

Effect of thermal and microwave processing on secondary structure of bovine β -lactoglobulin: A molecular modeling study

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Funding information

NSERC; Science and Engineering Research Council

Abstract

Milk allergy is known to cause severe allergic reactions in hypersensitive patients, especially in infants and children. β -Lactoglobulin is one of the major allergens in bovine milk. The influence of thermal and microwave processing on the structural deviations of β -lactoglobulin protein have been studied using molecular modeling techniques. The structural deviations are studied using root mean square deviations, radius of gyration, dipole moment, and solvent accessible surface area. STRIDE analysis showed significant changes in the β -lactoglobulin, especially when oscillating electric fields were applied along with heat. Root mean square fluctuations (RMSF) has been assessed for known epitopes in the β -lactoglobulin molecule. This showed that when the protein is exposed to certain thermal stress, it compacts by burying hydrophobic residues in the core. However, few allergic epitope residues also exhibit increased RMSF leading to higher reactive sites on the surface of the protein molecule.

Practical applications

This study showed that molecular modeling can be used to gain valuable insights regarding the structural changes during processing. In the future, with more computational capacity, it can be used to make comparison between results obtained from simulations and real-time experiments. The current techniques used in food industries such as Nuclear Magnetic Resonance Imaging, Fourier Transformation Infrared Spectroscopy, X-ray diffraction can analyze pre- and post-processing effects. Hence, it become necessary to understand the changes that takes place during the processing techniques. Molecular dynamic simulation could be a useful technique in analyzing the changes occurring during the processing.

KEYWORDS

milk allergy, molecular modeling, protein structure, RMSF, secondary structure, β -lactoglobulin

1 | INTRODUCTION

Cow milk allergy (CMA) is one of the major food allergies and is in the list of Big-8 allergens recognized by Food Allergy and Research Education which includes other food products such as peanut, wheat, egg, soy, tree nuts, fish, and shell fish (Crittenden & Bennett,

2005; Schäfer et al., 2001; Taylor & Hefle, 2001; Zuberbier et al., 2004). Almost 2%–6% children under the age of one suffer from CMA worldwide (Caffarelli et al., 2010; Høst, 2002). Many studies on CMA state that infants and adults develop a tolerance for cow milk after consuming it for a long period of time (Skripak, Matsui, Mudd, & Wood, 2007). Out of all the patients allergic to cow milk,

50% outgrow milk allergy during their first year of life whereas 80%–90% children do that within their fifth year (Høst et al., 2002; Wood, 2003).

There is a widespread confusion prevailing within the general population in regard to “Lactose Intolerance” and “Milk Allergy”. Lactose intolerance occurs when the human body is unable to digest the sugar “Lactose” due to absence of lactase enzyme. It can lead to abdominal pain, bloating, flatus, and diarrhea. Lactose intolerance is further classified into primary and secondary. The primary intolerance occurs when there is a reduction in the levels of lactase essential to break down the sugar present in milk and dairy products. It is usually observed in infants older than three and can be found in Asian and African population. Secondary lactose intolerance occurs when lactase production is decreased by the small intestine. This usually occurs when the small intestine is involved in any kind of surgery or illness (Heyman, 2006). It causes mucosal damage which is followed by severe gastroenteritis (Walsh, Meyer, Shah, Quekett, & Fox, 2016).

However, CMA can be defined as an abnormal adverse immunological reaction caused by human immune system to cow milk proteins which can lead to gastrointestinal discomfort, respiratory failure as well as anaphylactic shock which can lead to death (Bu, Luo, Chen, Liu, & Zhu, 2013; El-Agamy, 2007; Exl & Fritsché, 2001). CMA is classified into Immunoglobulin E (IgE)-mediated (Acute onset) and non-IgE-mediated (Delayed onset) or both (Fiocchi et al., 2010). IgE-mediated allergic reactions occur immediately after consumption of milk whereas non-IgE-mediated reactions occurs after 48 hr of consumption of milk. It should be noted that both the reactions involve the immune system (Walsh et al., 2016).

Cow milk proteins are primarily classified into casein and whey proteins. Casein constitutes to 80% of the cow milk protein whereas the remaining 20% is linked to whey. Caseins are further subdivided into α_{s1} , α_{s2} , β , and κ casein whereas whey protein contains β -lactoglobulin (β -lg), α -lactalbumin (α -la), bovine serum albumin (BSA), immunoglobulins, lactoferrin, and proteose-peptone fractions (Ig) (Chandan, 1997; El-Agamy, 2007; Farrell et al., 2004; Robin, Turgeon, & Paquin, 1993). β -lg, α -la, and caseins are considered as main allergens in cow milk. Bovine serum albumin and lactoferrin in small amount are also recognized as potential allergens (Bu et al., 2013; Fritsché, 2003; Sharma, Kumar, Betzel, & Singh, 2001). Many studies conducted on cow milk protein allergy state that β -lg and casein are major allergens which can cause adverse allergic reactions in hypersensitive patients (Busse, Järvinen, Vila, Beyer, & Sampson, 2002; Cocco, Järvinen, Sampson, & Beyer, 2003; El-Agamy, 2007; Järvinen et al., 2002).

β -lg occurs in dimeric form (represented as chains A and B) having a total molecular weight of 36 kD and each side chain corresponds to a 162-residue polypeptide chain (Järvinen, Chatchatee, Bardina, Beyer, & Sampson, 2001; Wal, 1998). It has two disulfide bridges along with one free cysteine (Järvinen et al., 2001). The structure of β -lg consists of eight strand antiparallel β -barrel with a 3-turn- α -helix located outside the core as shown in Figure 1. A ninth β -strand is also present flanking the first strand (Kontopidis, Holt, & Sawyer, 2004). Lipocalin

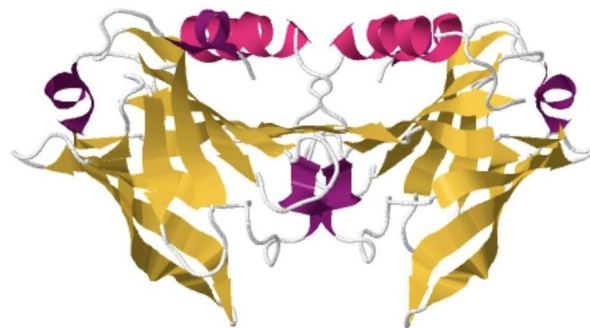


FIGURE 1 Three-dimensional structural view of bovine β -lg (PDB code: 2q2m)

fold consisting of eight-strand β -barrel is present in each subunit of β -lg. The barrel has a lining of hydrophobic residues which is useful when protein binds with hydrophobic molecules such as fatty acids and retinols (Kontopidis, Holt, & Sawyer, 2002; Qin, Creamer, Baker, & Jameson, 1998; Vijayalakshmi, Krishna, Sankaranarayanan, & Vijayan, 2008; Wu, Pérez, Puyol, & Sawyer, 1999).

To date, numerous food industries are trying to assess the effect of thermal and nonthermal processing techniques on milk proteins. It is well documented that majority of allergens in food products are proteins whose structure is vital in determining their functional properties. (Vanga, Singh, & Raghavan, 2017). Hence, investigating the effect of these processing techniques on structural aspects of milk proteins can further help researchers in understanding their functional properties including milk allergenicity. As mentioned previously, β -lg is a major allergen in milk and is responsible for most of the allergic reactions caused by milk in allergic patients (Bu et al., 2013; Fritsché, 2003; Sharma et al., 2001).

Molecular dynamic (MD) simulation techniques can be an innovative alternate for the above-mentioned techniques to evaluate the conformational changes occurring in protein due to processing techniques (Vagadia, Vanga, Singh, & Raghavan, 2016). MD simulation represents application of computational knowledge to bridge the gap between microscopic levels (in terms of length and time) and macroscopic levels (real-time experiments) (Allen, 2004). It can be used to analyze the dynamic properties of biomolecules under consideration that cannot be done by means of analytical tools. The dynamic state of a molecule can describe a wide array of thermally accessible states of a system. Hence, it can build a connection between sequence to structure and functionality of the molecule under consideration. Hence, by following MD simulations in space and time, a better understanding of dynamic and structural properties can be made. A huge amount of information can be obtained from such simulations such as geometry and energy of molecules; average atomic fluctuations; local fluctuations such as breakage and formation of hydrogen bonds, interaction trends between water, solute, and ion particles, rates of configurational change such as nucleic-acid sugar repuckering, diffusion and ring flips, and so on (Schlick, 2010).

