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Structural responses of kiwifruit allergen Act d 2 to thermal and electric field stresses based on molecular dynamics simulations and experiments

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Kiwifruit is considered to be the most common plant-based food causing allergic reactions, after peanuts, soybeans, and wheat. These responses are triggered by the proteins in kiwifruit. Modifying the structures of these allergenic proteins (epitopes) through external stresses may decrease their specific binding capacity with antibodies, resulting in a decrease in kiwifruit allergenicity. To provide a visual insight into the structural changes of Act d 2, we treated it with a thermal force and an oscillating electric field using molecular dynamics (MD) simulations. The results showed that Act d 2 is a heat-stable protein, while the electric field combined with thermal stress significantly affected its secondary structure and surface properties, which could result in conformational changes. The results obtained from ELISA tests showed that the combination of the thermal force and electric field significantly reduced the Ig-E binding capacity of Act d 2 by 75.3%, accompanied with obvious losses of alpha-helix and turn structures.

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1. Introduction

Food allergies are mainly caused by the exposure of patients to allergenic proteins in various food sources.¹ In 2008, the American Center for Disease Control and Prevention reported that an 18% increasing trend in the prevalence of food allergies was observed from 1997 to 2007 among children.² Nowadays, up to 10% of the total population suffers from food allergies worldwide.^{3,4} Among all food allergies, kiwifruit is considered to be the most common plant-based food source causing allergic reactions after the big eight.^{5,6} Kiwifruit has been recognized as the fourth most common food source causing allergy after beef, milk, and cocoa among young children (6–9 years old) in Turkey.⁷ Up to 50% of patients with food allergy histories are also allergic to kiwifruit due to cross-reactivity, according to a self-report analysis in Sweden and Denmark.⁸ In Spain, approximately 1.8% of the total population is allergic to kiwifruit.⁹ Furthermore, studies have reported that the prevalence of kiwifruit allergy is increasing worldwide because of the cross-reactivity between kiwifruit allergy and pollen (e.g., tree or grass) allergies or other plant-based food (e.g., avocado) allergies.^{10–14} Kiwifruit allergy can trigger various symptoms, which are classified as oral allergy syndrome (OAS) and life-threatening anaphylaxis.¹⁵ OAS is gen-

erally accompanied with some mild allergic reactions, such as itching and tingling in the skin, which can be improved in a natural way or by using antihistamines in a short time.¹⁰ However, the severe, life-threatening anaphylaxis reactions, including blood pressure decrease, heart rate increase, and loss of consciousness, may even result in death if there is no immediate treatment.¹⁶ Therefore, kiwifruit allergy is becoming a major issue related to food safety and human health.

To date, several studies have been reported regarding the modification of allergenic kiwifruit proteins through different food processing methods. For example, 10 min steam cooking at 100 °C decreased the concentration of two major kiwifruit allergens (Act d 1 and Act d 2) compared to untreated samples.¹⁷ This decrease in the allergen concentrations may be due to the changes in their tertiary and secondary protein structures under external thermal stress, which in turn can alter their ability to induce allergic responses.^{18,19} Moreover, the allergenicity of Act d 1 and Act d 2 can be averted after two-stage enzymic treatments, including pepsin and trypsin or chymotrypsin.²⁰ However, these processing methods, including steam cooking and enzymatic hydrolysis, function inefficiently but also result in high nutrient loss of the treated products during processing. Therefore, novel and more effective processing modifications to decrease kiwifruit allergenicity are needed.

Molecular dynamics (MD) simulations have shown potential applications in visually evaluating the structural and dynamical changes of proteins under external stresses (e.g., high temperatures) at the molecular level. Recently, the MD simu-

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lation technique has been used to observe structural changes in peanut¹ and soybean^{21,22} allergens under external stresses (e.g., high temperatures). This technique can contribute visually to understanding the folding and unfolding of protein structures under different external stresses in a system. However, no related studies have been performed on the modification of kiwifruit allergen Act d 2 under external stresses using MD simulations. In the present study, Act d 2 was selected as a simulation subject. During the MD simulation, Act d 2 was treated with thermal and electric field stresses. Hopefully, the results obtained from the simulations can provide a visual prediction of the structural changes of kiwifruit allergen Act d 2 and help identify a new strategy to address kiwifruit allergy-related issues.

2. Materials and methods

2.1. MD simulations

According to previous studies, MD simulations were performed using Groningen Machine for Chemical Simulations (GROMACS) software (version 5.0.4, Stockholm Center for Biomembrane Research, Stockholm, Sweden) based on the classical MD algorithm.¹ Act d 2, a major allergen present in kiwifruit, was used in the simulations. The protein data file was obtained from the Protein Data Bank (PDB) with access code 4BCT.²³ CHARMM27 and the TIP3P water model were selected as the force field and solvent, respectively.²⁴ The protein Act d 2 was transferred into a periodic water box with the dimensions $7.799 \times 7.799 \times 7.799$ containing 15 089 water molecules. The system was neutralized using the TIP3P water model and was then minimized for energy by a converging criterion at $10 \text{ kJ nm}^{-1} \text{ mol}^{-1}$ of force value using the steepest descent for 50 000 steps followed by 100 ps equilibrations to the constant pressure, constant temperature (NPT) and constant volume, constant temperature (NVT).

