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Study of the Interaction of Cinnamaldehyde with Alpha-lactalbumin: Spectroscopic and Molecular Docking Investigation

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ABSTRACT

Cinnamaldehyde is an important compound of the cinnamon essential oil, and it is responsible for the most of the health benefits of cinnamon. Enriching foods such as milk with cinnamaldehyde can lead to greater utilization of cinnamaldehyde properties. In this study, we investigated the interaction of cinnamaldehyde with bovine alpha-lactalbumin. Analyzing the spectrum of alpha-lactalbumin in the presence of different concentrations of cinnamaldehyde by ultraviolet-visible, fluorescence and circular dichroism spectroscopy showed that cinnamaldehyde is capable to bind to alpha-lactalbumin without changing its secondary structure. Conformational change in alpha-lactalbumin induced by interaction with cinnamaldehyde and the number of binding sites and binding constant were 1.1 and 5.88×10^5 M^{-1}, respectively. The molecular docking results showed one binding site which most of its interactions are hydrophobic. The ability of cinnamaldehyde to bind to alpha-lactalbumin suggests that alpha-lactalbumin can be used as a suitable vehicle for cinnamaldehyde especially for fortification of milk.

Keywords: Cinnamaldehyde, Alpha-Lactalbumin, Fluorescence spectroscopy, Circular Dichroism spectroscopy

INTRODUCTION

Medicinal plants have many beneficial compounds, while they usually don't show side effects of chemical drugs. Currently, 20 percent of drugs in advanced industrial countries and 80 percent of drugs in developing countries are attributed to medicinal plants. Use of medicinal plants in Iran, like most developing countries is common and has been increased during last decades. Cinnamon is an herb used in traditional medicine as an analgesic [1]. Some scientific studies have shown anti-viral effect of cinnamon, especially it has been confirmed that it is effective in patients with acquired immunodeficiency HIV (Human Immunodeficiency Virus) [2,3]. It has been reported that some components of cinnamon extract have a prevention properties against Alzheimer's disease in the mouse model [4]. Many *in vitro* and *in vivo* studies around the world have shown the beneficial health effects of cinnamon, for example anti-inflammatory, antimicrobial and anticancer activities [5].

Cinnamaldehyde, the major fraction of cinnamon bark oil, is a known natural antioxidant [6] that has antimicrobial activity against foodborne pathogens [7]. It is widely used as a flavoring component in foods and drinks such as beverage, ice cream, sweet and chewing gum. As shown in Fig. 1 trans-cinnamaldehyde molecule has a phenyl group attached to the unsaturated aldehyde. Thus, the molecule can be considered as a derivative of acrolein diethyl acetal [8]. Several biological activities such as peripheral vasodilatation, antitumor, antifungal, cytotoxic and anti-mutagenic effects have been attributed to cinnamaldehyde [9]. It has been observed that cinnamaldehyde has interaction with proteins. Although it is not clear that cinnamaldehyde react with which proteins in the *in vivo* condition, investigation in the *in vitro* condition showed that cinnamaldehyde binds to sulfur atoms of cysteine residues of bovine serum albumin [10].

Milk contains two major groups of proteins, caseins and milk serum proteins [11]. Milk serum proteins are a mixture of globular proteins that are known as whey proteins. The

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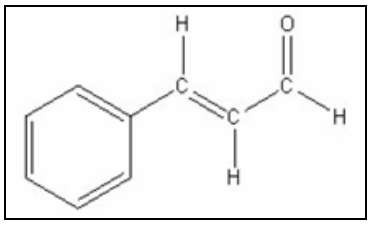

Fig. 1. Molecular structure of *trans*-cinnamaldehyde C_9H_8O.

serum proteins make up about 10% of total milk dry weight. These proteins are normally a mixture of beta lactoglobulin, alpha-lactalbumin, serum albumin, lactoferrin, immunoglobulin, lacto peroxidase enzyme and glycomacropeptides [12,13]. Milk serum proteins are marketed as dietary supplements [14]. Studies have shown that milk serum proteins and its components may reduce the risk of cancer in animals [15-17].

Extensive researches have done on the use of milk serum proteins as a source of amino acids and their effect on reducing the risk of heart disease, cancer, diabetes [18] and their anti-hypertensive [19], hypocholesterolemic, anti-microbial and opioidergic effects [16]. Milk serum proteins contain high concentration of leucine, isoleucine and valine amino acids which are used to arouse synthesis of protein [20]. In general, the milk serum proteins has higher concentration of all the essential amino acids rather than the vegetable protein sources such as soy and corn [13].

