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Fluorescence spectroscopy and molecular modeling studies on the interaction of aflatoxin B1 and G1 with bovine α-lactalbumin

Omid Soltanabadi[1], Maliheh Sadat Atri[1]*, Mohammad Bagheri[2]

[1] Department of Molecular and Cell Biology, Faculty of Basic Sciences, University of Mazandaran, Babolsar, Iran; [2] Department of Analytical Chemistry, Faculty of Chemistry, University of Mazandaran, Babolsar, Iran.

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Abstract

Aflatoxins are toxic chemicals produced by *Aspergillus fungi*. Reports exist on the relationship of aflatoxin exposure via contaminated food and feed to hepatotoxicity and liver cancer. Aflatoxin B1 (AFB1) and Aflatoxin G1 (AFG1) are two dangerous types of aflatoxins for human health. Bovine α-lactalbumin (ALA) is the second major whey protein in milk which bear diverse biological functions. In this study, the interaction of AFB1 and AFG1 with the ALA protein was studied using fluorescence spectroscopy, molecular docking and molecular dynamic (MD) simulation. The spectroscopy experiments showed that the interaction with AFB1 and AFG1significantly quenched the intrinsic fluorescence emission of ALA via a static quenching mechanism. The free energy of binding and binding constant (Ka) obtained from the intrinsic fluorescence results were –5.32 kcal per mol and 0.80×10^4 L mol^{-1} for AFB1 and -5.64 kcal per mol and 1.35×10^4 l mol^{-1} for AFG1, respectively. Molecular docking studies were conducted before and after the MD simulation to estimate the binding sites, Ka s and binding mode. Results from the molecular docking showed that AFB1 and AFG1 bound to ALA via hydrophobic interaction and hydrogen bond. After MD simulation, the precision of the Ka obtained from the docking results was improved and it was more similar to the experimental results of fluorescence spectroscopy. Other simulation results were aligned well with the molecular docking and fluorescence spectroscopy results. Accordingly, AFB1and AFG1 could form complex with ALA, however, AFG1 showed higher affinity for binding to ALA and more compact complex structure.

Introduction

Aflatoxins are toxic secondary metabolites made by some fungi species like *Aspergillus flavus*, *Aspergillus parasiticus* and the rare type *Aspergillus nomius*.[1] Four of these compounds that fungi produce are naturally found in foodstuffs: Aflatoxin B1 (AFB1), aflatoxin B2 , aflatoxin G1 (AFG1) and aflatoxin G2 .[2] When cows eat AFB1-containing feedstuffs, increased the AFB1 concentration in the blood causes lipid peroxidation and oxidative DNA damage. Different biotransformation pathways of AFB1 in liver lead to different metabolites. Aflatoxin M1 (AFM1) is major toxic metabolite secreted into milk. The AFB1 biotransformation to AFM1 in addition to the liver can also occur in the bovine mammary epithelial cells.[3,4] All aflatoxins absorb ultraviolet (UV) light with an absorption peak at the wavelength of 360 nm.[5] Under UV light excitation B type aflatoxins exhibit blue fluorescence at 425 nm and G type aflatoxins show green fluorescence around 450 nm. *Aspergillus flavus* and *A. parasiticus* molds produce aflatoxin B, however, aflatoxin G is made by second one.[6] Aflatoxins are carcinogens and they have significant human health hazard. Some of damaging human health dangers are related to dietary contamination of aflatoxins along with hepatotoxicity and liver most cancers. There are some reports on the relationship between aflatoxin and hepatoxicity and liver cancer especially AFB1 which is the most potent liver carcinogen.[3,7] The presence of aflatoxin within the food causes impaired growth in youngsters.[8] Most of the above-cited aflatoxin kinds, AFB1and AFG1, are more harmful to human health.[9] In the structure of Aflatoxin B, there is a cyclopentanol loop that is replaced with lactam coumarin in the structure of aflatoxin G.