During the MD simulations, the Berendsen thermostat was used to maintain the desired temperatures, and the Parrinello–Rahman barostat was applied to set the pressure at one bar. As shown in Table 1, the duration of the MD simulations was set at 2000 picoseconds to evaluate the structural response of Act d 2 to different temperatures (300 K as a control, 325 K, 350 K, and 375 K) and an external oscillating electric field with an intensity of 0.05 V nm^{-1} at a frequency of 2.45 GHz. Visual Molecular Dynamics (VMD) software (version 1.9.3, University of Illinois, Urbana-Champaign, USA) was used

to obtain the structural diagrams, and the STRIDE algorithm implemented in the software was utilized to characterize the changes in Act d 2 after thermal processing and electric field treatment.²⁵ Several parameters, such as the root mean square deviation (RMSD) of the backbone atoms, the root mean square fluctuations (RMSF) and surface hydrophobicity, were evaluated using GROMACS software.²⁶ VMD was also utilized to acquire snapshots of the conformational changes of the protein.¹

2.2. Analysis of root mean square deviation (RMSD)

Recently, the RMSD has been applied to a peanut protein (Ara h 6, peanut allergen) and soybean protein (1AVU, soybean trypsin inhibitor) to analyze and predict the arithmetical values of the deviation in their molecular structures under different external stresses.^{1,21} The following equation was used to calculate the RMSD of the kiwifruit protein Act d 2:²⁷

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

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

2.3. Analysis of root mean square fluctuations (RMSF)

The RMSF is a measurement of the deviation between the position of atom i and some reference position in a protein molecule.²⁸ During the MD simulations, the RMSF changes in Act d 2 were measured by the following equation:²⁹

$$\text{RMSF}_i = \sqrt{\frac{1}{T} \sum_{t_i=1}^T |r_i(t_i) - r_i^{\text{ref}}|^2} \quad (2)$$

where T is the time and r_i^{ref} is the reference position of atom i .

2.4. Measurement of solvent accessible surface area (SASA)

The SASA is the surface area of a molecule which is available to interact with solvents and molecules. Generally, the SASA is used as a tool to evaluate the interactions between a protein and solvents, which in turn can be applied to obtain the characteristics and functions of the protein.²¹ Recently, studies have been reported where the surface properties of a protein are highly dependent on its structural conformations, and changes in its functional properties can be caused by a slight

Table 1 Summary of parameters used in the MD simulated systems

Protein	Thermal	Combination of oscillating electric field and thermal	Duration
Act d 2 (kiwifruit allergen, PDB accession code: 4BCT)	300 K (control)	0.05 V nm^{-1} , 2450 MHz + 300 K	2000 picoseconds
	325 K	0.05 V nm^{-1} , 2450 MHz + 325 K	2000 picoseconds
	350 K	0.05 V nm^{-1} , 2450 MHz + 350 K	2000 picoseconds
	375 K	0.05 V nm^{-1} , 2450 MHz + 375 K	2000 picoseconds

change in its conformation.^{1,21,27} In this study, built-in commands present in the GROMACS software were used to evaluate SASA changes. The following equation was used to calculate the SASA of the protein Act d 2:¹

$$\text{SASA} = A = \sum \left(R / \sqrt{R^2 - Z_i^2} \times D \times L_i \right) \quad (3)$$

where A is the surface area, R is the radius of the atom, L_i is the length of the arc drawn on a given section i , and Z_i is the perpendicular distance of section i from the center of the sphere.

2.5. Thermal and electric field treatment

Recombinant Act d 2 (soluble powder purchased from Elabscience, USA) was dissolved in double distilled water at a concentration of 0.1 mg mL⁻¹. The protein solution was randomly divided into two groups, which were heated in water baths for one min at 325 K, 350 K, and 375 K, respectively. Untreated samples at room temperature (300 K) were set as controls. The samples were cooled to room temperature; then, one of two groups was transferred into a chamber and treated with an electric field (PPS 30, Waterloo, ON, Canada) for 60 s at 5 Hz and 15 V cm⁻¹. After treatment, the samples were stored at 4 °C until further analysis.

2.6. Circular dichroism (CD) spectroscopy analysis of Act d 2

A J-815 spectropolarimeter (JASCO, Tokyo, Japan) with a temperature-controlled cuvette holder (cuvette path length: 0.1 cm) was used to determine the secondary structure (α -helices, β -sheets, and turns) of Act d 2.³⁰ Each sample was scanned in the range of 260 nm to 190 nm, with a bandwidth of 1 nm and a pitch of 0.2 nm. The scanning speed was set at 50 nm min⁻¹ in a continuous model. Each spectrogram obtained was an average of five spectral accumulations. The CD analysis was performed using CDPro Software.