This study focuses on the effect of thermal and microwave processing on the secondary structure of bovine β -lg where properties

such as radius of gyration (R_g), root mean square deviation (RMSD), dipole moment, root mean square fluctuations (RMSF), and solvent accessible surface area (SASA) are taken into consideration during the study.

2 | MATERIALS AND METHODS

Molecular dynamics simulation was performed by GROMACS (Groningen machine for chemical simulation, Version 5.0.4) from the Stockholm Center for Biomembrane Research, Stockholm, Sweden (Hess, Kutzner, Spoel, & Lindahl, 2008). The protein structure of β -lg in PDB format (PDB code: 2Q2M) was downloaded from Protein Data Bank. Amber 99SB-ILDN force field was opted for the appropriate simulations. It also provides functions as well as parameters to every type of atom present in the system (Lindorff-Larsen et al., 2010). The protein molecule was enclosed in a box of cubical shape having dimensions 7.051 nm $\times$ 4.230 nm $\times$ 6.372 nm and 30,369 molecules of water were added in the box such that minimum image convention can be satisfied which indicates that a protein molecule should not be able to see its periodic image otherwise all the force calculated will give misleading results.

A temperature set of 333, 348, and 363 K was chosen for the study. These temperature sets were adopted because protein denaturation starts at 328 K initially with the loss of tertiary structure which aggravates to secondary structure loss starting at 343 K. There are many other losses which takes place at elevated temperature (Ishiwatari, Fukuoka, & Sakai, 2013; Vanga et al., 2017). The β -lg molecule contains $-14e$ charge based on its amino acid composition. To compensate the negative charge and neutralize the protein molecule, same number of Na^+ ions were added to the system and replaced by hydrogen atom such that the protein has no net negative or positive charge. This methodology is conducted at a neutral pH of 7 so that stability can be achieved. Before starting the simulation, it should be ensured that the system has no steric clashes or inappropriate geometry. This is achieved by a process called energy minimization. There are two conditions which decide whether energy minimization step was successful or not, namely E_{pot} (potential energy) should be negative and F_{max} (maximum force) should be less than 1,000 kJ mol $^{-1}$ nm $^{-1}$ (<http://www.mdtutorials.com/gmx/>).

After all the steps are processed, it should be ensured that solvent and ions around the protein must be in equilibrium. It is achieved through two steps; NVT (constant number of particles, volume, and temperature) and NPT (constant number of particles, pressure, and volume). The NVT step is initiated first to reach a similar temperature where simulation will take place and to properly orient the solvent molecules around the solute. After this, the pressure is applied to the system (protein and water molecules) so that it can reach a proper density.

Six molecular dynamic simulation were carried out by varying the temperature (thermal treatment) and applying oscillating electric field (microwave treatment) at 2.45 GHz. The electric field was set at 0.5 V/nm. Wang, Li, He, Chen, and Zhang (2014) performed

experiment on insulin and reported that the secondary structure remains intact when electric field of intensity 0.15 V/nm was applied. However, after applying an electric field of strength 0.25 V/nm or higher, there were some disruptions observed in the secondary structure. The authors concluded that increasing or decreasing the intensity of electric field could foster protein folding or might have a negative effect on secondary structure in terms of its damage. Marracino, Apollonio, Liberti, d'Inzeo, and Amadei (2013) also studied the effects of static and pulse electric field on myoglobin using MD simulations. Electric field intensities ranging from 1 to 10 V/nm were used for the study and it was observed that maximum structural deviation was observed at 10 V/nm. All simulations were performed for 2 ns (2000 ps). To analyze the effect of the treatment on secondary structure of protein molecule, properties such as RMSD, R_g , dipole moment, SASA, and RMSF of atoms were taken into consideration. STRIDE analysis was also conducted to gain insight of the secondary structural changes occurred in protein during the treatments using visual molecular dynamics (VMD) software (Humphrey, Dalke, & Schulten, 1996).

3 | RESULTS AND DISCUSSIONS

The main purpose of the study was to investigate the effect of thermal treatment with and without the oscillating electric field at 300, 333, 348, and 363 K on secondary structure of bovine β -lg. Various computational analytics were used to study the effect of thermal and microwave treatment on structural properties of β -lg including the deviations in the epitopes responsible for IgE-mediated allergic reactions.

3.1 | Secondary structure analysis

STRIDE algorithm introduced by Frishman and Argos (1995) is used to analyze secondary structural changes occurring in the protein. STRIDE employs a knowledge-based algorithm which uses combination of hydrogen bond energies and backbone torsional angle information to predict the structural change. Based on the information obtained, each residue is assigned the most probable secondary structure which can change with time in a given simulation (Frishman & Argos, 1995; Heinig & Frishman, 2004). STRIDE analysis is a tool to find out changes occurring in secondary structure during the treatment, the residues responsible for the change and the exact time where the change occurred.

STRIDE can be plotted graphically by utilizing the inbuilt sequence viewer option present in the VMD package. The graph represents time frame on X-axis and amino acid residues on Y-axis as shown in Figure 2. As the simulation time progresses indicating additional stress on protein, different color bands can be observed representing the variations occurring within the protein secondary structure. In addition to this, a comparison can be carried out based on the initial and final structural changes observed during the analysis.



FIGURE 2 STRIDE analysis shows the secondary structural deviations in 2q2m with no oscillating electric field (a) 300 K, (b) 333 K, (c) 348 K, and (d) 363 K

β -lg exists in the form of dimer with side chain A and B (Järvinen et al., 2001; Wal, 1998). From Figure 2a,b, it can be observed that there is a dislocation of 3/10 helix (1 LEU A–8 LYS A) with an increase in temperature. Further, conversion of turns into 3/10 helix ((15 VAL A–36 SAR A) and (3 VAL B–17 GLY B)) and disruption of turns (108 GLU B–122 LEU B) can be observed as the temperature is increased from 300 to 333 K. When the temperature was increased further from 348 to 363 K (Figure 2c,d), there was a continued disruption of turns and 3/10 helix between 1 LEU A–8 LYS A and 3 VAL B–17 GLY B. There is also a relocation of turns (24 MET B–38 PRO B), conversion of α -helix into 3/10 helix and introduction of new turn structures between 122 LEU B and 143 LEU B.

When oscillating electric field is applied in combination with thermal treatment (Figure 3), there was an increased conversion of 3/10 helix into turns (1 LEU A–15 VAL A) and (24 MET B–38 PRO B). Newly ordered turn structures were observed between the residues 108 GLU B–115 GLN B as the temperature increased from 300 to 333 K (Figure 2a,b). On further increasing the temperature from 348 to 363 K (Figure 2c,d), there was conversion of ordered turns into α -helix (1 LEU A–15 VAL A). However, between residues 120 GLN A and 148 ARG A, the α -helix structure was converted into turns. Further, conversion of turns into 3/10 helix (31 LEU B–38 PRO B), disruption of turns (108 GLU B–115 GLN B) and conversion of α -helix into turns (122 LEU B–143 LEU B) were observed with the temperature increasing to 363 K in combination with oscillating electric field.