***Correspondence:**

Maliheh Sadat Atri. PhD
Department of Molecular and Cell Biology, Faculty of Basic Sciences, University of Mazandaran, Babolsar, Iran
E-mail address: m.atri@umz.ac.ir

Bovine α-lactalbumin (ALA) is a calcium-binding, small acidic protein which is the second major whey protein in milk. The concentration of ALA in blood is associated with mammary growth and development. In pregnant heifers serum ALA concentrations become detectable in the last trimester of pregnancy with modest increase until just before calving when concentrations increase significantly.[10] The ALA structure and function are important from several points of view. It has been reported that ALA protein (14.20 kDa) has diverse biological and pharmaceutical functions such as lactose biosynthesis in the mammary gland and induction of apoptosis in tumor cells.[11] The ALA can interact to hydrophobic substances such as retinol,[12] vitamin D3,[13] hydrophobic peptides, model lipid membranes and fatty acids.[14,15]

The ALA is genetically and structurally homologous to lysozyme.[16] It is a two-domain protein that consists of a single polypeptide chain of 123 amino acid residues. The α-domain of ALA is comprised of two short 3_{10} (residues 18 - 20 and 115 - 118) and three major α-helices (residues 5 - 11, 23 - 24, and 86 - 98). One short 3_{10} helix (residues 77 - 80) and three-stranded antiparallel β-sheet (residues 41 - 44, 47 - 50 and 55 - 56) are the components of the smaller β-domain.[17] The α and β domains are separated by a calcium-binding loop. The structure of ALA is stabilized by four disulfide bridges. One of the essential characteristics of this protein is its putative ability to bind to metal cations.[18,19]

The interaction of milk proteins with mycotoxins is an important area of study in dairy science. The interaction of AFB1 and AFG1 with bovine ALA has not been previously investigated. In this research, the molecular details of the interaction of AFB1 and AFG1 with bovine ALA is investigated using fluorescence spectroscopy technique, molecular docking and molecular dynamics simulation. The binding sites of AFB1 and AFG1 on ALA are determined and the free energy of binding (ΔG°) and binding constant (Ka) is calculated. The effect of aflatoxins on ALA conformation is examined. This study aimed to determine the mechanism of AFB1 and AFG1 binding to ALA and explore mycotoxin effect on ALA protein structure and function. The present study could be helpful in understanding the role of ALA protein in mitigating aflatoxin toxicity.

Materials and Methods

Materials. Bovine α-lactalbumin (code: L5385) and AFB1 and AFG1were purchased from Sigma-Aldrich (St. Louis, USA). Tris buffer and acetonitrile were obtained from Merck (Darmstadt, Germany).

Fluorescence quenching measurements. Fluorescence spectroscopy is a valuable approach for analyzing structural changes of proteins and calculation of the thermodynamic parameters of ligand binding to the protein in solution. Fluorescence measurements were performed on a fluorescence spectrophotometer (FP-8300 JASCO, Tokyo, Japan) and 1.00 cm quartz cuvette. Stock solution of ALA (30.00 nM) was prepared in Tris-HCl buffer solution (Tris 20.00 mM, pH 7.40). Protein concentration was determined spectrophotometrically using absorption at 280 nm by an Epoch microplate spectrophotometer (BioTek Instruments, Winooski, USA). Stock solutions of aflatoxins (1.00 ppm) were dissolved in acetonitrile. The excitation wavelength was 280 nm and the scanning range was 295 to 550 nm. The excitation and emission slit widths were 10.00 nm. The ALA (30.00 nM) solution were titrated with the AFB1 and AFG1 stock solutions in a way that the concentration of AFB1 and AFG1 was 0.00, 15.00, 30.00, 45.00, 60.00, and 75.00 nM (equal to 0.00, 4.90, 9.80, 14.80, 19.70, 24.60 ng mL^{-1} for AFG1 and 0.00, 4.70, 9.40, 14.00, 18.70, and 23.40 ng mL.00 for AFB1) in the cuvette. It was carried out at room temperature. Following each step of titration the sample was incubated for three minutes and then the fluorescence emission spectrum was recorded.