2.7. Allergen binding capacity determination using an ELISA test

Biotin-conjugated rabbit polyclonal antibodies and recombinant Act d 2 were purchased from Elabscience (USA). Immunodetection of the protein (Act d 2) was performed by a direct ELISA test. After the reaction, the optical density (OD) was measured spectrophotometrically at a wavelength of 405 nm according to the protocol of the kit.³¹ The binding capacity of Act d 2 with its specific antibody was calculated according to the following equation:

$$\text{Act 2 binding capacity (\%)} = (C_0 - C_1)/C_0 \times 100 \quad (4)$$

where C_0 is the initial concentration of Act d 2 and C_1 is the final concentration of Act d 2 after thermal and electric field processing.

2.8. Statistical analysis

The data obtained from MD simulations under external thermal and electric field stresses were analyzed by one-way

analysis of variance (ANOVA) followed by Duncan's multiple range test ($P < 0.05$) using SPSS 22.0 analytical software (SPSS Inc., Chicago, USA). All the MD simulations and experimental studies were performed in triplicate.

3. Results and discussion

3.1. Secondary structure of Act d 2

Act d 2 is a member of the thaumatin-like protein (TPL) family and has a molecular weight of 20 to 26 kDa.³² In the plant kingdom, TPLs are very common proteins which can be highly expressed when plants undergo stresses (*e.g.*, drought) or pathogen infections to activate their defense systems.^{33–35} From the Protein Data Bank, it can be seen that the secondary structure of act d 2 contains 38% β -sheets (18 strands; 77 residues) and 13% helices (6 helices; 28 residues) with 201 amino acid residues (Fig. 1). The six helices consist of four α -helices and two 3/10 helices. As shown in Fig. 1a, four α -helices are present between residues 150–153 (Iso-Glu), 167–171 (Pro-Phe), 174–177 (Asp-Cys), and 187–195 (Asp-Arg). The two 3/10 helices are formed between residues 156–158 (Asp-Leu) and 206–208 (Asp-Thr). β -Sheets can be found between residues 26–31 (Thr-Asn), 36–42 (Thr-Val), 44–49 (Gly-Leu), 53–57 (Gln-Ile), 66–75 (Ala-Asn), 82–86 (Gly-Thr), 104–113 (Thr-Phe), 116–122 (Leu-Ser), 130–135 (Ala-Pro), 144–146 (Ile-Cys), 210–214 (Thr-Pro), and 218–223 (Asn-Phe).

The visual molecular dynamics (VMD) STRIDE algorithm has been applied to a peanut protein (Ara h 6) and a soybean protein (trypsin inhibitor) to visualize their secondary structure changes under different external stresses, such as thermal or electric field stresses.^{1,21,22} In the present study, the VMD STRID technique was used to evaluate the structural responses of kiwifruit allergen Act d 2 to external thermal and electric field stresses. The values of the Y and X-axes represent the residues of the protein and the time/frames, respectively (Fig. 2). Each band with distinct colors matches the different residues present in the secondary structure of Act d 2 under various simulation conditions. The results showed that Act d 2 is quite stable under different external stresses, which can be attributed to the formation of disulphide bonds between the cysteine residues 33–224, 74–84, 89–95, 140–213, and 146–1961.^{1,22} As presented in Fig. 2a and b, some obvious disruption was observed between residues 91–103 (frame 55–176), 175–181 (frame 67–112), and 199–211 (frame 144–200) when the temperature increased from 300 K to 325 K. A higher amount of loss of the turn structures was observed in the protein when the temperature was raised further. Specifically, the turn structure between residues 121–127 was replaced with a large amount of 3/10 helices under 375 K thermal stress (Fig. 2d). At the same temperature, more 3/10 helix structures were produced between residues 175–187 compared to at 300 K. This indicates that the turns present in Act d 2 are sensitive to heat; once the temperature reaches the required level, the turns are replaced with 3/10 helix structures. Similar results were obtained for the secondary structure of an electric field-treated

