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Study on the Molecular Interaction of Anthocyanin-rich Eggplant Extracts with Bovine α-Lactalbumin

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Nasunin is the main phenol-based constituent of the eggplant peels, which is famous for its multiple advantages such as antioxidant activity. The α-lactalbumin (α-lac) is a milk protein with great nutritional importance due to its necessary amino acids' contents. Mechanism of the interaction of α-lac with nasunin was followed by the in silico and spectroscopy studies. The results of the fluorescence quenching experiments demonstrated a reduction in the fluorescence intensities due to the bonding with the fluorescence residues. The particle size of the α-lac and nasunin complex was considerably bigger than the native protein. Far ultraviolet circular dichroism outcomes indicated that the second structure of the protein was rearranged in presence of nasunin. Molecular docking pointed out that the hydrogen binding had a significant influence on the constitution of nasunin-α-lac complex. The results suggested that α-lac could be a perfect carrier for nasunin loading for additional uses in nutrition field.

Keywords: Anthocyanins, α-Lactalbumin, Nasunin, Fluorescence spectroscopy, Molecular modeling

INTRODUCTION

Polyphenols are plentiful in nature and possess various classes, *e.g.*, phenolic acids, stilbenes, lignans, and flavonoids [1]. Previous researches have shown that eating foods in high polyphenols reduces the risk of neurodegenerative and cardio-vascular illnesses, osteoporosis, and cancer [2]. Anthocyanins, a category of phenolic compounds, are the colorants of the fruits and flowers [3].

Anthocyanins are glycosides of polymethoxy and polyhydroxy derivatives of flavilium salts or 2-phenyl benzopyrylium, and they have a significant role on human health [4]. Because anthocyanins are very sensitive to the factors such as temperature, pH, light, the presence of enzymes, and oxygen, they simply degrade during storage and food preparation [5]. So, due to the low stability of anthocyanins and their interaction with other components in food, the utilization of them as health functional elements and food colorants has been restricted. To enhance the bioavailability of phenolic compounds, their complex formation with various carriers such as α-lactalbumin, β- lactalbumin, serum albumin, and whey proteins have been described. As a result, binding studies are required as a prerequisite for further understanding of ligand-protein interactions [5-9]. Eggplant (*Solanum melongena* L.) is a popular and useful foodstuff [10]. Delphinidin-3-(*p*-coumaroylrutinoside)-5-glucoside (nasunin), the main ingredient of eggplant anthocyanin colorant, has an antioxidant property that can prevent peroxidation caused by a linoleic acid-lipoxygenase system (Scheme 1) [11]. Furthermore, the inhibiting effects of nasunin on lipid peroxidation in rat brain homogenate were described [12]. Bovine α-lactalbumin as a ubiquitous calcium-binding protein, is the second significant whey protein in bovine milk. This protein has a significant role on the synthesis of lactose in the mammary gland [13]. It contains a single polypeptide chain of 123 amino acid residues with 4 disulfide bonds, which is assembled by a large α-domain