Quenching can seem due to various inter and intramolecular interactions, i.e., molecular collisions (dynamic quenching), complex formation (static quenching), energy transfer, and conformational exchange.[20] The fluorescence quenching data showed that the ALA conformation was changed and that an intermolecular energy transfer occurred between ALA and aflatoxins. Fluorescence quenching can be described by Stern–Volmer equation as follows:[21]

$$F_0/F = 1 + K_{sv}[Q] = 1 + k_q \tau_0 [Q]$$

where, F_0 is the fluorescence intensity of ALA alone, F is the fluorescence intensity of ALA in the presence of the ligand, K_{SV} is the Stern - Volmer quenching constant $[Q]$ is the ligand concentration, k_q is the bimolecular quenching constant and τ_0 is the lifetime of the fluorescent molecule in ALA without quencher (2.60 nsec).[21]

The Ka and number of binding sites can be obtained using the below formula when quenching is static:

$$\log [(F_0-F)/F] = \log K_a + n \log [Q]$$

where, n is the number of binding sites and their values obtained from the plot of log $[(F_0- F)/F]$ versus log [Q].[22]

Molecular docking. Molecular docking study was carried out using AutoDock Software package (version 4.2; Scripps Research, California, USA) based on default parameters by the genetic algorithm to obtain the three-dimentional structure of the ALA-aflatoxin complexes and their binding affinities.[23] The crystal structure of ALA (PDB ID: 1HFZ) was obtained from the Research Collaboratory for Structural Bioinformatics Protein Data Bank.[24] Water molecules and heteroatoms removed from the protein structure using ArgusLab Software (version 4.0.1; Planaria Software, Seattle, USA).[25] Polar hydrogens were added to

the protein structure by Auto-DockTools. The three-dimensionalstructure of AFB1and AFG1 was created and optimized geometrically using HyperChem (version 8.0; hypercube, Gainesville, USA).[26] Population size was 150, the maximum number of evaluations was 2,500,000, maximum number of generations was 27,000 and the rate of the crossover were 0.80. Subsequently, binding sites of ALA were defined by a docking box of 50.00 × 50.00 × 50.00 and a grid spacing of 0.375 Å. The best conformer was used for further analysis. To analyze the hydrogen bond and hydrophobic interactions of the complexes, the Ligplot program (version 2.2; European Bioinformatics Institute, Cambridgeshire, UK) was used.

Molecular dynamic (MD) simulations. The conformational changes of the complexes were estimated using the MD method by GROMACS Software package (version 5.1.2; Royal Institute of Technology and Uppsala University, Uppsala, Sweden) and GROMOS96 43a1 force field.[27,28] The initial conformation was one of the lowest binding energy docked conformations. The topology parameters of ALA were defined by the Gromacs Software. The coordinate of ligands was converted to Gromacs topology using PRODRG2.5 server. The ALA protein and both complexes were solvated in three the same triclinic boxes (2.996 × 3.453 × 4.722 nm). The Simple Point Charge (SPC) water molecules surrounded the complex and Na^+ and Cl^- ions were used to neutralize the charge.[29] The steepest descent method was used to minimize system energy. The equilibration phase of the system (NVT/NPT) was performed at 298 K by 100,000 steps for 200 psec.[30] Reduce-off distances for calculating Coulomb and van der Waals interactions had been 1.00 nm and the time step became two fs for all levels. Subsequently, 20 nsec manufacturing MD was run at constant temperature and pressure. The atom coordinates were recorded every 40 psec during the simulation for later analysis. To understand the MD process better, the root mean square deviation (RMSD) was measured. To have a rough measure of the compactness of protein structure, radius of gyration (Rg) was calculated.[31] Structural analysis was done using Chimera Software (Chimera Technologies, Bengaluru, India). The VMD Software (version 1.9.3; University of Illinois, Champaign, USA) was used to visualize the consequences.

Results

Fluorescence spectroscopy. The fluorescence emission spectra of ALA in the presence of different concentrations of AFB1 and AFG1 are shown in Figure 1A and 1B. The intrinsic fluorescence emission of ALA was quenched as a result of adding of aflatoxins. The wavelength of the maximum emission of ALA was about 310 nm which was shifted (about 3.00 nm) to a shorter wavelength in the presence of AFB1 and AFG1. Based on the Stern-Volmer plot slope (Fig. 1C and 1D), the value of K_{sv} was equal to 3.30×10^6 L mol^{-1} and 3.2×10^6 L mol^{-1} for AFB1 and AFG1, respectively. Previous studies have shown that where the k_q value was greater than 2.00×10^{10} L mol^{-1} per sec, the quenching was static and where smaller, the quenching was of a dynamic type.[32] Since the obtained k_q value was 1.23×10^{15} and 1.26×10^{15} L mol^{-1} per sec for AFB1 and AFG1, it could be concluded that the fluorescence quenching mechanism was static and the complex formation happened.