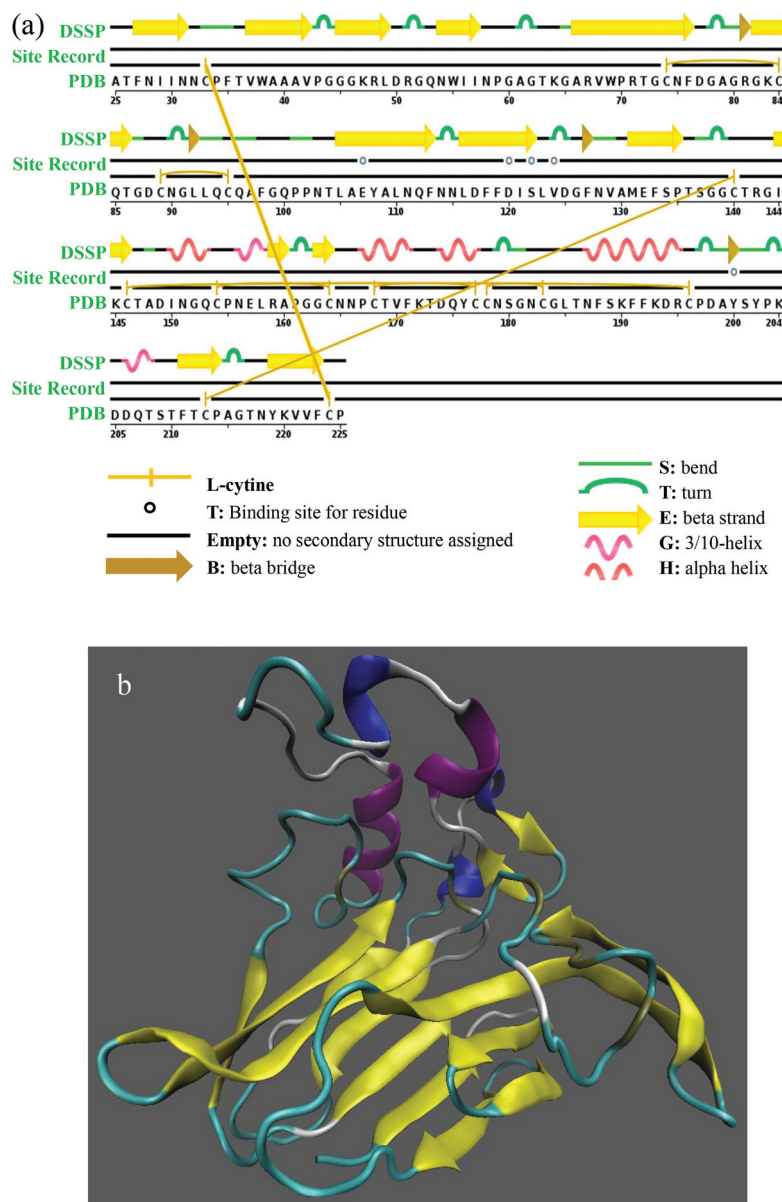


Fig. 1 (a) FASTA sequence of kiwifruit allergen Act d 2 (PDB accession code: 4BCT); (b) secondary structure of kiwifruit allergen Act d 2 (source: Protein Data Bank, α -helix: purple, 3/10 helix: blue, η -helix: red, β -sheets: yellow, turns: cyan, coils: white).

soybean allergen molecule.²² Moreover, as shown in Fig. 2e and f, a significant disruption of turn structures between residues 55–67 was observed under electric field treatment (0.05 V nm^{-1} , 2.45 GHz) at 325 K compared to at 300 K . Furthermore, the turn structure transformed into a beta strand between residues 55–67, and a 3/10 helix formed between residues 151–157 (Fig. 2f). The α -helix transformed into turn and 3/10 helix structures between residues 169–175 (frame 112–184) and 205–211 (frame 91–136) when simulated with the electric field at 325 K . More transformations of turn (Asn (residue: 151)-Glu (residue: 157)) and alpha-helix (residues: 169–175) structures into 3/10 helices were observed when the temperature increased from 300 K to 350 K and 375 K under the electric

field (Fig. 2g and h). Similar results were obtained for Ara h 6 (peanut allergen) and trypsin inhibitor (soybean allergen) when simulated under a combination of thermal and electric field stresses.^{1,21} Therefore, it is clear that the combination of thermal and electric field treatment showed potential impacts on the secondary structure of Act d 2 through changing the turn and helix structures in the protein.

3.2. Root mean square deviation (RMSD) of Act d 2

As shown in Fig. 3, no obvious differences were observed in the RMSD of Act d 2 when simulated under thermal stress at 325 K and 375 K compared to at 300 K , while the RMSD values