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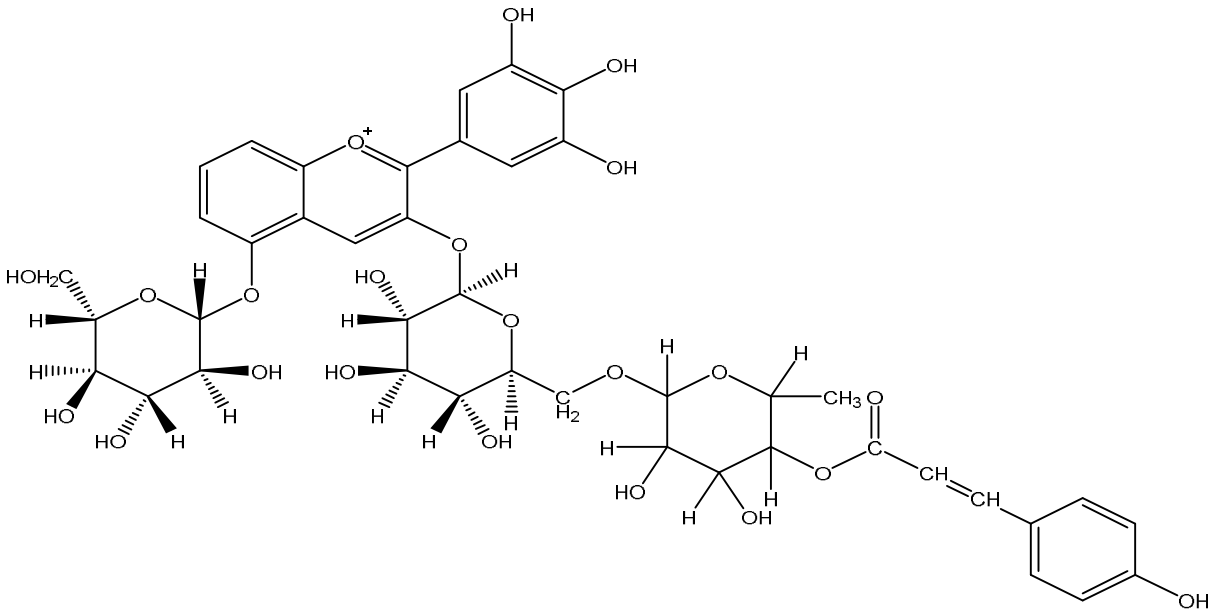

Scheme 1. Chemical structure of nasunin, delphinidin-3-(*p*-coumaroylrutinoside)-5-glucoside

and a small β-domain (14.2 kDa). The α-domain possesses 3 main α-helices and 2 short 310, and the smaller β-domain contains a series of loops, a single short 310 helix, and three-stranded antiparallel β-sheet. The one and the only strong calcium binding site, which is called the calcium binding loop, is in a profound cleft that disconnects the α and β domains. The α-lac actively binds to Ca^{2+} and is an excellent protein for use in medicinal applications. The induction of apoptosis in tumor cells is another significant and representative biologic activity of α-lac [14]. The nasunin and α-lac exist in our current diets, so it might be interesting to explore the binding mechanism of nasunin with α-lac. The interaction of nasunin extract from eggplant and bovine α-lac was investigated using fluorescence quenching experiments. Furthermore, the active sites were anticipated *via* the molecular docking approach. The purpose of our study is to develop novel functional composites for food applications.

MATERIALS AND METHODS

Materials

α-Lac from bovine milk was bought from Sigma Aldrich (St. Louis, MO, USA). Nasunin was extracted as described by Kuroda and Wada [15,16]. Other chemicals used were analytical in purity.

Methods

Fluorescence spectroscopy. All the fluorescence spectra were registered on a FP-6200 spectrofluorometer (Jasco, Japan).

An excitation wavelength of 280 nm was utilized, and emission spectra were registered in the range of 300 nm to 450 nm with 1.0 nm incrementations in all cases. The excitation and emission slits widths were fixed to five nanometers. The α-lac protein solution (10 μM) was prepared by dissolving in buffer phosphate solution (20 mM) comprising of EDTA (3.5 mM) eliminating calcium (apo state of protein) and was titrated with various ratios of nasunin solution (the molar ratio of 0-10-fold of nasunin in respect to α-lac). Fluorescence spectroscopy measuring was accomplished after incubating for 3 min at RT. The fluorescence quenching data (Binding constants, Stern-Volmer constants, number of binding sites) were evaluated by the Stern-Volmer equation.

UV-Vis absorption spectroscopy. The UV-Vis spectrophotometer (UV-1800, Shimadzu, Japan) was used to scan the UV absorption of α-lac (10 μM) at different

molar ratios of 0-10 folds of nasunin in respect to α-lac at a wavelength range from 200 to 400 nm and at RT. Concentration of α-lac solution was specified by ultraviolet absorbance investigation using recognized molar absorption coefficient value of 28, 540 M^{-1} cm^{-1} at 280 nm [13].

Circular dichroism (CD) spectroscopy. 500 μl of α-lac (5 μM) in phosphate buffer at pH = 7.4 was titrated with nasunin at various molar portions of 0, 1, and 10 folds of nasunin related to α-lac, and incubated for 3 min at RT. Then, induced conformational changes of nasunin were measured at the RT using far ultraviolet-circular dichroism in the J-815 automatic recording spectropolarimeter (Jasco, Tokyo, Japan).