Alpha-lactalbumin is a protein presents in milk of almost all species of mammals [21]. Alpha-lactalbumin is one of the main proteins in the mother milk. It constitutes about 20-25% of maternal milk serum protein and 22% of bovine milk serum protein and as an available source of essential amino acids contains a wide range of amino acids [22]. Bovine alpha-lactalbumin amino acids have a high similarity to human alpha-lactalbumin amino acids [16]. Alpha-lactalbumin is known as one of the great sources of natural antioxidative peptides [23]. The expression of alpha-lactalbumin in primates is upregulated in response to the prolactin hormone and it contributes to the synthesis of lactose [24]. Alpha-lactalbumin is a milk serum protein that can interact with hydrophobic ligands [25] and can be used as a carrier for hydrophobic vitamins such as vitamin D [26]. The interaction between vitamin D and apo alpha-lactalbumin has been investigated and confirmed the competency of alpha-lactalbumin as a vehicle for carrying vitamin D [27]. Casein and beta lactoglobulin are other milk proteins that have been proposed as a carrier for vitamin D [26]. A study on the cheddar cheese enriched with vitamin D has shown that whey protein compared to casein, lose less vitamin D [25], so the milk serum proteins may be a better carrier for vitamin D. In spite of the high concentration of beta lactoglobulin in the milk serum it is allergen, therefore it can be said that alpha-lactalbumin the second abundant protein in the milk serum, is the best option for carrying vitamin D and enrichment of foods [28,29]. The complex of partially folded form of human alpha-lactalbumin and oleic acid is called HAMLET (Human Alpha-lactalbumin Made Lethal to Tumor cells). HAMLET, reduces apoptosis in the tumor cells and immature cells [30].

Alpha-lactalbumin is a very valuable milk serum protein in terms of nutrition. This broadly accessible protein is considerable due to its ability to bind to different ligands, enzyme activity and anti-cancer activity. Furthermore, cinnamaldehyde, widely used food flavour, has many health benefits. Cinnamaldehyde has not been used to enrich milk yet, and many medicinal properties attributed to it. This study carried out on the interaction of cinnamaldehyde and alpha-lactalbumin, to investigate the use of alpha-lactalbumin as a natural vehicle for cinnamaldehyde in foods. Alpha-lactalbumin and cinnamaldehyde complex can be used for food enrichment. So far, the possibility of interaction between alpha-lactalbumin and cinnamaldehyde is not reported. The purpose of this study was to investigate possibility of the interaction between alpha-lactalbumin and cinnamaldehyde and the effect of cinnamaldehyde binding on the conformation of alpha-lactalbumin using UV spectroscopy (Ultraviolet spectroscopy), fluorescence spectroscopy and circular dichroism techniques. The interaction and the probable binding site also were computed by molecular docking.

MATERIALS AND METHODS

Material

Alpha-lactalbumin from bovine milk (L5385) was purchased from Sigma-Aldrich. Tris (hydroxymethyl)

amino methane buffer (108382), Ethanol (100971) and *Trans*-cinnamaldehyde (802505) were obtained from Merck.

Ultraviolet Spectroscopy

To study the interaction of cinnamaldehyde with alpha-lactalbumin, changes in absorption of alpha-lactalbumin in the absence and presence of various concentrations of cinnamaldehyde was determined using an Annalytik Jana (Germany) UV-Vis spectrophotometer at room temperature and the wavelength range of 230-330 nm. The concentration of stock solution of alpha-lactalbumin was measured by its optical density at 280 nm, using molar extinction coefficient of 28540 M^{-1} cm^{-1} [31]. Alpha-lactalbumin 10 µM (in Tris buffer 100 mM, pH 7.5) was titrated with small volumes of cinnamaldehyde stock solution (5 mM) in ethanol, so that the molar ratios of cinnamaldehyde to alpha-lactalbumin were 0.5, 1, 2, 3 and 4. Before each measurement, the mixture of alpha-lactalbumin and cinnamaldehyde was incubated for 3 minutes.