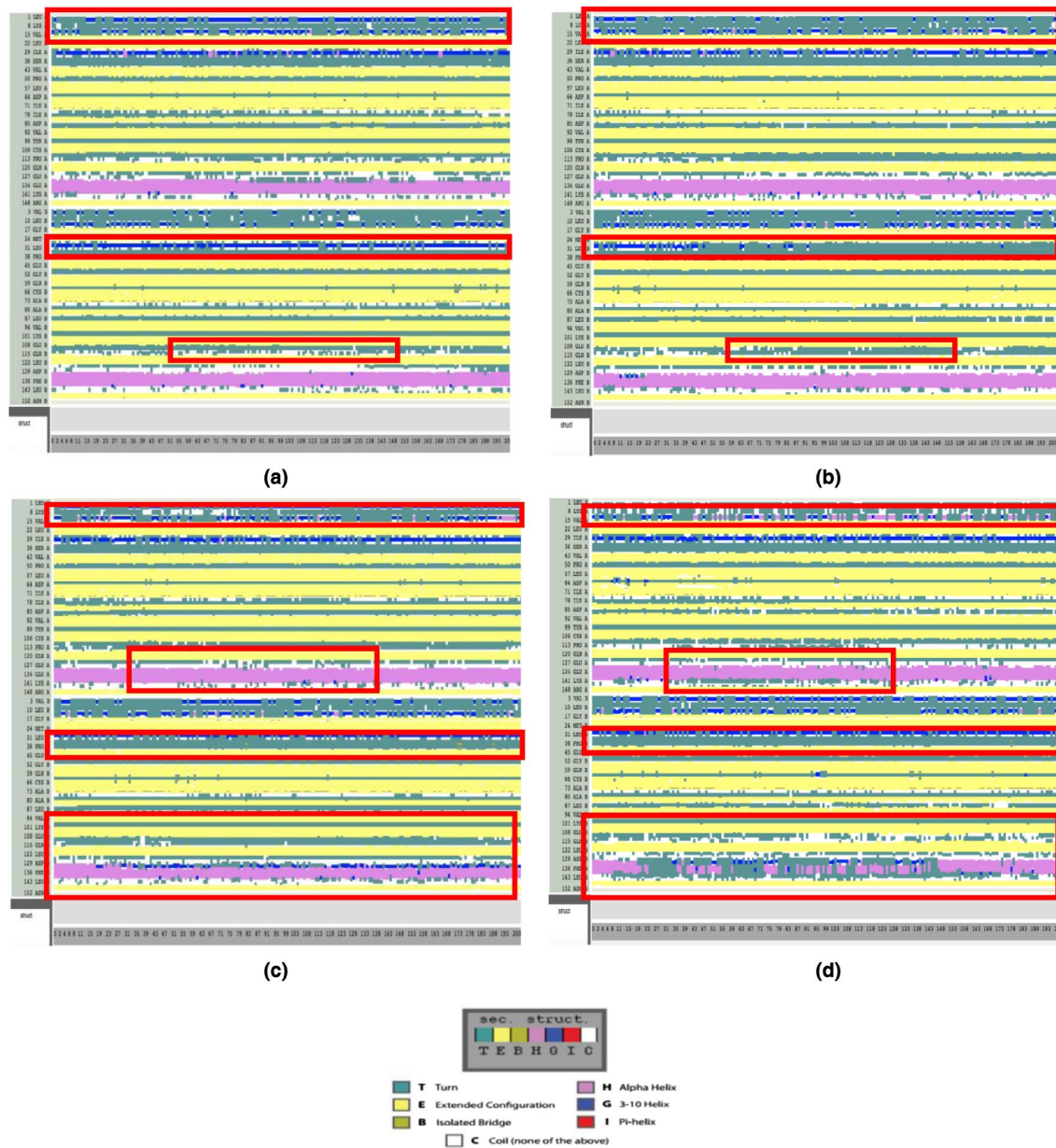


FIGURE 3 STRIDE analysis shows the secondary structural deviations in 2q2m with oscillating electric field (a) 300 K, (b) 333 K, (c) 348 K, and (d) 363 K

3.2 | Root mean square deviation

The RMSD is the measure of deviations in the structure of molecule in terms of atomic movement when subjected to external stress (Vanga, Singh, & Raghavan, 2015). The RMSD is calculated by evaluating the change in structure of protein molecule with reference to a molecule that is not under any external stress. The control molecule can be taken as the reference molecule (Budi, Legge, Treutlein, & Yarovsky, 2004). The following equation is used to calculate the RMSD of protein molecule (Singh, Orsat, & Raghavan, 2013).

$$\text{RMSD} = \sqrt{\frac{1}{N} \sum_{i=0}^n |r_{\text{final}}(i) - r_{\text{initial}}(i)|^2} \quad (1)$$

where $r_{\text{final}}(i)$ is the final coordinates of an atom, and $r_{\text{initial}}(i)$ is the initial coordinate of the atom i , and N is the number of atoms.

From Figure 4a, it was observed that there was an increase in RMSD values as the temperature increased from 300 to 363 K. There were some high peaks observed between 700 and 1500 ps at 348 K and 1700–2000 ps at 363 K which could be due to some significant structural changes. In case of application of heat treatment with oscillating electric waves at a frequency of 2,450 MHz, it was observed

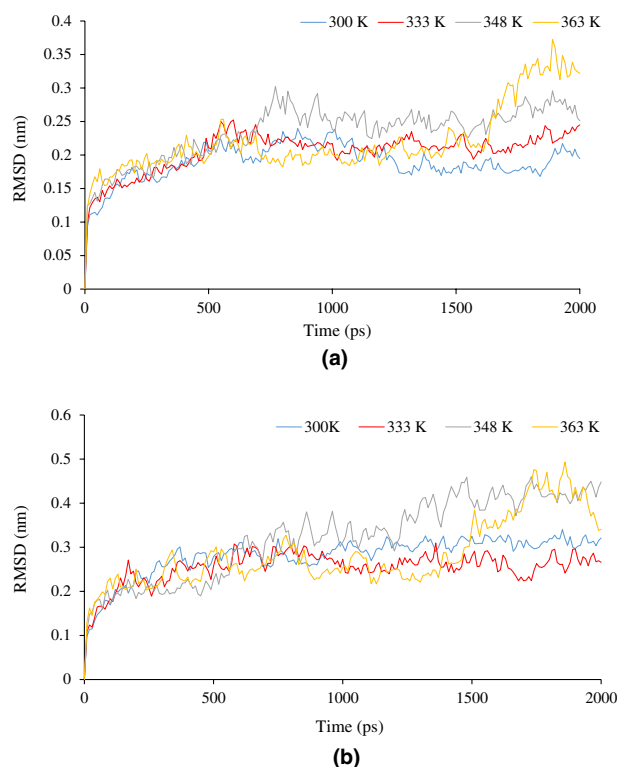


FIGURE 4 Root mean square variations observed in bovine β -Ig: (a) heat treatment and (b) heat treatment + oscillating electric field at 2,450 MHz

that the RMSD values decreased at 333 K compared to 300 K which is the control temperature. There were again some high peaks observed between 810–2000 ps and 1,500–2000 ps at 348 K indicating significant movements in the residues (Figure 4b). It can also be observed from Table 1 that average values for RMSD for thermal treatment and combination of thermal treatment and oscillating electric field at 2,450 MHz is maximum for 348 K which is the initiation temperature for denaturation of proteins (Vanga et al., 2017).

3.3 | Radius of gyration

The R_g is the distribution of atoms in the protein molecules with respect to its center of mass (Budi, Legge, Treutlein, & Yarovsky, 2005, 2008; Singh, Orsat, et al., 2013). R_g is an index of measuring the compactness of protein molecule. It is accepted that lower R_g suggests that number of atoms packed around the center of protein molecule is higher (Lobanov, Bogatyreva, & Galzitskaya, 2008). R_g is calculated by the following equation (Singh, Orsat, et al., 2013):

$$R_g = \sqrt{\frac{1}{N} \sum_{i=1}^N |r(i) - r_{\text{center}}|^2} \quad (2)$$

where $r(i)$ is the coordinates of an atom (i) and r_{center} is the coordinates of protein's center of mass, and N is the number of atoms.

It was observed that R_g first increased when the temperature increased from 300 to 333 K (Figure 5a) but decreased as the

TABLE 1 RMSD values observed for β -Ig

Temperature	Thermal treatment (pm)	Thermal treatment + OEF (pm)
300 K	189 ± 29	280 ± 47
333 K	205 ± 30	254 ± 38
348 K	234 ± 41	322 ± 91
363 K	221 ± 52	284 ± 78

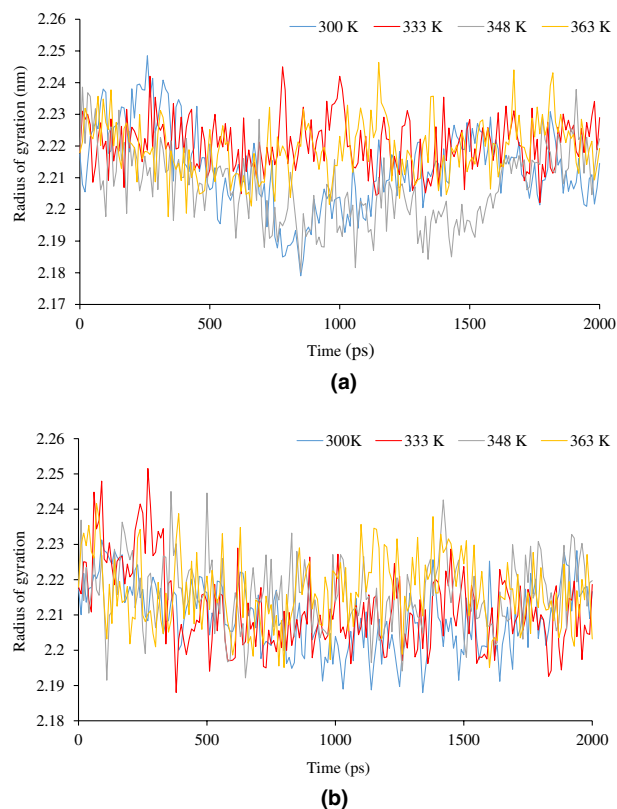


FIGURE 5 Radius of gyration (R_g) variations observed in bovine β -Ig: (a) heat treatment and (b) heat treatment + oscillating electric field at 2,450 MHz