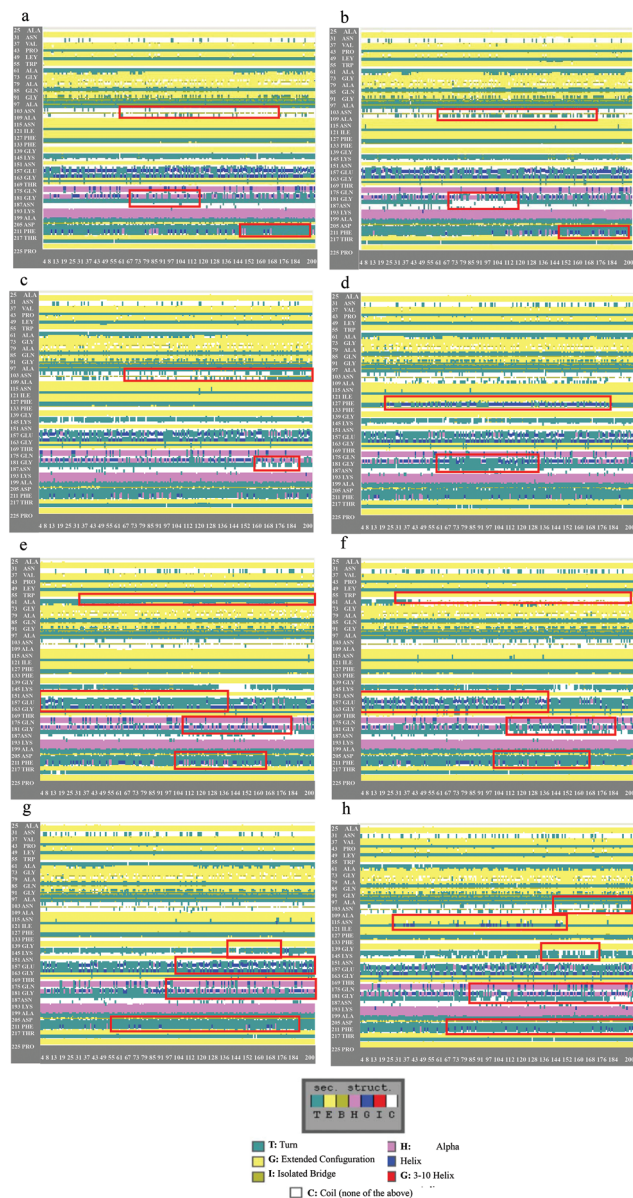


Fig. 2 Secondary structure changes of Act d 2 after thermal treatment at (a) 300 K, (b) 325 K, (c) 350 K, (d) 375 K and thermal + oscillating electric field treatment at 2450 MHz: (e) 300 K, 0.05 V nm^{-1} ; (f) 325 K, 0.05 V nm^{-1} ; (g) 350 K, 0.05 V nm^{-1} ; (h) 375 K, 0.05 V nm^{-1} through STRIDE analysis. Note: color code: Magneta denotes α (alpha)-helix, red denotes π (pi)-helix, cyan denotes turn, blue denotes 3/10 helix.

obtained at 350 K were obviously higher than those obtained at 300 K and with other treatments (Fig. 3a–c). This significant enhancement may be due to the increased thermal sensitivity of Act d 2 during thermal simulation in the range of 300 to 350 K. No further increase of the RMSD was observed even when the processing temperature was increased to 375 K, which can be attributed to the fact that the effects of external thermal stress on the RMSD of Act d 2 reached a maximum level at 350 K.¹ Similar results were reported for Ara h 6 peanut allergen¹ and soybean trypsin inhibitor protein²¹ subjected to

simulated thermal stress at 300 to 425 K. In comparison to thermal stress alone, the combination of the electric field at 0.05 V nm^{-1} , 2.45 GHz with thermal stress significantly increased the RMSD values with increasing treated temperature (Fig. 3d–f). Specifically, the RMSD of Act d 2 showed a significant jump when the temperature increased further to 375 K (10.41 nm) from 300 K (9.65 nm) (Fig. 3f). These continual increases in the RMSD of Act d 2 within a specific temperature range may be due to the cumulative effects of thermal and electric field stresses.²¹

3.3. Root mean square fluctuation (RMSF) determination of Act d 2

The fluctuation of atoms present in Act d 2 increased with increasing applied temperature from 300 K to 375 K during the thermal simulation (Fig. 4a). Specifically, two sharp peaks were observed around the atoms 1750 and 2250 under 325 K thermal treatment compared to the control (300 K). Several sharp peaks were found under 350 K thermal simulation at atoms 300, 600, 800, 1100 and 1800. As the temperature further increased, more sharp peaks formed during the 375 K thermal simulation compared to the others. These sharp peaks were also obtained when a milk allergen (bovine β -lactoglobulin) was treated with an electric field combined with high temperature.³⁶ The formation of these sharp peaks can be attributed to secondary structure changes, such as turns and helices (Fig. 2a–h). As shown in Fig. 4b, similar results were obtained during the simulation of the oscillating electric field at 2.45 GHz combined with thermal stress. The fluctuation of atoms in the protein molecule showed the highest frequency at 375 K, followed by 350 K, 325 K, and 300 K. This indicates that the mobility of atoms in a protein molecule can be effectively improved by increasing the applied temperature under electric field stress, which may enable modification of the structure of the protein under certain temperatures.

3.4. Solvent accessible surface area (SASA) measurements of Act d 2

The SASA of Act d 2 showed a fluctuating trend with increasing applied temperature (Fig. 5). Specifically, a significant increase in the surface area of Act d 2 was observed under the thermal simulation at 325 K compared to that at 300 K. This enhancement can be attributed to the new binding sites (Fig. 6b) which became available on the surface of the molecule of Act d 2 under appropriate thermal conditions (325 K).²¹ The surface area of Act d 2 returned to the initial level with increasing temperature from 300 K to 350 K; then, its value showed a decreasing trend during thermal treatment at 375 K (Fig. 5a). In comparison to the 300 K thermal treatment, the surface area of Act d 2 showed a significant increase during the simulation of the electric field at 0.05 V nm^{-1} , 2.45 GHz, 300 K (Fig. 5b). This indicates that the electric field produced additional stresses on the protein structure during the simulation, which in turn could lead to volatile properties in the