Fluorescence Spectroscopy

Intrinsic fluorescence intensity of alpha-lactalbumin was measured in the absence and in the presence of various concentrations of cinnamaldehyde at room temperature by Carry Eclipse (Varian, Australia) spectrofluorometer using a 500 microliter quartz cuvette with 1 cm light path. The excitation wavelength was set at 280 nm and the emission spectrum in the wavelength range of 295-410 nm was investigated. Both excitation and emission slits were 5 nm. 400 µl of alpha-lactalbumin (10 µM) dissolved in Tris buffer pH 7.5 was titrated with small aliquots of cinnamaldehyde stock solution (2.5 mM) in ethanol, so that the molar ratios of cinnamaldehyde to alpha-lactalbumin were 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5 and 3. The mixture of alpha-lactalbumin with cinnamaldehyde was incubated for 3 min before fluorescence intensity measurement.

Circular Dichroism Spectroscopy (CD)

500 µl of alpha-lactalbumin (5 µM) in Tris buffer at pH 7.5 was incubated for 3 min with different concentrations of cinnamaldehyde and then measurements were made in the far ultraviolet region (195 to 260 nm) by Aviv 215 Spectropolarimeter (Lakewood, New Jersey, USA). The molar ratios of cinnamaldehyde to protein were 0, 1, 2, 3 and 5 and a cuvette of 0.1 cm path length was used.

The buffer spectrum was subtracted of each spectrum and results converted to molar ellipticity by Aviv software. By using the molar ellipticity in the wavelength range of 195-260 nm and CDNN2.1 software, percentage of the elements of secondary structure in the protein structure was determined in each experiment [32].

The limitation of this study was the use of ethanol as a solvent for cinnamaldehyde, because cinnamaldehyde has a low solubility in water. In all experiments, the ethanol concentration at the end of titration was less than 1% (v/v) in the sample cell. Ethanol less than 2% (v/v) does not affect alpha-lactalbumin structure [33], so we can say that the observed changes were due to the cinnamaldehyde binding to alpha-lactalbumin not the ethanol present in the sample.

Molecular Docking

Molecular Docking calculations were performed using AutoDock Vina [34]. Alpha-lactalbumin crystal structure was obtained from the RCSB Protein Data Bank (http://www.rcsb.org), PDB ID 1hfz [35] and the ligand-free structure (chain A) was used as initial protein structure. The cinnamaldehyde structure retrieved from Zinc database and optimized by HyperChem 8.0.6 using amber force filed [36]. AutoDock Tools 4 [37] was applied for preparing protein files such as hydrogen atoms addition, Gasteiger charges calculation and set up of rotatable bonds of ligand. Grid box of 30 × 35 × 48 point numbers and 1 Å spacing were used to cover the whole protein structure. The docking results were analyzed and visualized using AutoDock Tools 4, LigPlot+ v.1.4.5 program [38], and VMD package [39].

RESULTS

Ultraviolet Spectroscopy

Increased alpha-lactalbumin absorption in the presence of cinnamaldehyde (Fig. 2) shows that binding of cinnamaldehyde causes the tryptophan, tyrosine and phenylalanine residues become more accessible and therefore interaction of cinnamaldehyde with alpha-lactalbumin causes conformational changes in the protein.

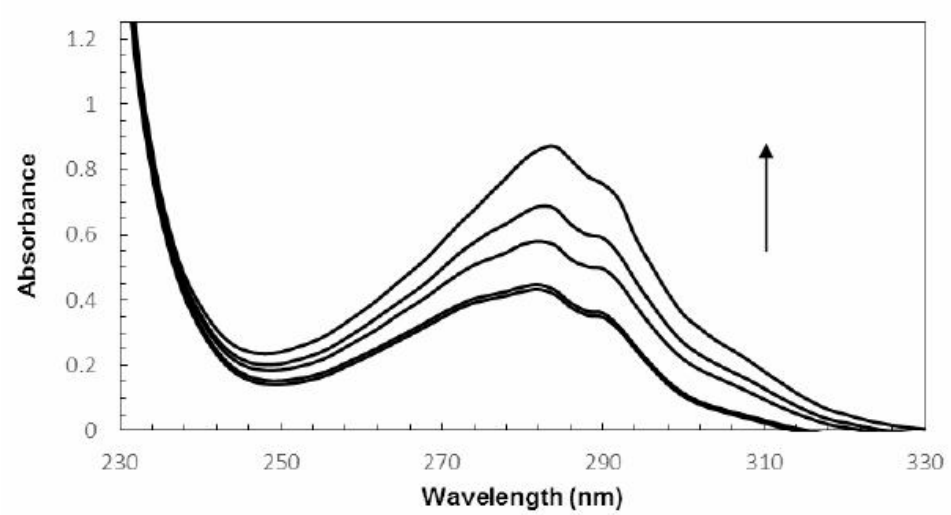

Fig. 2. Effect of increasing of the concentration of cinnamaldehyde on absorption spectrum of alpha-lactalbumin (5 μM) in Tris buffer, pH 7.5 at 25 °C. The lowest curve is alpha-lactalbumin in the absence of cinnamaldehyde and the arrow shows the direction of increased concentration of cinnamaldehyde. The molar ratio of cinnamaldehyde to alpha-lactalbumin was 0.5, 1, 2, 3 and 4 respectively.