Dynamic Light Scattering (DLS)

Size and size distribution of the complexes formed by the adding of the nasunin stock solution to the protein solution (10 mM) at different ratios in phosphate buffer and at 25°C were measured by a Zetasizer (Malvern Instruments Inc., Malvern, U.K.).

Molecular Docking and Molecular Dynamic Simulation Studies

Molecular docking investigations of the interaction between nasunin and α-lac were carried out using AutoDockTools-1.5.6rc3. The PDB format of α-lactalbumin was downloaded from the Protein Data Bank (ID: 1F6S).

The structure of nasunin was built up and the energy was optimized by Chem3D Ultra 8.0. To determine the preferable binding sites, the nasunin molecule was allowed to move overall the whole regions of α-lac with 100 runs to obtain all the probable binding sites, and the binding mode of the nasunin-α-lac complex with the lowest energy value was represented. The distance between bound nasunin and protein residues were estimated using PyMol [17]. LigPlot+ (v.2.2.4) was used to create the 2D representations of α-lac-nasunin complexes using PDB input file [18]. Molecular dynamic simulation was carried out by using GROMACS software package version 5.1.2 with the GROMOS 43A1 force field and the TIP3P water model [19-21]. The topological pattern of nasunin was created by the PRODRG server [22]. The complex was centralized in a three-dimensional box solvated with water molecules; the final charge of the system was balanced by adding of enough ions with opposite charges. Thereafter, the system was equilibrated at 300 K and 1 bar pressure. The simulating process was run for 50 ns. Electrostatic interactions were characterized by means of the particle-mesh Ewald procedure [23]. We applied the LINCS algorithm to constrain all the bonds [24]. Dispersion interactions were considered using a Lennard-Jones potential with a 1 nm cut-off.

RESULTS AND DISCUSSION

Fluorescence Quenching Mechanism

To decrease the inner filter effect, absorption spectra of α-lac at 280 nm (excitation wavelength) were measured, and the fluorescence intensities used in this research were all corrected using the equation [25]:

$$F_c = F * anti\log\left\{\frac{A_{ex} + A_{em}}{2}\right\} \quad (1)$$

where F and F_c are the initial and corrected intensities of fluorescence, respectively; A_{ex} is the absorption of the ligand at the excitation wavelength, and A_{em} is the absorption of the nasunin at the emission wavelength.

Because of the attendance of the four tryptophan residues (Trp-118, 60, 104, and 26), α-lac is an intrinsically fluorescent protein which leads to the maximum emission at 333 nm at an excitation wavelength of 280 nm. The effect of an increase in concentration of nasunin on the fluorescence intensity spectra was tracked, and the interactions between α-lac and nasunin were measured to obtain new sights with affinity of forming bind between the two components. In Fig. 1, the fluorescence of the α-lac was remarkably quenched with an increase in the concentration of the ligand.

This result suggested that nasunin interacted/bound with α-lac caused the fluorescence quenching of protein. The addition of nasunin to α-lac led to a blue shift of 12 nm in the maximal spot (λ_{max}). These findings indicate that interactions between α-lac and nasunin cause alterations in the polarity of the environment around Trp residues of the protein [26]. Moreover, the considerable spectral blue shift indicates that Trp residues in the protein are more subjected to the hydrophobic solvent due to the interaction with