temperature further increased to 348 K. The lower peaks were observed between 690–1020 ps at 300 K and 1,280–1570 ps at 348 K indicating that during these time span molecules has compacted due to external stress. The R_g values reported at 363 K were close to 333 K when the temperature was further increased. This can also be observed in Table 2 where the average R_g values are very close to each other. This indicates that as the temperature is increasing, the protein molecule first compacts to a lower energy state and then expands as the temperature increases to 363 K (Figure 5a). When combined heat treatment and microwave at 2,450 MHz are applied (Figure 5b), the R_g at 333 K is similar to that of the control at 300 K which implies that increasing temperature to 333 K did not have any effect on the core packing of the molecule. When the temperature is further raised to 348 K and 363 K, the R_g values showed no significant change (Table 2). From the combined heat and microwave

TABLE 2 R_g values observed for β -lg

Temperature	Thermal treatment (pm)	Thermal treatment + OEF (pm)
300 K	2,213 $\pm$ 13	2,209 $\pm$ 9
333 K	2,220 $\pm$ 8	2,211 $\pm$ 11
348 K	2,207 $\pm$ 11	2,216 $\pm$ 10
363 K	2,219 $\pm$ 9	2,216 $\pm$ 10

treatment results, where we can see a gradual increase in R_g , it can be inferred that protein molecule is trying to unfold as the temperature is increased from 300 to 363 K, respectively.

3.4 | Dipole moment

Due to the amino acid orientations, the protein secondary structures possess dipole moment which as a result of application of external electric fields changes when protein molecule tries to align in the direction of the applied field (Singh, Munshi, & Raghavan, 2013; Vanga et al., 2015). The following equation can be used to represent dipole moment of a protein molecule (Singh, Munshi, et al., 2013):

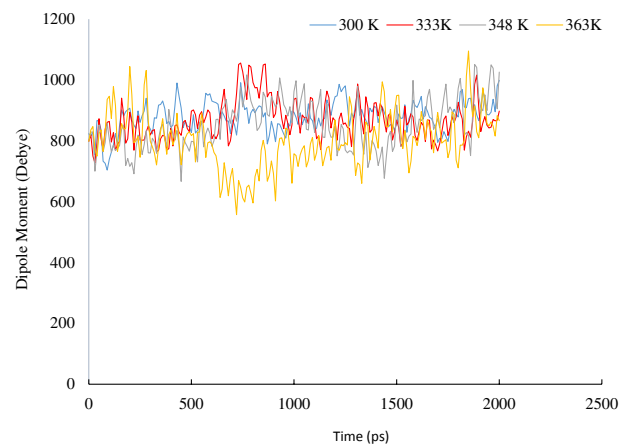
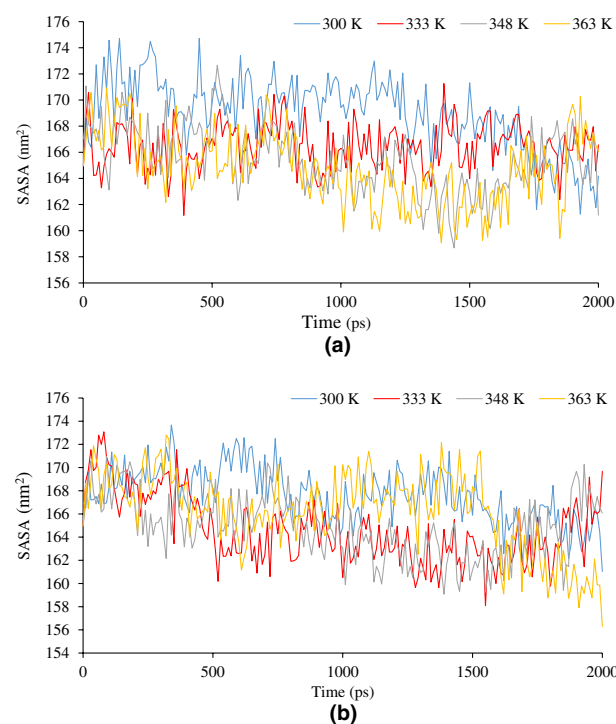
$$\vec{\mu}_d = \sum_{i=1}^n q_i(i) \vec{r}_i \quad (3)$$

where $\vec{\mu}_d$ is the dipole moment, q_i is the charge of the atom, i , $\vec{r}_i$ is the directional vector of each atom, and N represents the number of atom.

Figure 6 shows dipole moment when oscillating electric field is applied in combination with heat treatment at different temperatures. The variations were minimal in dipole moments at 300, 333, and 348 K. The values at 363 K showed variations as the dipole moment increased initially within the first 350 ps and then dropped by almost 200 Debye between 500 and 100 ps. Thereafter, a gradual increasing trend was observed. This variation in the dipole moments could be the result of higher stresses with the application of electric field in combination with high temperature. The secondary structures, especially the helical structures with dipole moment try to align in the direction of the electric field, and in the process the protein molecule can lose its stability (Budi et al., 2004).

3.5 | Solvent accessible surface area

SASA is an important parameter for analyzing the interactions between protein with other molecules and solvents. These properties depend on the structural confirmations of the protein and a slight deviation in the confirmation could lead to a change in surface properties and in turn affect the protein intermolecular interactions (Vagadia et al., 2016; Wellner et al., 2005). Lee and Richards (1971) proposed an equation for determining the SASA. During their study, they suggested that accessible surface area can be defined as an

**FIGURE 6** Dipole moment variations observed in bovine β -lg: (a) heat treatment and (b) heat treatment + oscillating electric field at 2,450 MHz**FIGURE 7** Solvent accessible surface area (SASA) variations observed in bovine β -lg: (a) heat treatment and (b) heat treatment + oscillating electric field at 2,450 MHz

area where center of the solvent molecule can be placed without disturbing the vicinity of other atoms. The following equation can be used to evaluate SASA:

$$SASA = A = \sum \left(R / \sqrt{R^2 - Z_i^2} \right) \times D \times L_i \quad (4)$$

where A denotes the surface area, R denotes the radius of atom, L_i denotes the length of the atom drawn on a given section $\odot$, and z_i

denotes the perpendicular distance of section © from the center of the sphere. It is worth mentioning that this study takes only static accessibility under consideration and does not have any relation with the potential flexibility of the structure.

It was observed that during heat treatment (Figure 7a), SASA decreased at 300 K after 1,500 ps under the stress whereas other temperatures decreased within the first 500 ps. The surface area at 333 K remained constant through processing with no significant changes. There were decreasing trend observed for both 348 and 363 K, respectively, after 750 ps of processing. It could be due to the rearrangement in the surface residues at high temperatures such as 348 and 363 K which results in reduction of SASA of molecule to interact with other molecules. When oscillating electric field is applied in combination with thermal treatment (Figure 7b), there were high peaks observed at 300 K between 530–770 ps and 1,050–1580 ps. A decrease in SASA values was observed for 333 and 348 K at around 500 ps and then it almost remained constant before slightly increasing after 1,700 ps. Higher peaks were observed at 363 K between 970 and 1560 ps after which the SASA values dropped drastically from 172 to 160 nm². Overall, the trend is decreasing for combined heat and oscillating electric field treatment at 2,450 MHz for all the temperatures used during the study. It is interesting to note that bovine β -lg structure is stable under the influence of heat treatment and combination treatment used in this study. This can be attributed to the fact that this structure exists in the form of dimer and it is stabilized due to the presence of opposite charges at both the ends of major helix. This can also bring dissociation in the dimeric structure transforming it into a monomer based on the pH which can induce some structural changes in the protein molecule (Fessas, Iametti, Schiraldi, & Bonomi, 2001; Uhrínová et al., 2000). The two-helix present in the structure run in antiparallel directions, resulting in close interactions between opposite charges present on both the monomers at neutral pH. There are hydrophobic residues present on the β -strand of the protein structure which are engaged in hydrophobic interaction and bind the two helical regions together, thus increasing the stability of protein molecule (Fessas et al., 2001).