Fluorescence Spectroscopy

As the intrinsic fluorescence emission is highly sensitive to the polarity of surrounding environment of fluorophores, cinnamaldehyde concentration dependent conformational changes of alpha-lactalbumin were followed using fluorescence spectroscopy. Intrinsic fluorophores in proteins include tryptophan, tyrosine and phenylalanine residues, which have hydrophobic nature and usually are buried inside the globular proteins. The intrinsic fluorescence emission spectrum of alpha-lactalbumin in Tris buffer at pH 7.5 and excitation wavelength of 280 nm was measured. As seen in Fig. 3a, the wavelength of maximum emission of the alpha-lactalbumin is 326 nm. In the presence of cinnamaldehyde with molar ratios of 0, 0.25, 0.5, 0.75, 1, 1.5, 2 2.5 and 3 emission intensity was quenched. The fluorescence intensity reduction is because of exposure of fluorophores of the protein such as tryptophan residues to the solvent and conformational changes. Although wavelength of maximum emission did not shift considerably (only about 5 nm red shift), so we can say that there is no remarkable unfolding observed in this range of cinnamaldehyde concentration. Figure 3b shows the maximum emission in the presence of various molar ratios of cinnamaldehyde to alpha-lactalbumin.

Intrinsic fluorescence quenching can provide information about the mechanism of protein-ligand binding. In general three types of quenching mechanism are dynamic, static and both static and dynamic at the same time. The amount of quenching determined by Stern Volmer equation [40-42]:

$$F_0/F = 1 + K_{sv}[Cin.] = 1 + k_q \tau [Cin.] \quad (1)$$

Where F_0 and F are fluorescence intensities in the absence and presence of quencher, respectively. K_{sv} is the Steme-Volmer quenching constant. k_q is the biomolecular quenching constant and τ is the fluorescence average lifetime of the fluorophore in the absence of quencher, that has been reported to be 2.6 ns [27].

According to Stern Volmer plot (Fig. 4), the K_{sv} is equal 1.15×10^5 M^{-1} and k_q is 4.42×10^{13}. Previous studies have shown that if k_q is greater than 2×10^{10} mol per second the quenching is static and if it is smaller than 2×10^{10} mol per second the quenching is dynamic [41]. The quenching is static, so reducing of fluorescence intensity was observed due to binding of cinnamaldehyde to alpha-lactalbumin.