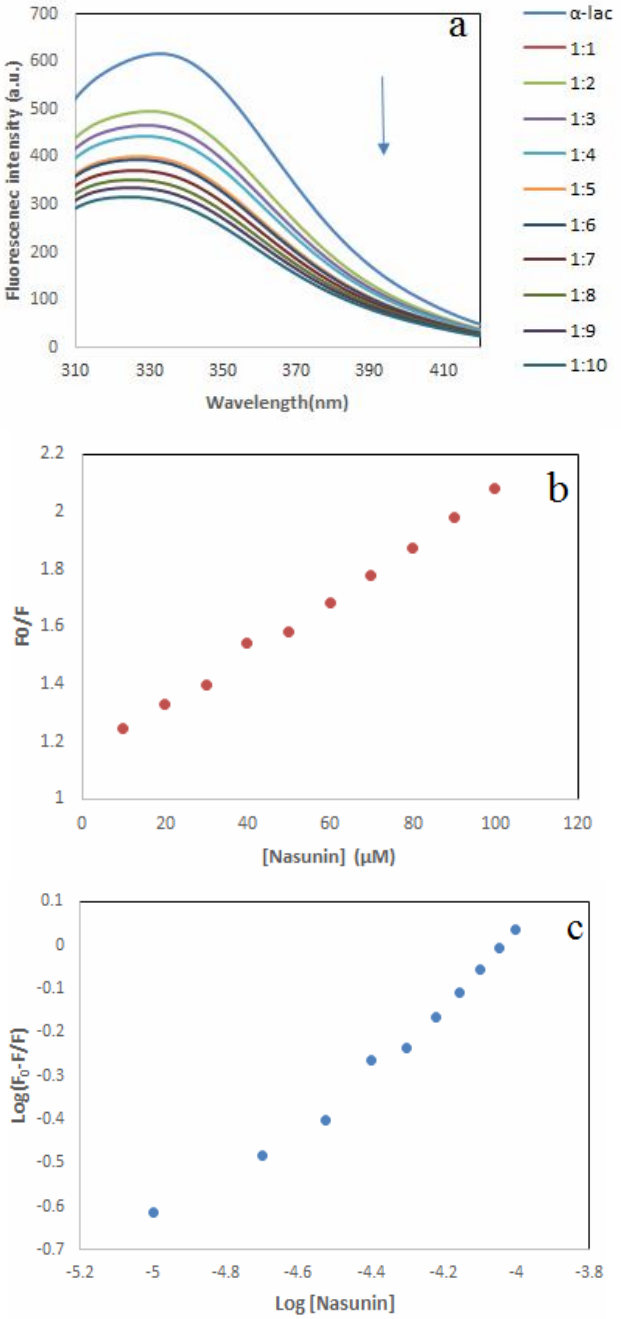

Fig. 1. (a) Fluorescence emission spectra of α-lac-nasunin complex at 280 nm, pH = 7.4, and temperature of 298 K, and the downward arrow indicates the increase of nasunin level. (b) The Stern-Volmer plot and (c) Modified Stern-Volmer plot for quenching fluorescence intensity of α-lac (10 μM) with nasunin at T = 298 K.

nasunin [27]. Furthermore, the Trp fluorescence of α-lac was essentially motivated by the emission from Trp-104, as the signals from Trp-60 and Trp-118 were noticeably extinguished by the S–S bonds in their proximity; hence, the extinguishing operation was probably the consequence of nasunin linkage in the residue of Trp-104 [28]. The cleft area of α-lac separating the β-sheet and α-helical areas counted Trp-60 and Trp-104, which emphasized the possible bonding of nasunin to the cleft region of α-lac.

The fluorescence quenching of a protein by small molecules can be static and/or dynamic in nature. To better clarify the quenching mechanisms between α-lac and nasunin, the classical Stern-Volmer equation Eq. (2) was employed [29]:

$$\frac{F_0}{F} = 1 + K_q \tau_0 [nasunin] = 1 + K_{sv} [nasunin] \quad (2)$$

where F_0 and F display relative fluorescence units without and with nasunin, respectively; kq is the extinguishing rate constant for the α-lac, K_{SV} is the Stern-Volmer dynamic quenching constant, and τ_0 is the mean lifespan of the fluorophore in the absence of nasunin (τ_0 = 2.6 ns). As it is clear, the process of fluorescence quenching can proceed *via* two mechanisms: dynamic (collisional, diffusion-limited) and static (diffusion-independent). The Stern-Volmer plots, which are linear within a certain concentration, may either expose the presence of a single type of quenching, or show the occurrence of only a single binding site for quencher in the fluorophore neighborhood [30]. Figure 1b represents the Stern-Volmer plot of α-lac fluorescence intensities at 333 nm (the peak maximum) quenched by nasunin. The curve is linear, suggesting the existence of a single type of quenching (dynamic or static) and/or a single binding site for nasunin in the α-lac neighborhood. If kq is smaller than 2×10^{10}, the interacting process is dynamic and if that larger than 2×10^{10}, the interacting process is static. According to the Fig. 1b, Ksv is equal to 0.93×10^4 M^{-1} and consequently, kq will be 3.57×10^{13} l s^{-1} mol^{-1} which indicates the static interacting process ($k_q > 2 \times 10^{10}$ l s^{-1} mol^{-1}). This demonstrates that nasunin molecule can be bound to α-lac, resulting in the fluorescence quenching of the proteins tryptophan residues [30].