3.6 | Root mean square fluctuations

RMSF is a measurement of the average atomic mobility of atoms present in protein molecules (Vendome et al., 2011). The following equation can be used to calculate RMSF of an atom © (Welford, 1962):

TABLE 3 Allergen epitopes present in bovine β -lg

Allergen epitopes in bovine β -lg	Reference
58–77	Cerecedo, Zamora et al. (2008)
47–56, 57–76, 75–84, 127–136 and 141–150	Järvinen et al. (2002)
127–140	Wang, Lin et al. (2010)
51–64, 67–88, 129–144 and 139–156	Järvinen et al. (2001)

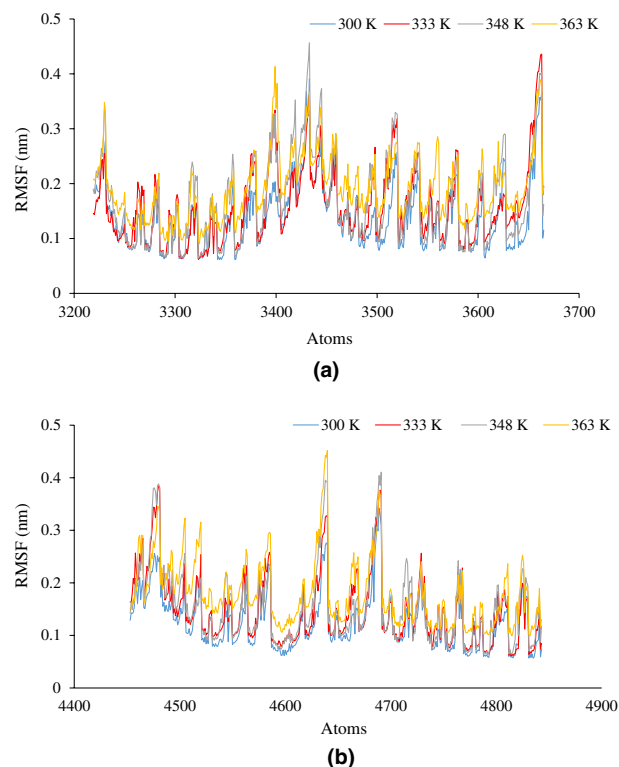


FIGURE 8 Root mean square fluctuations (RMSF) observed in bovine β -lg during heat treatment for allergen epitopes (a) 51–77 and (b) 127–150

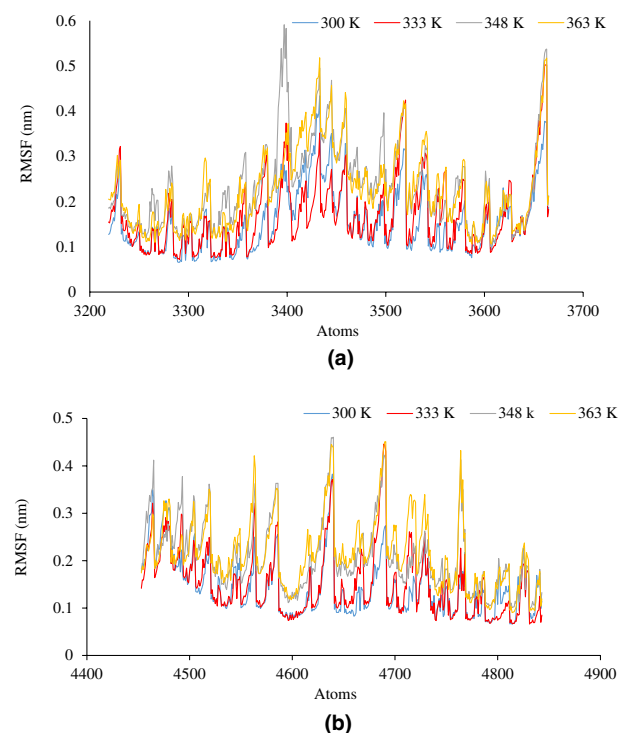


FIGURE 9 Root mean square fluctuations (RMSF) observed in bovine β -lg during combination of heat treatment and oscillating electric field at 2,450 MHz for allergen epitopes (a) 51–77 and (b) 127–150

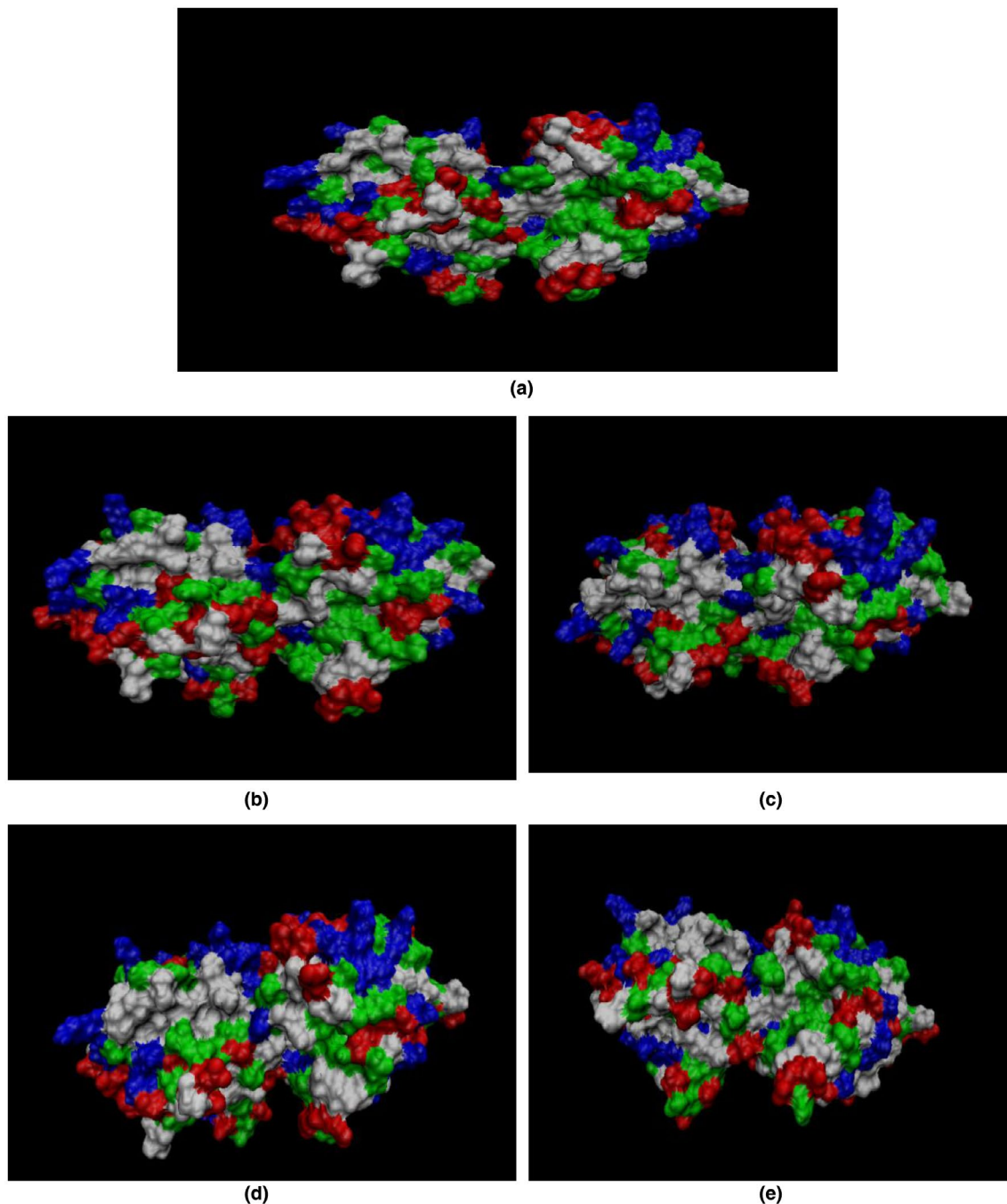


FIGURE 10 Snapshot of surface properties of β -lg (a) 300, (b) molecule at 300 K and 940 ps, (c) molecule at the end of 333 K, (d) molecule at 348 K and 1580 ps, and (e) molecule at the end of 363 K. Nonpolar residues (white), basic (blue), acidic (red), and polar residues (green)

$$\text{RMSF} = \sqrt{\left\langle (x_i - \langle x_i \rangle)^2 \right\rangle} \quad (5)$$

where x_i denotes the position of atom with respect to time.