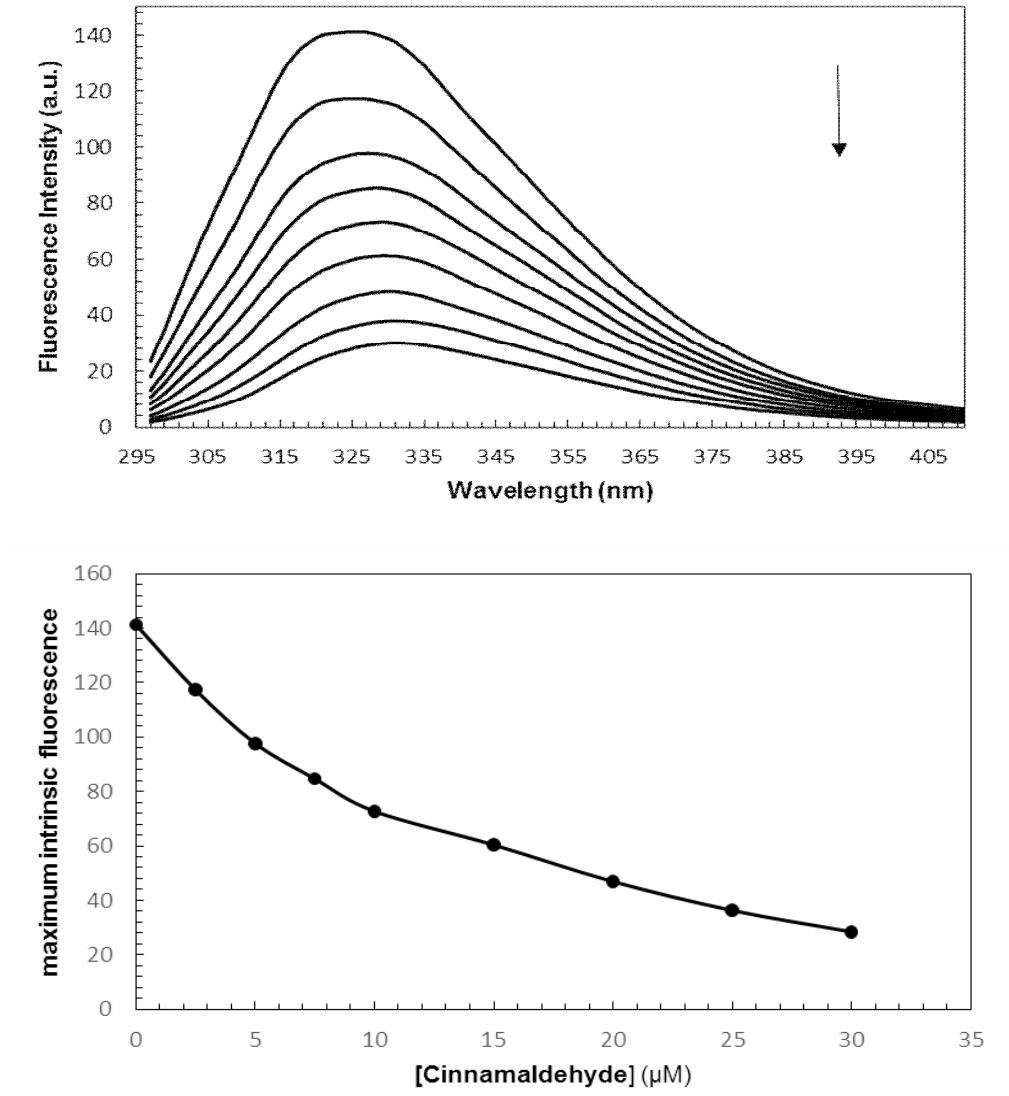

Fig. 3. Intrinsic fluorescence emission spectrum of alpha-lactalbumin in Tris buffer, pH 7.5 in the different concentration of cinnamaldehyde with molar ratios of 0, 0.25, 0.5, 0.75, 1, 1.5, 2 2.5 and 3, the arrow shows the direction of increased concentration of cinnamaldehyde. The excitation wavelength was 280 nm (a). Changes of maximum emission of alpha-lactalbumin in the presence of above mentioned molar ratios of cinnamaldehyde (b).

$$\log[(F_0 - F)/F] = \log K_a + n\log[Q] \quad (2)$$

Analysis of the fluorescence quenching data using the Eq. (2) [27,41,42] showed that the number of binding sites (n) is equal to 1.1 and binding constant (K) is $5.88 \times 10^5\ M^{-1}$ (Fig. 5). So we can say almost one cinnamaldehyde molecule binds to each alpha-lactalbumin molecule.

Circular Dichroism Spectroscopy

Secondary structure of proteins and its changes can be investigated by far UV circular dichroism spectroscopy experiments. Alpha-lactalbumin circular dichroism