Also, the number of binding sites (*n*) and binding constant

(Ka) were obtained using improved version of the Stern-Volmer equation, Eq. (3) [31]:

$$\log \frac{(F_0 - F)}{F} = \log K_a + n \log[Q] \quad (3)$$

Accordingly, n and k_a are 0.66 and 4.48 × 10^4 M^{-1}, respectively. Furthermore, at 25 °C, the ΔG° of the interaction between nasunin and α-lac (ΔG° = -RTlnk_a) was -6.317 kcal mol^{-1}, indicating that the binding process was spontaneous.

UV-Vis Absorption Spectroscopy

UV-Vis absorption analysis is a useful technique to study the structural changes of proteins as a result of complex formation [32,33]. The absorption spectra of α-lac with and without increasing concentrations of nasunin are shown in Fig. 2. An increase in the absorbance peak and a slight blue shift in the presence of nasunin indicates that binding of nasunin let the Trp, Tyr and Phe residues to be more achievable, and hence, interaction of nasunin with α-lac causes the conformational alterations in the α-lac [34].

Circular Dichroism (CD) Spectroscopy

To investigate alterations in the secondary construction of α-lac upon interaction with nasunin, far UV CD was utilized at various concentrations. The outcomes are shown in Fig. 3 and Table 1.

The structure of the α-lac consists of α-helix as well as β-sheet structures, as analyzed by CDSD and CDNN software.

As stated in Table 1, in the presence of nasunin ([α-lac]/[nasunin]→1:10), the percent of α-helix construction in α-lac reduced from 36.2% to 28.6%, whereas the β-sheet portion enhanced from 22.1% to 27.5%, and β-turn

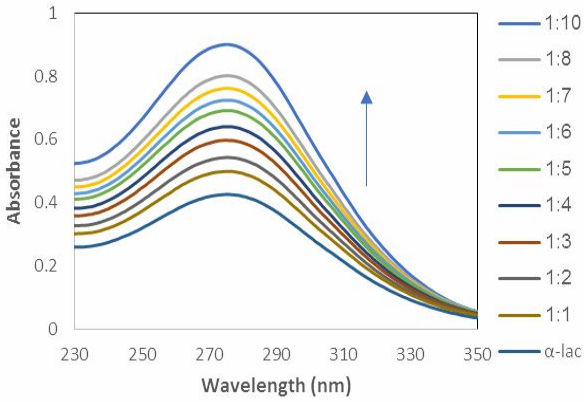

Fig. 2. Evolution of the α-lac UV-Vis absorption spectra when increasing the concentration of nasunin at pH = 7.4 and T = 298 K. The upward arrow indicates an increase in the nasunin concentration.

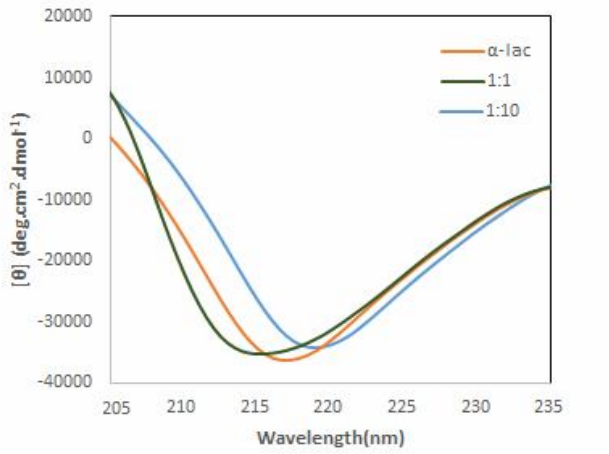

Fig. 3. Far-UV-CD spectra of α-lac and α-lac-nasunin at two different ratio concentrations; at pH = 7.4 and T = 298 K.