There are many allergen epitopes present in bovine milk β -lg which are the amino acid sequence (Aas) responsible for IgE-mediated reactions. Table 3 lists the allergenic epitopes present in milk as reported by various studies. Two common Aas sequence (51–77 and 127–150) were chosen and RMSF of atoms present in the selected

Aas were calculated in this study. Figure 8a,b shows RMSF values of the selected epitopes when thermal treatment was applied. There were some peaks observed at 363 K in both allergen epitopes mentioned. It indicates that as the applied temperature is increased, the fluctuation of atoms increased. As higher temperatures are applied, allergen epitopes lost their rigidity leading to higher fluctuations. This could further open the ways of studying the behavior of allergic molecules within the scope of an epitope range. It can also be used to predict the exposure of new binding sites which could further

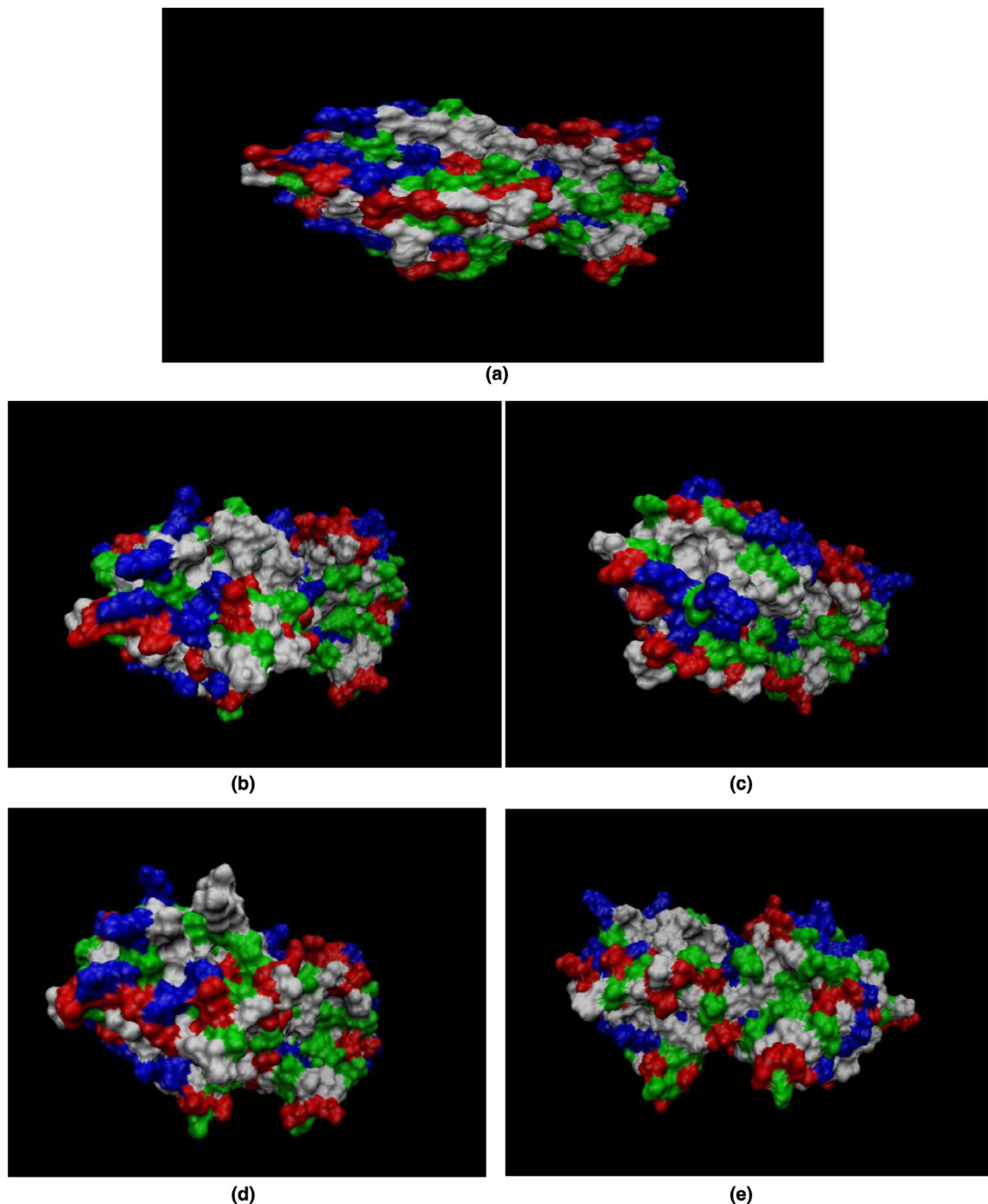


FIGURE 11 Snapshot of surface properties of β -lg with application of thermal treatment and oscillating electric field (a) at the start of 300 K, (b) at the end of 333 K, (c) at 348 K and 1580 ps, (d) at 363 K and 1,090 ps, and (e) at the end of 363 K. Nonpolar residues (white), basic (blue), acidic (red), and polar residues (green)

react with IgE antibodies increasing the chances of an allergic reaction. There were some peaks observed at 348 K in epitopes (57, 59, 62–64, and 69) which were even reported higher than 363 K. A significantly higher peak was observed at 333 K for epitope 77 (Figure 8a). As the temperatures were raised, the fluctuations in the molecule decreased. This is an indication that the residues that were free to react were moving into the molecule, away from the reactive surface of β -lg becoming more rigid. In general, the higher peaks observed with rising temperature suggests that fluctuation of allergen

epitopes are increasing as the stress due to temperature increases. Hence, there occurs a possibility that epitopes might react with antibodies present in immune system and causes allergen reactions. However, when the fluctuation peaks disappear with increasing temperatures, in this case, peaks observed at 348 K compared to 363 K suggests that the epitopes have less fluctuations at higher temperatures and there occurs a possibility that these epitopes are burying themselves in the hydrophobic core and hence would not be able to bind with IgE.

When oscillating electric field was applied with thermal treatment, the same trend was observed (Figure 9). There were higher peaks observed at 363 K (Figure 9a) suggesting that these epitopes are becoming unstable and are deviating further compared to control. Moreover, peaks were observed at 348 K for epitope range (54–55, 58–59, 61, 66–69, and 77) which could be due to the burying of epitopes at such high temperature. This is the same trend observed in the case of application of 348 K thermal stress in absence of oscillating electric field. There were peaks observed at 363 K in epitope range 127–150 (Figure 9b) suggesting that in this region, epitopes might be becoming reactive. Furthermore, in epitope range (127–130), 348 K has higher peaks compared to 363 K which might be due to the reason that in this range, epitopes are moving into the core and becoming unavailable on the surface.

Figure 10 shows the snapshot of β -lg molecule at different temperature and time. It can be observed that β -lg exists in the form of dimer at 300 K (Figure 10a). The dimeric form started converting into monomeric form at 940 ps and 300 K as shown in Figure 10b. It can be observed that as the temperature is increasing, the molecule is turning into a monomer as shown in Figure 10c–e, respectively. It can also be observed that as the temperature is increasing, basic residues which were protruded outward initially are shifting inward gradually. Figure 11 shows the snapshot of β -lg molecule when oscillating electric field is applied in combination to thermal treatment. It can be observed that at 300 K (Figure 11a), the protein molecule has changed its orientation which might be the movement in the molecule due to dipole moment. Under the influence of electric field, the molecule tries to align itself in the direction of applied electric field (Figure 11b). A significant change is also observed at 348 K and 1580 ps where the residues present in the molecule are found to be compacting due to the rearrangement (Figure 11c). This can be attributed to the fact that R_g values is decreasing at higher temperatures which is evident from Table 2. Moreover, it can be observed that at 363 K and 1,090 ps (Figure 11d), a nonpolar residue (white in color) is protruding outward which again shifts inward, into the core of the protein as the treatment time proceeds. At the end of 363 K as shown in Figure 11e, the molecule is again changing its orientation. The surface properties need to be studied as they can affect the functions and properties of proteins.

4 | CONCLUSION

β -Lactoglobulin is a major protein present in milk. Evaluating the structural deviations in this molecule due to thermal and microwave processing could be vital in understanding the changes in various functional properties including its allergenicity. The analysis performed shows that the increasing temperature causes significant secondary structure deviations in the molecule. There are increased number of deviations observed at 348 K with and without the application of oscillating electric fields. The molecule was found to stabilize once the temperature is increased further which is evident from the RMSD and RMSF values reported. The molecule which is

a dimer with two side chains was showed to merge, thereby reducing the available surface area on which the enzymes can act which could reduce the digestibility, but at the same time make epitopes unavailable for IgE-mediated allergic reactions. Further studies have to be conducted on evaluating the implications of these structural changes on the functional properties like allergenicity and digestibility of the protein.