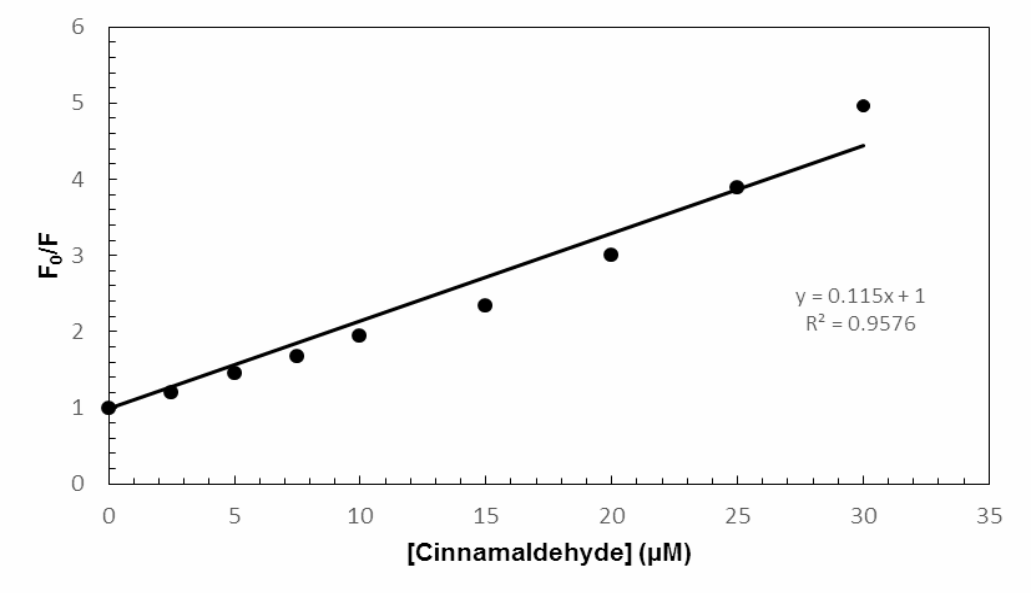

Fig. 4. Stern Volmer plot for quenching of fluorescence intensity of alpha-lactalbumin with cinnamaldehyde.

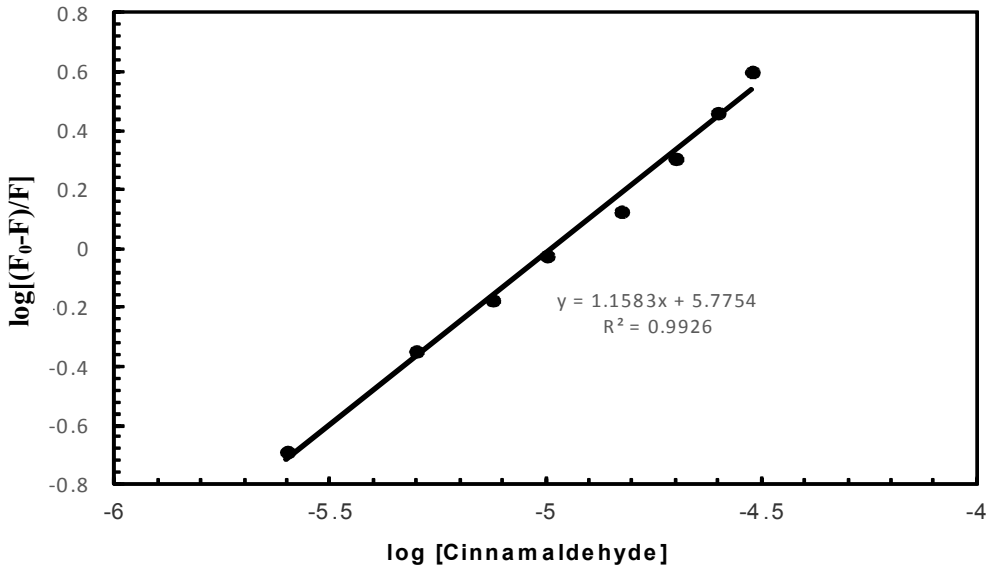

Fig. 5. Modified Stern-Volmer plot for quenching of fluorescence intensity.

spectrum in the various concentrations of cinnamaldehyde with molar ratios of 0, 1, 2 and 3 were investigated (Fig. 6). Using the CDNN software percentage of each secondary structure was obtained. There was no considerable change in the percentage of secondary structures in the presence of cinnamaldehyde, even at high concentration of the ligand.

Molecular Docking

The molecular docking techniques are often used to predict the preferential orientation of ligand to target and