Binding parameters. The Ka and number of binding sites obtained from the plot of log $[(F_0 - F)/F]$ versus log [Q] (Fig. 1E and 1F). These results showed that the values of n were almost equal to 1, hence, there was one binding site for the AFB1 and AFG1 on ALA molecule. Following calculating the Ka, ΔG° was obtained by using $\Delta G° = -RT \ln K_a$[33] (Table 1). In this equation, R (gas constant) is equal to 8.314 J K^{-1} mol^{-1} and T (temperature) is 298.1 K.

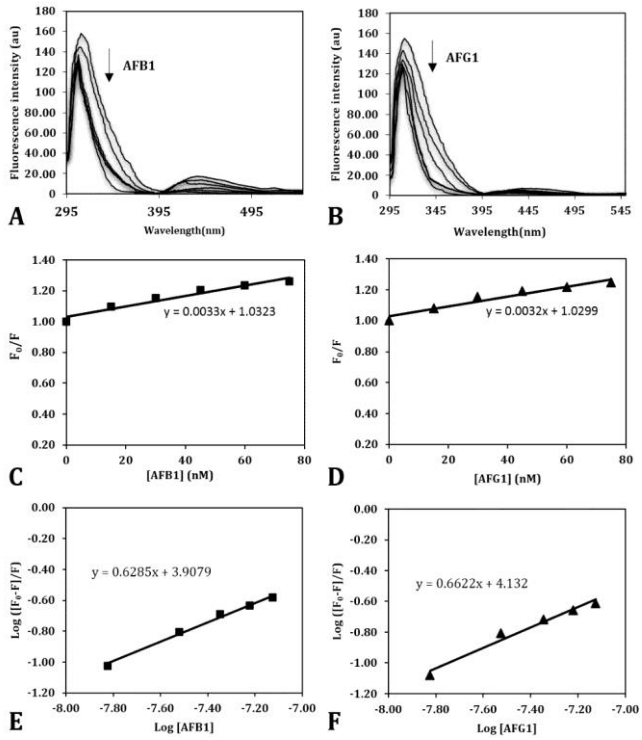

Fig. 1. The fluorescence quenching results. **A** and **B)** Intrinsic fluorescence spectra of 30.00 nM α-lactalbumin (ALA) in Tris buffer alone and in the presence of increasing concentration of aflatoxin B1 (AFB1) and aflatoxin G1 (AFG1; 15.00 - 75.00 nM) at room temperature and the excitation wavelength of 280 nm. The arrow shows the concentration increase of aflatoxins; **C** and **D)** Stern-Volmer plot; **E** and **F)** log $[F_0 - F / F]$ *vs*. log [Q] for the measurement of binding constant of AFB1and AFG1by ALA.

Molecular docking analysis. The molecular docking was employed to estimate the Ka and binding site of AFB1 and AFG1 on ALA to expand the data obtained from the experimental results. In the first step of molecular docking, binding site and Ka were detected. Then, the

best conformation of complexes which showed the lowest binding free energy was selected after performing MD and the molecular docking was accomplished on these optimal MD conformers of ligand and protein. The highest-scoring results for binding of AFB1 and AFG1 to ALA before and after MD and residues involved in binding are shown in Table 1. The best conformation of the ALA-AFB1 and ALA-AFG1 complexes obtained by molecular docking before and after MD is shown in Figure 2 and the main amino acid residues of binding sites before and after MD are shown in Figure 3. The results indicated that these aflatoxins bound to the surface area of ALA. The binding site for AFB1 and AFG1 on ALA was located on α domain and between α and β domain of the protein, respectively. The obtained Ka values were consistent with the fluorescence studies. However, these values became much closer to the experimental results after performing MD simulation. The docking results showed that hydrogen bond and hydrophobic interactions played a significant role in the binding of AFB1 and AFG1 to the ALA. After performing MD simulation, the number of hydrogen bonds was increased.