Table 1. The Secondary Structure Composition for Alone α- Lac, 1:5, and 1:10 Ratios of α-lac: Nasunin Complexes at T = 298 K and pH = 7.4

Complex	α-helix (%)	β-sheet (%)	β-turn (%)	Random coil (%)
α- Lac	36.2	22.1	14.8	26.9
([α-lac]/[nasunin]→1:5	30.4	26.2	15.1	28.3
([α-lac]/[nasunin]→1:10	28.6	27.5	16.2	27.7

constructions increased from 14.8% to 16.2%. Also, the amount of random coil constructions showed a little change. These findings indicate that the nasunin binding changes the secondary structures of protein.

Dynamic Light Scattering (DLS)

Zeta potential is a significant method for anticipating the steadiness of protein's systems by estimating the superficial charge. Also, the protein size provides us with beneficial data for studying the denaturation of the protein, the aggregations, and conformation conditions [35]. The complete data are briefly shown in Table 2.

DLS consequences showed that the sizes of the α-lac in the absence of nasunin and its complex with nasunin at a molar ratio of 1:1 were 156 nm and 234 nm, respectively. According to the results in Table 2, the size of the complex was increased by adding nasunin to the protein. Nevertheless, at greater concentrations of nasunin, *viz*. 1:5 and 1:10 (α-lac:nasunin mole ratio), the size was less than the 1:1 molar ratio, which was 85.43 nm and 153.98 nm. This result could be attributed to the falling of α-lac upon linking to the greater ratios of nasunin. The conformational changes may be able to explain the reduction in the size by displaying a concentration-dependent mode [36].

The findings of Delavari *et al*., Abbasi *et al*., and Katouzian *et al.* are also in agreement with the current results [37-39]. As reported by them, this alteration is ascribed to both the protein interactions and the adjustment of the ligand in the protein's hydrophobic and hydrophilic sites, which leads to the formation of bigger α-lac complexes [40].

Molecular Docking Analysis

Acording to the result of the docking modeling, the governing interaction was hydrophobic, apart from the hydrogen bonds (Fig. 4). The α-lac-nasunin complex was principally stabilized by hydrophobic interactions with amino acid residues, such as Ser34, Ala106, His32, Leu10, Phe31, Gly35, Ala40, Val42, Glu49, Gln54, Thr33, Val99, Trp104, and Tyr103. Furthermore, the amidic moieties of Leu105 and Asp37 are accountable for the constitution of hydrogenic bonds with the three hydroxyl moieties of nasunin. The decrement in the fluorescence intensiveness of α-lac could be described by the hydrophobic interactions

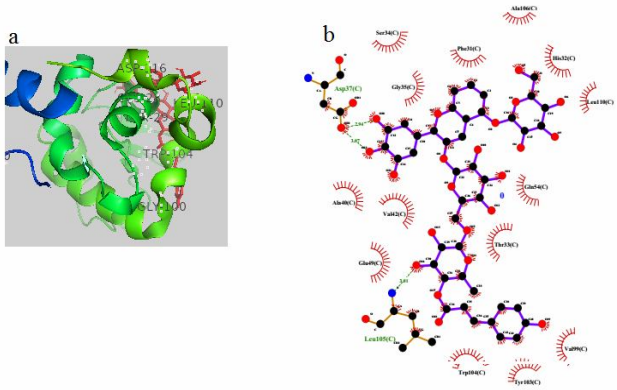

Fig. 4. (a) The molecular docking analysis for the binding of nasunin molecule to the α-lac, (b) LigPlot^{+} diagram of the interaction between α-lac and nasunin. The hydrogen bonds are shown by the dashed green line between Asp37, Leu105, and nasunin.