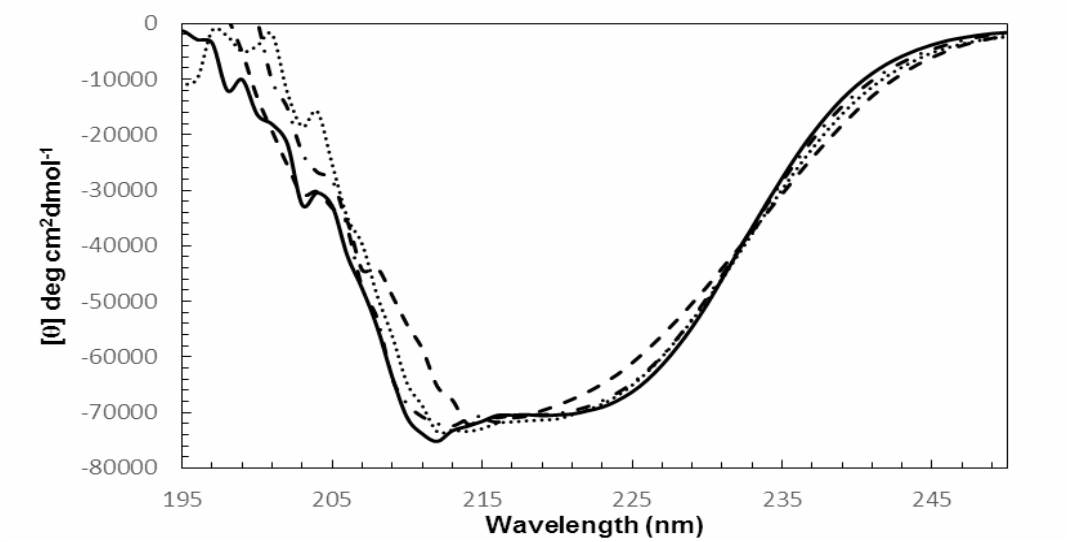

Fig. 6. Far-UV CD spectra of alpha-lactalbumin (10 µM) (___) in Tris buffer, pH7.5 and alpha-lactalbumin in the presence of cinnamaldehyde with molar ratios 1 (…), 2 (- - -) and 3 (- . -).

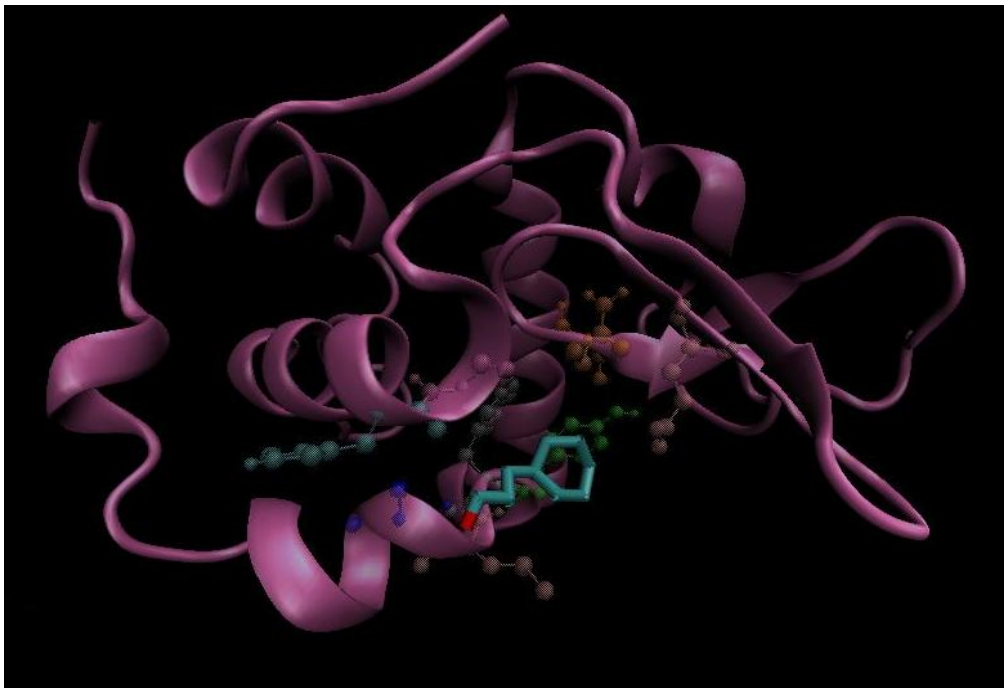

Fig. 7. Model of cinnamaldehyde binding site on alpha-lactalbumin. The important interacting residues are shown.

also mode and energy of the binding [43]. Molecular docking results showed the best probable binding site of cinnamaldehyde on alpha-lactalbumin. According to Fig. 7 cinnamaldehyde binds near the flexible loop of alpha domain of alpha-lactalbumin. This flexible loop (Leu 105 to Leu 123) takes up an extended conformation in HAMLET

