distance was suitable for both constructs. Antigenic and IL-16 domains would not create functional interference with each other. Also, these results showed the superiority of construct 1 because the distance between the two domains (Ag-IL-16) was more in construct 1. Hence, the effect of the two domains on each other in construct 1 was less than that in construct 2. On the other hand, the distance between the center of mass of MOG and MBP epitopes in both constructs were suitable, so MOG and MBP would not create functional interference with each other (data not shown). The center of mass of two epitopes in construct 2 is more than that in construct 1. Of course, separation between antigenic domain (MOG + MBP) and IL-16 is more important than separation between MOG and MBP epitopes because these epitopes perform the same function, but IL-16 has different function with antigenic domain in fusion protein. In conclusion, inflexible enterokinase linker can prevent the interaction between the two epitopes. Therefore, it can provide a suitable epitope presentation for antigen processing. However, the results of this analysis could be compared with the results of the analysis of the number of contacts (less than 0.6 nm) between antigenic domain and IL-16 domain in both constructs for more certainty (Table 1 and Fig. 3b).

The RMSF plot of two constructs shows that compound 1 is more flexible than compound 2 (Fig. 4). Also, the total area under the RMSF plot of fusion proteins and $F_{Overall}$ (following equation) can be related to their overall flexibility of protein (35,36).

$$F_{\text{overall}} = [\frac{1}{n} \sum_{i=1}^{n} rmsf_i^2]^{1/2}$$

In this equation, "i" is residue number. These results are displayed in Table 2.

The comparison of the results of the area under the RMSF curves and $F_{overall}$ showed that construct 1 had more flexibility than construct 2. These results are consistent with the results of the distance of the mass center of the antigenic domain and IL-16 domain and the number of contacts between them. In other words, more distance between IL-16 domain and antigenic domain in construct 1 would lead to less contact and less spatial prohibition between them and so more overall flexibility in construct 1. Also, this analysis was in agreement with the results of RMSD and Rg analysis. When the area under the RMSF plot and $F_{overall}$ of the IL-16 domain of these constructs were compared to their free IL-16, it was revealed that the flexibility of IL-16 domain in fusion proteins was less than that of free IL-16. The difference between IL-16 flexibility in construct 2 and free IL-16 was more than the difference between IL-16 flexibility in construct 1 and free IL-16 and would probably impair binding construct 2 to its receptor. The effect of the antigenic domain on the decrease in the flexibility of IL-16 domain in construct 1 was reduced and IL-16 domain was able to maintain its flexibility; because construct 1 had more distance between two antigenic domain and IL-16 domain as well as smaller number of contacts between them than construct 2. Overall flexibility of antigen epitopes (sum of the surface area under RMSF curve of MOG and MBP or sum of $F_{overall}$ of them) in construct 1 was more than that in construct 2 and in free antigen (Table 2); because in construct 1 epitopes had less overlap with each other compared to free mode and construct 2. In general, the results of this analysis indicated the superiority of construct 1 over construct 2. Thus, it seems reasonable to say that the epitopes' fragments arrangement in the design of these vaccines is important and probably the number of antigenic fragment is important, too.

The more negative solvation free energy of construct 1 indicated that construct 1 was more soluble and stable than construct 2. The number of inter-molecular hydrogen bonds between protein and water were consistent with solvation free energy in Table 1.

The higher score of Pep Cleave-cd4 server indicates that the peptide could be cut with more accuracy and specificity and less sensitivity. As a result, construct 1 could be cut better in epitopic sites (MOG and MBP) than construct 2.

CONCLUSION

The results of the present study can help us to select an appropriate fusion vaccine for MS disease. In summary, all computational analyses showed that both fusion vaccines had the necessary structural stability and suitable distance between domains. However, between two fusion vaccines, construct 1 (MOG-MBP-IL16) had more acceptability and better antigen presentation. In addition, the flexibility and the solvent accessible surface area and binding power of construct 1 to IL-16 domain were better than the other construct. The results of the analysis of the PepCleave cd4 server, which predicts precision position in a protein structure for $CD4^+$ T cells, revealed that epitope fragments could appropriately be cut in protein structures and the designed vaccine could produce more proper antigen segment and subsequently more antigen presentation capability. In general, the comparison of two fusion proteins indicated that construct 2 had two unsuitable properties (the flexibility and the solvent accessible surface area of its IL-16 domain was less than that of free IL-16) (Tables 1 and 2). The results of this computational study showed, both designed constructs were appropriate in terms of structure stability, and they were expected to have a good capability in antigen presentation. The epitope order in different arrangements of epitopes affects the structures and functions of proteins. For example, in this study, MOG-MBP-IL16 (construct 1) appeared to be a more efficient vaccine. The method employed in the current study helps to accelerate the analysis of the designed fusion proteins before conducting any expensive, time-consuming, and high risking experimental methods. It can also be used to select the best fusion protein among some proposed fusion vaccines. It is recommended that other fusion proteins be designed by using

other neuroantigen epitopes related to MS. We also recommend that their structure and function and binding affinity to their receptor should be predicted by docking and molecular dynamics simulation studies. The advantage of this work is modification of MOG and MBP epitopes. We hope that this work will shed light on the remedy of the MS disease.

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