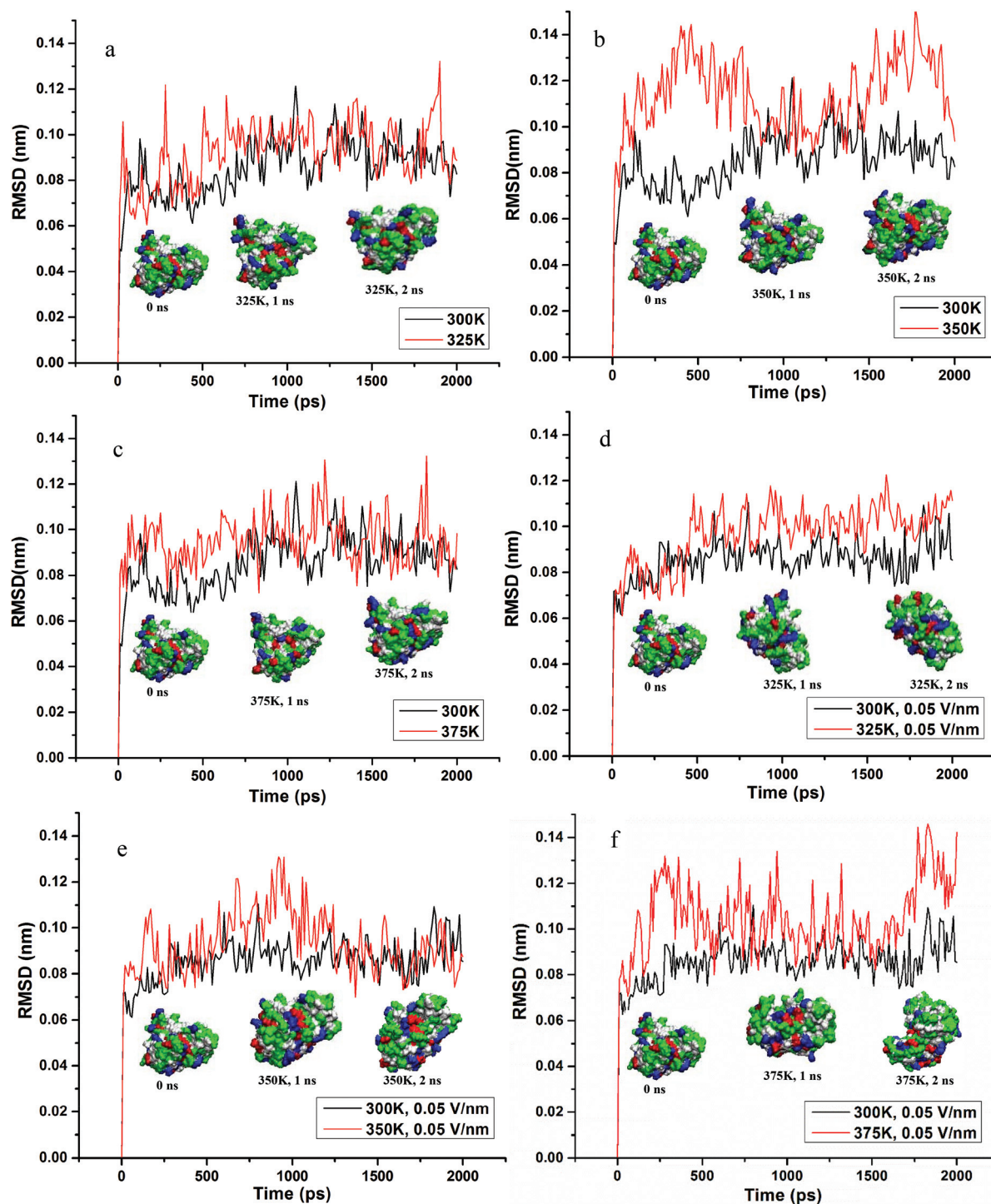


Fig. 3 RMSD variations observed in kiwifruit allergen Act d 2 under (a–c) thermal treatment and (d–f) thermal + oscillating electric field treatment at 2450 MHz.

protein. However, there was a decreasing trend in the SASA of Act d 2 when the applied temperature increased from 300 K to 350 K (Fig. 5b). Moreover, the surface area values of Act d 2 remained almost constant at 375 K. Similar results have been observed with a soybean protein under thermal and electric field simulations.²¹ These fluctuations may be due to the

application of the electric field, which can force the amino acid residues of Act d 2 to turn depending on the direction of the applied electric field.²¹

Surface properties have been used to measure changes in the functional characteristics of proteins in peanut¹ and soybean.²⁷ In the present study, we obtained snapshots of the