Molecular dynamic simulation. The structural behaviors of the best docking results of ALA-AFB1 and ALA-AFG1 complexes were investigated using the RMSD. The trajectory stability was confirmed by the analysis of the RMSD as a function of time for the complexes and ALA protein. As shown in Figure 4A, the RMSD of ALA, ALA-AFB1 complex and ALA-AFG1 complex were reached to stability and oscillated around average value after approximately 14.00 nsec simulation time. The mean RMSD values of backbone atoms in ALA, ALA-AFB1 complex and ALA-AFG1 complex were calculated from the last six ns trajectories, where the values were fluctuated for ALA (0.219 ± 0.014 nm), ALA-AFB1 (0.213 ± 0.009 nm) and ALA-AFG1 (0.179 ± 0.007 nm). The best conformer of ALA-AFB1 and ALA-AFG1 complexes in this RMSD range was selected for additional molecular docking.

For post-MD docking step, the ALA-AFB1 and ALA-AFG1 complexes were selected at 15.90 and 14.80 nsec, respectively. Docking was then performed on these structures. As can be seen in Table 1, the binding constants of AFB1 and AFG1 became closer to the fluorescence spectroscopy results after performing MD simulations. This result showed that the protein flexibility in its natural soluble state affected the binding energy and should be considered in complex simulations.

The analysis of molecular structures was performed by Chimera software to verify that the structure of ligands obtained from the MD were the final structure and the flexibility of ligands in AutoDock did not considerably influence the calculated Ka. RMSD values of AFB1 and AFG1 were obtained at 0.308 and 0.132 Å from docking after MD. Therefore, in the final stage, the fluctuations of ligand structures were minor indicating that ligand structures obtained from the MD were the final structures.

In order to evaluate the local protein flexibility and rigidity, the time-averaged root means square fluctuation values of free ALA and ALA–AFB1 complex and ALA-AFG1 complex were calculated. The results were plotted against residue numbers over all time scale of simulations trajectory (Fig. 4B). As seen in Figure 4B, amino acid residues of the binding site especially those with hydrogen bonds with ligand showed more rigid behavior in comparison with residues which were far from the ligand binding pocket. Therefore, the ligands strongly bound to the amino acid residues of the binding site and it confirmd the molecular docking results.

The Rg values of ALA, ALA-AFB1 and ALA-AFG1 complexes were plotted against the simulation time (Fig. 4C). The primary values of Rg for ALA alone, ALA-AFB1 complex and ALA-AFG1 complex were about 1.37 nm. Generally, the Rg values became stable at 8.00 nsec representing that the MD simulation reached to an equilibrium after 8.00 nsec. The Rg of protein and the two complexes reached to a constant value at approximately 8.00 nsec. The Rg value of free ALA, ALA-AFB1 and ALA-AFG1 were 1.329 ± 0.009, 1.338 ± 0.008, and 1.323 ± 0.008 nm, respectively. The attained results showed the decrease in Rg value of the ALA-AFG1 complex after complex formation and the Rg value of ALA-AFB1 was increased. It showed that the compactness of ALA structure was increased upon binding of AFG1. However, the higher mean value of Rg of ALA-AFB1 complex represented the increase of protein flexibility because of AFB1 binding.

Table 1. Comparison of thermodynamic parameters. Binding constant (K_a) and gibbs free energy of binding (ΔG°) for the complexes according fluorescence spectroscopy and docking studies before and after performing molecular dynamic (MD).

Parameters	ΔG° (kcal mol^{-1})	K_a (×10^4 M^{-1})	No.	Hydrogen bonds	Hydrophobic interactions
α-lactalbumin - aflatoxin B1					
Fluorescence	-5.32	0.80	0.628	-	-
Docking	-6.12	3.20		Gln117	Trp118, Cys120, Tyr36, Lys5, Thr4, Cys6, Phe31
post-MD docking	-4.84	0.36		His32, Gln43	Tyr36, Gly35, Ser34, Val42, Ile41
α-lactalbumin - aflatoxin G1					
Fluorescence	–5.64	1.35	0.662	-	-
Docking	-6.07	2.94		Leu105	Tyr103, Trp104, Gln54, Ala106, Thr33
post-MD docking	-5.47	1.06		Leu105, Ala106, Gln54	Glu49, Thr33, His32