Table 2. Size Distribution and Polydispersity Index (PDI) for Alone α- Lac, 1:1, 1:5, and 1:10 Ratios of α- Lac:nasunin Complexes; T = 298 K and pH = 7.4

Complex	Size (nm)	PDI[a]
α- Lac	85.4 ± 3.1	0.124 ± 0.011
([α-lac]/[nasunin]→1:1	153.9 ± 2.5	0.088 ± 0.006
([α-lac]/[nasunin]→1:5	124.7 ± 7.1	0.075 ± 0.022
([α-lac]/[nasunin]→1:10	116.3 ± 3.6	0.068 ± 0.038

[a]PDI = Polysidpersity index.

with the Trp104, Phe31, and Tyr103 residues, which are instinctively fluorescent. α-Lac includes 2 hydrophobic pieces: 1) it is composed of His32, Phe31, Trp118, and Gln117 from helix 2 and the 310 helix known as the first aromatic cluster and 2) aromatic cluster named the hydrophobic pocket comprising of Trp104, Trp60, and Trp26 residues [41]. Nasunin was incorporated into the aromatic cluster II and the interaction was motivated by hydrogenic bonds.

Molecular Dynamic Simulation Analysis

The stability of the α-lac-nasunin complex was studied

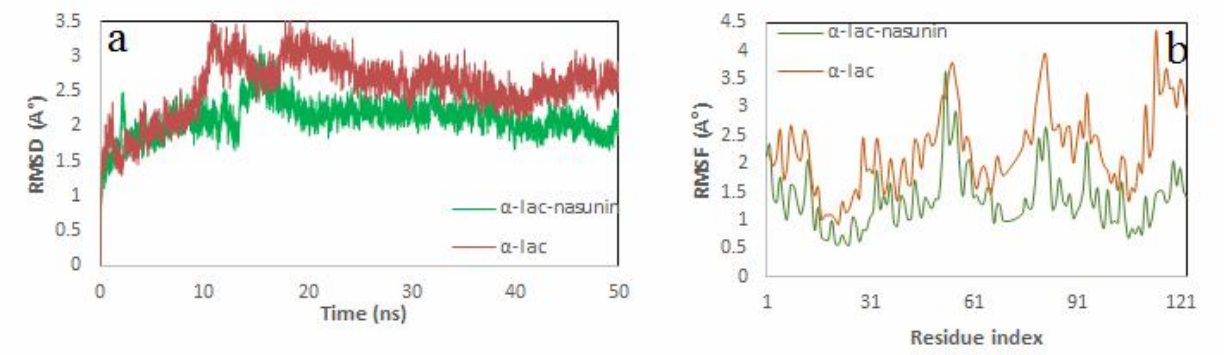

Fig. 5. Characterization of the α-lac-nasunin interaction parameters as a function of molecular dynamics simulation time. (a) RMSD of the α-lac C_a-atoms without and with nasunin; (b) RMSF deviation of the α-lac without and with nasunin.

by performing a molecular dynamics simulation for 50 ns at 300 K. The RMSD of a protein presents information on the conformation of the protein for the total period of simulation. The RMSDs of the α-lac Cα-atoms in the presence and absence of nasunin are shown in Fig. 5a. The RMSD of the α-lac in the presence of nasunin indicates that it was stabilized after around 20 ns. The RMSD values revealed that the nasunin bound to the active site of the α-lac and the α-lac-nasunin complex was stable. The conformational changes of the α-lac chain were explored by analyzing the root mean square fluctuation (RMSF) with respect to the simulation time (Fig. 5b) [42]. The loop areas fluctuated the most throughout simulation time, but α-helices and β-sheets were inflexible.

CONCLUSIONS

Interactions between α- lac and nasunin were studied by both the experiment and computational methods. Fluorescence results demonstrated that nasunin quenched the intrinsic fluorescence of α-lac. A reduction in the fluorescence intensity was possibly associated with the conformational alterations of α-lac, and the substantial fluorescent residue was Trp104. Secondary structure changes were studied by the far UV circular dichroism spectroscopy. The outcomes revealed that the linking of nasunin to the α-lac caused a decrease in the percentage of helix. Molecular dynamics simulation and molecular docking results confirmed that nasunin binds to theα-lac, and the hydrophobic interaction involved in the stabilization of the α-lac-nasunin complex. The results of this work give new insight into the interactions between α-lac and nasunin, which might be useful in promoting the usage of nasunin as a functional and natural colorant in the food manufacturing.

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DECLARATION OF COMPETING INTEREST

All authors declare that there is no conflict of interest.

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