ACKNOWLEDGMENT

The authors acknowledge the support of NSERC (National Science and Engineering Research Council) for the study.

CONFLICT OF INTEREST

The authors report no conflict of interest.

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REFERENCES

- Allen, M. P. (2004). Introduction to molecular dynamics simulation. *Computational Soft Matter: From Synthetic Polymers to Proteins*, 23, 1–28.
- Bu, G., Luo, Y., Chen, F., Liu, K., & Zhu, T. (2013). Milk processing as a tool to reduce cow's milk allergenicity: A mini-review. *Dairy Science & Technology*, 93(3), 211–223. <https://doi.org/10.1007/s13594-013-0113-x>
- Budi, A., Legge, S., Treutlein, H., & Yarovsky, I. (2004). Effect of external stresses on protein conformation: A computer modelling study. *European Biophysics Journal*, 33(2), 121–129. <https://doi.org/10.1007/s00249-003-0359-y>
- Budi, A., Legge, F. S., Treutlein, H., & Yarovsky, I. (2005). Electric field effects on insulin chain-B conformation. *The Journal of Physical Chemistry B*, 109(47), 22641–22648. <https://doi.org/10.1021/jp052742q>
- Budi, A., Legge, F. S., Treutlein, H., & Yarovsky, I. (2008). Comparative study of insulin chain-B in isolated and monomeric environments under external stress. *The Journal of Physical Chemistry B*, 112(26), 7916–7924. <https://doi.org/10.1021/jp800350v>
- Busse, P. J., Järvinen, K.-M., Vila, L., Beyer, K., & Sampson, H. A. (2002). Identification of sequential IgE-binding epitopes on bovine α s2-casein in cow's milk allergic patients. *International Archives of Allergy and Immunology*, 129(1), 93–96.
- Caffarelli, C., Baldi, F., Bendandi, B., Calzone, L., Marani, M., Pasquinelli, P., & Ewpgag, O. B. O. (2010). Cow's milk protein allergy in children: A practical guide. *Italian Journal of Pediatrics*, 36(1), 5. <https://doi.org/10.1186/1824-7288-36-5>
- Cerecedo, I., Zamora, J., Shreffler, W. G., Lin, J., Bardina, L., Dieguez, M. C., ... Sampson, H. A. (2008). Mapping of the IgE and IgG4 sequential epitopes of milk allergens with a peptide microarray-based immunoassay. *Journal of Allergy and Clinical Immunology*, 122(3), 589–594.
- Chandan, R. (1997). *Dairy-based ingredients*. Minnesota: Eagan Press Handbook series.
- Cocco, R. R., Järvinen, K.-M., Sampson, H. A., & Beyer, K. (2003). Mutational analysis of major, sequential IgE-binding epitopes in α s1-casein, a major cow's milk allergen. *Journal of Allergy and*

- Clinical Immunology*, 112(2), 433–437. <https://doi.org/10.1067/mai.2003.1617>
- Crittenden, R. G., & Bennett, L. E. (2005). Cow's milk allergy: A complex disorder. *Journal of the American College of Nutrition*, 24(sup6), 582S–591S. <https://doi.org/10.1080/07315724.2005.10719507>
- El-Agamy, E. (2007). The challenge of cow milk protein allergy. *Small Ruminant Research*, 68(1), 64–72. <https://doi.org/10.1016/j.smallrumres.2006.09.016>
- Exl, B.-M., & Fritsché, R. (2001). Cow's milk protein allergy and possible means for its prevention. *Nutrition*, 17(7), 642–651. [https://doi.org/10.1016/S0899-9007\(01\)00566-4](https://doi.org/10.1016/S0899-9007(01)00566-4)
- Farrell, H. M., Jimenez-Flores, R., Bleck, G. T., Brown, E. M., Butler, J. E., Creamer, L. K., ... Swaisgood, H. E. (2004). Nomenclature of the proteins of cows' milk—Sixth revision. *Journal of Dairy Science*, 87(6), 1641–1674. [https://doi.org/10.3168/jds.S0022-0302\(04\)73319-6](https://doi.org/10.3168/jds.S0022-0302(04)73319-6)
- Fessas, D., Iametti, S., Schiraldi, A., & Bonomi, F. (2001). Thermal unfolding of monomeric and dimeric β -lactoglobulins. *The FEBS Journal*, 268(20), 5439–5448. <https://doi.org/10.1046/j.0014-2956.2001.02484.x>
- Fiocchi, A., Schünemann, H. J., Brozek, J., Restani, P., Beyer, K., Troncone, R., ... Lockey, R. F. (2010). Diagnosis and Rationale for Action against Cow's Milk Allergy (DRACMA): A summary report. *Journal of Allergy and Clinical Immunology*, 126(6), 1119–1128.e1112. <https://doi.org/10.1016/j.jaci.2010.10.011>
- Frishman, D., & Argos, P. (1995). Knowledge-based protein secondary structure assignment. *Proteins: Structure, Function, and Bioinformatics*, 23(4), 566–579. <https://doi.org/10.1002/prot.340230412>
- Fritsché, R. (2003). Role for technology in dairy allergy. *Australian Journal of Dairy Technology*, 58(2), 89.
- Heinig, M., & Frishman, D. (2004). STRIDE: A web server for secondary structure assignment from known atomic coordinates of proteins. *Nucleic Acids Research*, 32(suppl_2), W500–W502. <https://doi.org/10.1093/nar/gkh429>
- Hess, B., Kutzner, C., Spoel, D., & Lindahl, E. (2008). GROMACS 4: Algorithms for highly efficient, load-balanced, and scalable molecular simulation. *Journal of Chemical Theory and Computation*, 4(3), 435–447.
- Heyman, M. B. (2006). Lactose intolerance in infants, children, and adolescents. *Pediatrics*, 118(3), 1279–1286. <https://doi.org/10.1542/peds.2006-1721>
- Høst, A. (2002). Frequency of cow's milk allergy in childhood. *Annals of Allergy, Asthma & Immunology*, 89(6), 33–37. [https://doi.org/10.1016/S1081-1206\(10\)62120-5](https://doi.org/10.1016/S1081-1206(10)62120-5)
- Høst, A., Halken, S., Jacobsen, H. P., Christensen, A. E., Herskind, A. M., & Plesner, K. (2002). Clinical course of cow's milk protein allergy/intolerance and atopic diseases in childhood. *Pediatric Allergy and Immunology*, 13, 23–28. <https://doi.org/10.1034/j.1399-3038.13.s.15.7.x>
- Humphrey, W., Dalke, A., & Schulten, K. (1996). VMD: Visual molecular dynamics. *Journal of Molecular Graphics*, 14(1), 33–38. [https://doi.org/10.1016/0263-7855\(96\)00018-5](https://doi.org/10.1016/0263-7855(96)00018-5)
- Ishiwatari, N., Fukuoka, M., & Sakai, N. (2013). Effect of protein denaturation degree on texture and water state of cooked meat. *Journal of Food Engineering*, 117(3), 361–369. <https://doi.org/10.1016/j.jfoodeng.2013.03.013>
- Järvinen, K.-M., Beyer, K., Vila, L., Chatchatee, P., Busse, P. J., & Sampson, H. A. (2002). B-cell epitopes as a screening instrument for persistent cow's milk allergy. *Journal of Allergy and Clinical Immunology*, 110(2), 293–297. <https://doi.org/10.1067/mai.2002.126080>
- Järvinen, K.-M., Chatchatee, P., Bardina, L., Beyer, K., & Sampson, H. A. (2001). IgE and IgG binding epitopes on α -lactalbumin and β -lactoglobulin in cow's milk allergy. *International Archives of Allergy and Immunology*, 126(2), 111–118. <https://doi.org/10.1159/000049501>
- Kontopidis, G., Holt, C., & Sawyer, L. (2002). The ligand-binding site of bovine β -lactoglobulin: Evidence for a function? *Journal of Molecular Biology*, 318(4), 1043–1055. [https://doi.org/10.1016/S0022-2836\(02\)00017-7](https://doi.org/10.1016/S0022-2836(02)00017-7)
- Kontopidis, G., Holt, C., & Sawyer, L. (2004). Invited review: β -lactoglobulin: Binding properties, structure, and function. *Journal of Dairy Science*, 87(4), 785–796. [https://doi.org/10.3168/jds.S0022-0302\(04\)73222-1](https://doi.org/10.3168/jds.S0022-0302(04)73222-1)
- Lee, B., & Richards, F. M. (1971). The interpretation of protein structures: Estimation of static accessibility. *Journal of Molecular Biology*, 55(3), 379–400. [https://doi.org/10.1016/0022-2836\(71\)90324-X](https://doi.org/10.1016/0022-2836(71)90324-X)
- Lindorff-Larsen, K., Piana, S., Palmo, K., Maragakis, P., Klepeis, J. L., Dror, R. O., & Shaw, D. E. (2010). Improved side-chain torsion potentials for the Amber ff99SB protein force field. *Proteins: Structure, Function, and Bioinformatics*, 78(8), 1950–1958. <https://doi.org/10.1002/prot.22711>
- Lobanov, M. Y., Bogatyreva, N., & Galzitskaya, O. (2008). Radius of gyration as an indicator of protein structure compactness. *Molecular Biology*, 42(4), 623–628. <https://doi.org/10.1134/S0026893308040195>
- Marracino, P., Apollonio, F., Liberti, M., d'Inzeo, G., & Amadei, A. (2013). Effect of high exogenous electric pulses on protein conformation: Myoglobin as a case study. *The Journal of Physical Chemistry B*, 117(8), 2273–2279. <https://doi.org/10.1021/jp309857b>
- Qin, B. Y., Creamer, L. K., Baker, E. N., & Jameson, G. B. (1998). 12-Bromododecanoic acid binds inside the calyx of bovine β -lactoglobulin. *FEBS Letters*, 438(3), 272–278. [https://doi.org/10.1016/S0014-5793\(98\)01199-5](https://doi.org/10.1016/S0014-5793(98)01199-5)
- Robin, O., Turgeon, S., & Paquin, P. (1993). Functional properties of milk proteins. In *Dairy science and technology handbook* (Vol. 1, pp. 277–353). New York, NY: VCH Publishers.
- Schäfer, T., Böhrer, E., Ruhdorfer, S., Weigl, L., Wessner, D., Heinrich, J., ... Ring, J. (2001). Epidemiology of food allergy/food intolerance in adults: Associations with other manifestations of atopy. *Allergy*, 56(12), 1172–1179. <https://doi.org/10.1034/j.1398-9995.2001.00196.x>
- Schlick, T. (2010). *Molecular odelling and simulation: An interdisciplinary guide: An interdisciplinary guide* (Vol. 21). Berlin, Germany: Springer Science & Business Media.
- Sharma, S., Kumar, P., Betzel, C., & Singh, T. P. (2001). Structure and function of proteins involved in milk allergies. *Journal of Chromatography B: Biomedical Sciences and Applications*, 756(1), 183–187. [https://doi.org/10.1016/S0378-4347\(01\)00107-4](https://doi.org/10.1016/S0378-4347(01)00107-4)
- Singh, A., Munshi, S., & Raghavan, V. (2013). Effect of external electric field stress on gliadin protein conformation. *Proteomes*, 1(2), 25–39. <https://doi.org/10.3390/proteomes1020025>
- Singh, A., Orsat, V., & Raghavan, V. (2013). Soybean hydrophobic protein response to external electric field: A molecular odelling approach. *Biomolecules*, 3(1), 168–179. <https://doi.org/10.3390/biom3010168>
- Skripak, J. M., Matsui, E. C., Mudd, K., & Wood, R. A. (2007). The natural history of IgE-mediated cow's milk allergy. *Journal of Allergy and Clinical Immunology*, 120(5), 1172–1177. <https://doi.org/10.1016/j.jaci.2007.08.023>
- Taylor, S. L., & Hefle, S. L. (2001). Food allergies and other food sensitivities. *Food Technology-Champaign Then Chicago*, 55(9), 68–84.
- Uhrinová, S., Smith, M. H., Jameson, G. B., Uhrin, D., Sawyer, L., & Barlow, P. N. (2000). Structural changes accompanying pH-induced dissociation of the β -lactoglobulin dimer. *Biochemistry*, 39(13), 3565–3574.
- Vagadia, B. H., Vanga, S. K., Singh, A., & Raghavan, V. (2016). Effects of thermal and electric fields on soybean trypsin inhibitor protein: A molecular modelling study. *Innovative Food Science & Emerging Technologies*, 35, 9–20. <https://doi.org/10.1016/j.ifset.2016.03.004>
- Vanga, S. K., Singh, A., & Raghavan, V. (2015). Effect of thermal and electric field treatment on the conformation of Ara h 6 peanut protein allergen. *Innovative Food Science & Emerging Technologies*, 30, 79–88. <https://doi.org/10.1016/j.ifset.2015.03.003>
- Vanga, S. K., Singh, A., & Raghavan, V. (2017). Review of conventional and novel food processing methods on food allergens. *Critical*