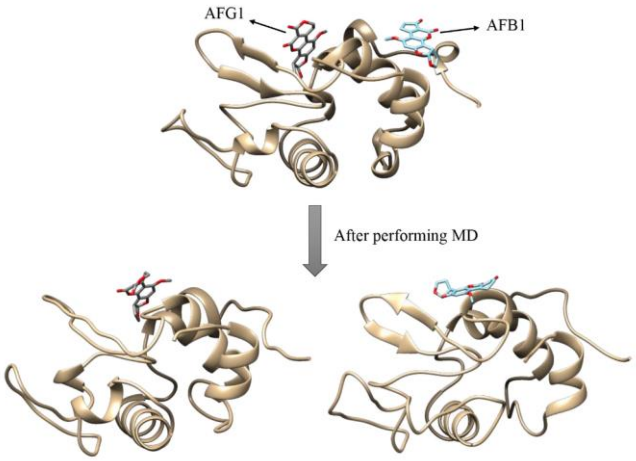

Fig. 2. The best conformation of the α-lactalbumin (ALA)- aflatoxin B1 (AFB1) and ALA- aflatoxin G1 (AFG1) complexes obtained by molecular docking before and after molecular dynamic (MD).

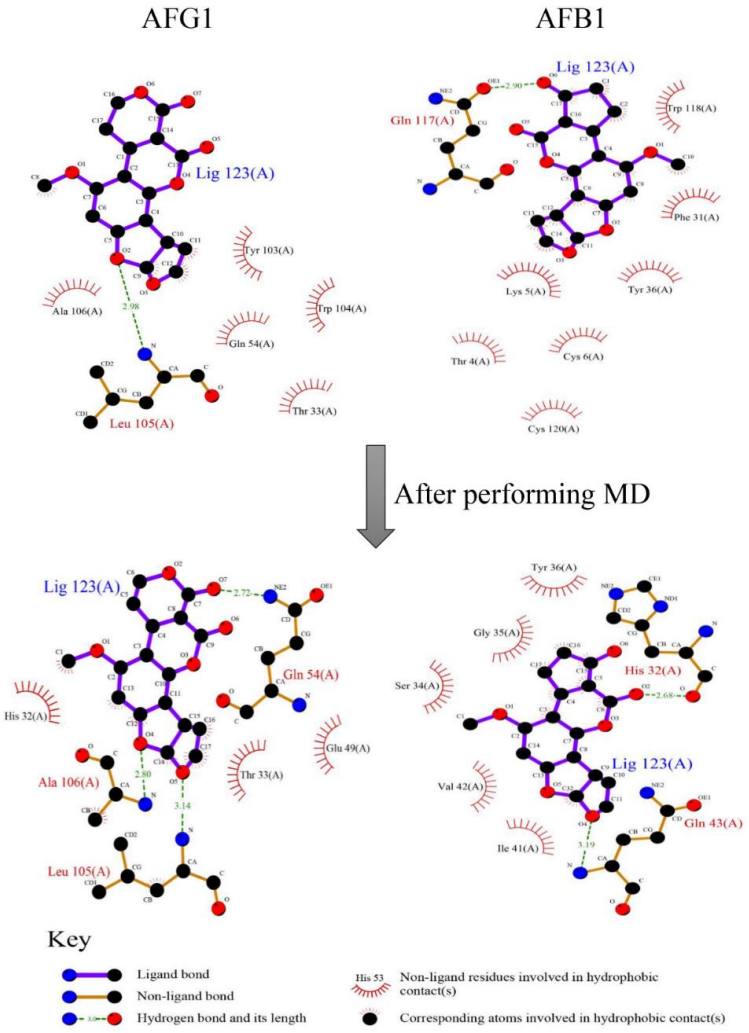

Fig. 3. Two-dimensional schematic representation of protein-ligand interaction by Ligplot Software. The binding mode of aflatoxin B1 (AFB1) and aflatoxin G1 (AFBG1) to α-lactalbumin before and after molecular dynamics simulations.