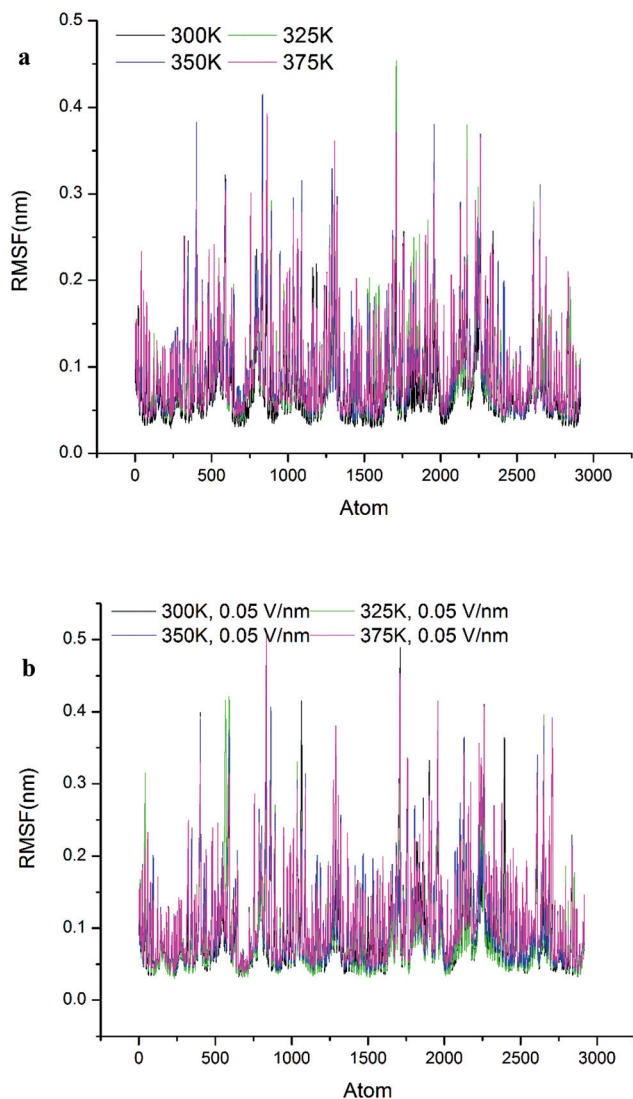


Fig. 4 Root mean square fluctuations (RMSF) observed in kiwifruit allergen Act d 2 under (a) thermal treatment and (b) thermal + oscillating electric field treatment at 2450 MHz.

molecular structures of the protein (Act d 2) treated with two external stresses, namely thermal and electric field, using VMD. As presented in Fig. 6a–d, there was a slight change in the molecule structure of Act d 2 under thermal simulation from 300 K to 375 K. This indicates that thermal stress alone does not effectively change the surface properties of the protein molecule due to its heat-stable properties. However, a significant change in the molecular structure of Act d 2 allergen was observed after an oscillating electric field (0.05 V nm^{-1} , 2.45 GHz) was applied at 300 K (Fig. 6e) compared to standalone thermal stress at 300 K (Fig. 6a). This change in the molecular structure increased the surface area of Act d 2 (Fig. 5). The secondary structure of Act d 2 was completely modified by the end of the simulation at 0.05 V nm^{-1} , 2.45 GHz , and 375 K (Fig. 6h), which led to a significant decrease in the surface area of Act d 2 compared to the initial level

(Fig. 5b). These significant changes in the molecular structure of the Act d 2 allergen can be attributed to the application of the electric field combined with thermal stress. Many studies have reported that there is a strong relationship between the surface properties and the structures of molecules.^{1,27} The surface properties of proteins highly rely on their molecular structures. Changes in structure under external stresses can cause changes in the surface properties, leading to significant changes in the functional properties of the related proteins or allergens.

3.5. Secondary structure and Ig-E binding capacity of Act d 2

In the present study, we analyzed the differences in the secondary structure of Act d 2 after thermal and electric field treatment using circular dichroism (CD) spectroscopy. The results indicated that the contents of alpha helix and beta sheet structures in Act d 2 showed increasing trends with increasing temperature under thermal simulation; meanwhile, the turn structure showed a decreasing trend, and higher turn structure loss was observed under thermal simulation at 350 K compared to the other treatments (Fig. 7a). The unordered structure of Act d 2 remained at the initial level. These experimental findings are in accordance with the observations obtained from MD simulations under thermal stress (Fig. 2a–d). The turn structures present in Act d 2 are sensitive to heat force and are prone to be replaced by other structures, such as 3/10 helices (Fig. 2d); meanwhile, other structures (*e.g.*, alpha helix) are thermally stable without significant differences due to the presence of disulphide bonds in Act d 2.³⁷ In comparison to thermal modification, the electric field combined with thermal stress obviously modified the secondary structure of Act d 2. Specifically, the alpha helix content significantly decreased from 9.2% to 3.2% with increasing temperature from 300 K (control) to 375 K under electric field simulation (Fig. 7b). A similar trend was observed in the turn structure, and an obvious decrease was found under electric field treatment at 325 K; this agrees with the results observed in the MD simulations (Fig. 2f). However, the beta sheet content showed an increasing trend with increasing temperature (Fig. 7b). The decreased amount of alpha helices present in Act d 2 can be attributed to the application of the electric field, which provides additional force to break or modify its structures under the high temperatures.³⁸ Similar observations have been reported for a milk allergen (bovine β -lactoglobulin),³⁶ a soybean allergen (7S-globulin),³⁹ and an egg white protein (avidin);³⁰ these studies found that external stresses (*e.g.*, ultrasound) can potentially modify the secondary structures of the proteins (alpha-helix, beta-sheet and turn).