- Reviews in Food Science and Nutrition*, 57(10), 2077–2094. <https://doi.org/10.1080/10408398.2015.1045965>
- Vendome, J., Posy, S., Jin, X., Bahna, F., Ahlsen, G., Shapiro, L., & Honig, B. (2011). Molecular design principles underlying β -strand swapping in the adhesive dimerization of cadherins. *Nature Structural & Molecular Biology*, 18(6), 693–700. <https://doi.org/10.1038/nsmb.2051>
- Vijayalakshmi, L., Krishna, R., Sankaranarayanan, R., & Vijayan, M. (2008). An asymmetric dimer of β -lactoglobulin in a low humidity crystal form—Structural changes that accompany partial dehydration and protein action. *Proteins: Structure, Function, and Bioinformatics*, 71(1), 241–249. <https://doi.org/10.1002/prot.21695>
- Wal, J. M. (1998). Cow's milk allergens. *Allergy*, 53(11), 1013–1022. <https://doi.org/10.1111/j.1398-9995.1998.tb03811.x>
- Walsh, J., Meyer, R., Shah, N., Quekett, J., & Fox, A. T. (2016). Differentiating milk allergy (IgE and non-IgE mediated) from lactose intolerance: Understanding the underlying mechanisms and presentations. *British Journal of General Practice*, 66(649), e609–e611. <https://doi.org/10.3399/bjgp16X686521>
- Wang, J., Lin, J., Bardina, L., Goldis, M., Nowak-Wegrzyn, A., Shreffler, W. G., & Sampson, H. A. (2010). Correlation of IgE/IgG4 milk epitopes and affinity of milk-specific IgE antibodies with different phenotypes of clinical milk allergy. *Journal of Allergy and Clinical Immunology*, 125(3), 695–702.
- Wang, X., Li, Y., He, X., Chen, S., & Zhang, J. Z. (2014). Effect of strong electric field on the conformational integrity of insulin. *The Journal of Physical Chemistry A*, 118(39), 8942–8952. <https://doi.org/10.1021/jp501051r>
- Welford, B. (1962). Note on a method for calculating corrected sums of squares and products. *Technometrics*, 4(3), 419–420. <https://doi.org/10.1080/00401706.1962.10490022>
- Wellner, N., Mills, E. N. C., Brownsey, G., Wilson, R. H., Brown, N., Freeman, J., ... Belton, P. S. (2005). Changes in protein secondary structure during gluten deformation studied by dynamic Fourier transform infrared spectroscopy. *Biomacromolecules*, 6(1), 255–261. <https://doi.org/10.1021/bm049584d>
- Wood, R. A. (2003). The natural history of food allergy. *Pediatrics*, 111(Supplement 3), 1631–1637.
- Wu, S.-Y., Pérez, M. D., Puyol, P., & Sawyer, L. (1999). B-Lactoglobulin binds palmitate within its central cavity. *Journal of Biological Chemistry*, 274(1), 170–174. <https://doi.org/10.1074/jbc.274.1.170>
- Zuberbier, T., Edenharter, G., Worm, M., Ehlers, I., Reimann, S., Hantke, T., ... Niggemann, B. (2004). Prevalence of adverse reactions to food in Germany—A population study. *Allergy*, 59(3), 338–345. <https://doi.org/10.1046/j.1398-9995.2003.00403.x>

How to cite this article: Saxena R, Vanga SK, Raghavan V. Effect of thermal and microwave processing on secondary structure of bovine β -lactoglobulin: A molecular modeling study. *J Food Biochem*. 2019;43:e12898. <https://doi.org/10.1111/jfbc.12898>