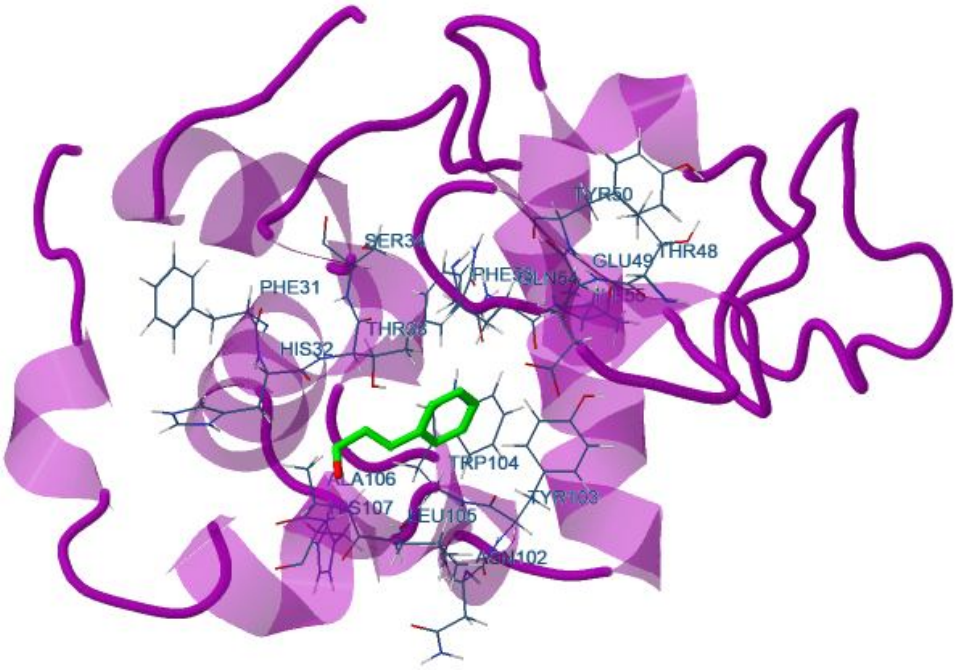

Fig. 8. Molecular docking result using dockingserver, it shows that cinnamaldehyde interacts with Trp104, Ala106, Thr33, Tyr103, Gln54, Glu 49, Leu 105 and His 32 residues of alpha-lactalbumin.

by forming a tail [44]. Cinnamaldehyde interacted with Trp 104, Ala 106, Thr 33, Tyr 103, Gln 54, Glu 49, Leu 105 and His 32 and hydrophobic interaction played the major role in the binding (Fig. 8). All of these amino acids contribute to vitamin D binding to alpha-lactalbumin too [27], so we can say that these amino acids play a prominent role in alpha-lactalbumin interaction with hydrophobic compounds. The Gibbs free energy (ΔG^0) of binding was equal to -18.81 kJ mol^{-1} which is less than experimental results (ΔG^0 = -32.88 kJ mol^{-1}). It may be due to the difference between the X-ray structure of the protein and the protein in the aqueous medium or the role of water and ethanol molecules in the hydrophobic interaction of cinnamaldehyde with protein. The results obtained from the docking study are in agreement with those from fluorescence spectroscopy measurement in which the binding process is spontaneous.

DISCUSSION

According to the results, alpha-lactalbumin has one binding site for cinnamaldehyde. CD spectrum shows that binding of cinnamaldehyde does not cause a considerable change in the secondary structure of alpha-lactalbumin and the secondary structure remains intact. Increasing of absorption of alpha-lactalbumin and quenching of its intrinsic fluorescence emission in the presence of cinnamaldehyde shows that local environment of chromophores changes. Four tryptophan residues of the bovine alpha-lactalbumin contribute to the overall intrinsic fluorescence intensity, though it has been stated in the literature [45] that in the native state, the signal of Trp 60 and Trp 118 remarkably quenched by the disulfide bonds in their proximity. Trp 104 and Trp 26 plays more prominent role in the fluorescence signal of the native alpha-lactalbumin. More likely, these tryptophan residues are partially accessible to the quencher and quenching can be due to local environment change of Trp 104 and Trp 26 of the protein because of cinnamaldehyde binding. With molecular docking study using Autodock vina we found that Trp 104 is one of interacting residues in cinnamaldehyde binding, so its role in the fluorescence signal of alpha-lactalbumin can be more prominent.

CONCLUSIONS

It can be concluded that conformation of alpha-lactalbumin changes without remarkable unfolding. This complex that consists of natural milk protein and non-synthetic antioxidant cinnamaldehyde can be used in the food industry, especially food and drink enrichment.

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