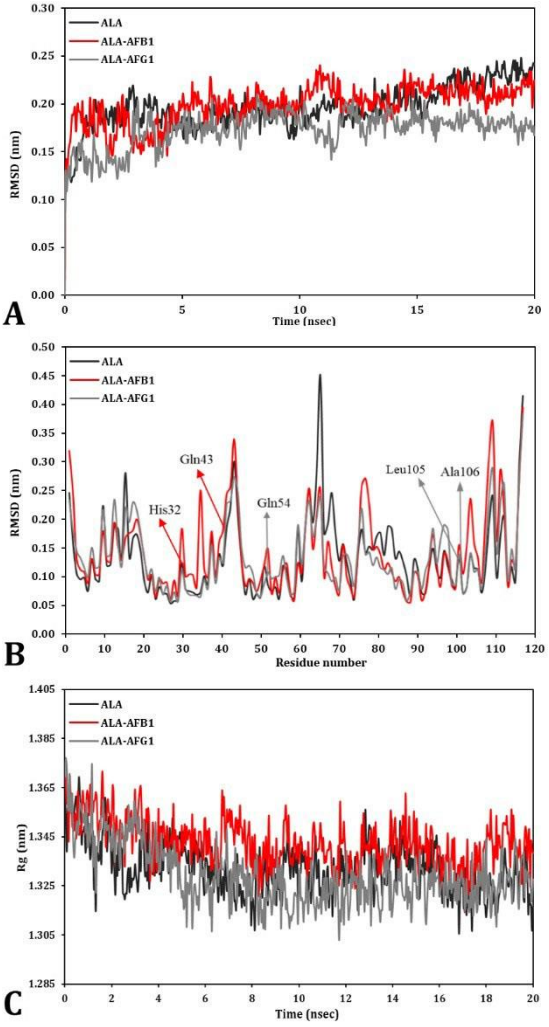

Fig. 4. A) Root mean square deviation (RMSD) of α-lactalbumin (ALA) alone and ALA-aflatoxin complexes as obtained from molecular dynamic (MD) simulation. **B)** The root means square fluctuation (RMSF) values of ALA and ALA-Aflatoxin B1 (AFB1), Aflatoxin G1 complexs plotted against residue numbers. **C)** Time dependence of the radius of gyration (Rg) for the backbone atoms of ALA during the MD simulation in the absence and presence of AFB1and AFG1.

Discussion

In the present work, the binding properties of AFB1 and AFG1 to ALA protein were examined using fluorescence spectroscopy and computational methods. The ALA fluorescence emission was reduced due to AFB1 and AFG1 binding resulting from static quenching which indicated complex formation. Molecular docking results showed that ALA-AFB1 and ALA-AFG1 complexes structure were stabilized by hydrophobic interactions and hydrogen bonds were formed through residues on the protein surface. The Ka of AFB1 and AFG1 to ALA was calculated by molecular docking and the results after performing MD simulation complemented fluorescence spectroscopy results. Results suggested that ALA could bind to AFB1, AFG1 without altering the secondary structure of ALA, however, compactness of its tertiary structure might be changed a little. The lower RMSD value of both complexes showed that the binding of AFB1 and AFG1 to the ALA decreased the degree of freedom of protein.[34] The reported Rg value of ALA explored by small-angle X-ray scattering measurements was 1.56 nm.[35] It showed that the simulation result was very close to the experimental results. However, 20 ns was used for the MD simulations because of the high computational cost and increase in MD time could generate further information about structure of complexes. Previous studies showed that AFM1 could bind to ALA through hydrophobic interactions and hydrogen bonds with the Ka of 2.12×10^3 M^{-1}.[36] It could be suggested that the interaction of AFB1 and AFG1 with ALA was one of their potential detoxification pathways and the ALA protein could play a role in mitigating aflatoxin toxicity especially when the liver did not work properly. Our results showed that binding affinity of AFG1 for ALA was higher than the AFB1, maybe because of one more hydrogen bond it forms, and binding affinity of AFB1 were higher than AFM1, maybe because of stronger hydrophobic interaction with ALA**.** It seems that the binding site of AFG1 on ALA was similar to AFM1 and located in the hydrophobic pocket between α-helix and β-sheet, however, AFB1 bound to another binding site on the α-helices. Therefore, it could be stated that different mycotoxins did not necessarily bind to the same binding site on the protein. The binding of AFB1 and AFG1 to ALA could decrease the bioavailability of the toxins in the bovine blood stream and mammary epithelial cells, reducing their harmful effects.

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Conflict of interest

The authors have no competing interests.

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