Further, many studies have reported that the allergenicity of some foods can be decreased by changing the structures of their proteins under external stresses (*e.g.*, high temperature). For example, soy protein isolate was treated with high hydrostatic pressure (HHP) at 200 to 500 MPa for 15 min. After HHP processing, the allergen content of soybean was decreased by

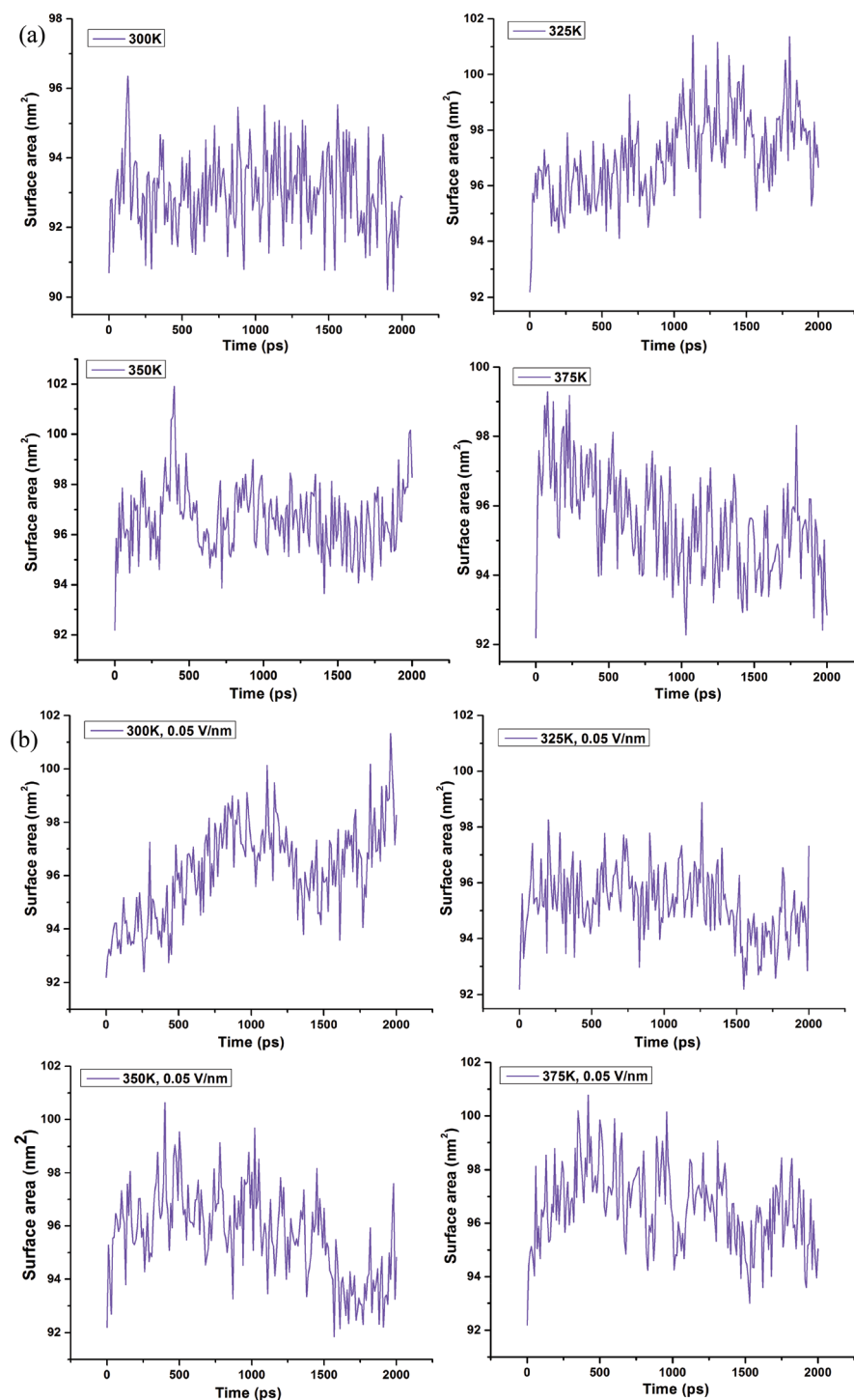


Fig. 5 Solvent accessible surface area changes of kiwifruit allergen Act d 2 under (a) thermal treatment and (b) thermal + oscillating electric field treatment at 2450 MHz.

45%, accompanied by increases in the turn and alpha-helix contents and decreased amounts of beta-sheet structures.³⁹ In the present study, we determined the allergen binding capacity of Act d 2 with a specific antibody after thermal and electric field treatments. As shown in Fig. 7c, the Ig-E binding capacity

of Act d 2 showed a decreasing trend with increasing temperature under thermal processing from 300 K to 375 K. Specifically, the lowest binding capacity of Act d 2 was observed after treatment at 350 K for one min compared to the untreated sample (300 K) and other treatments. Although

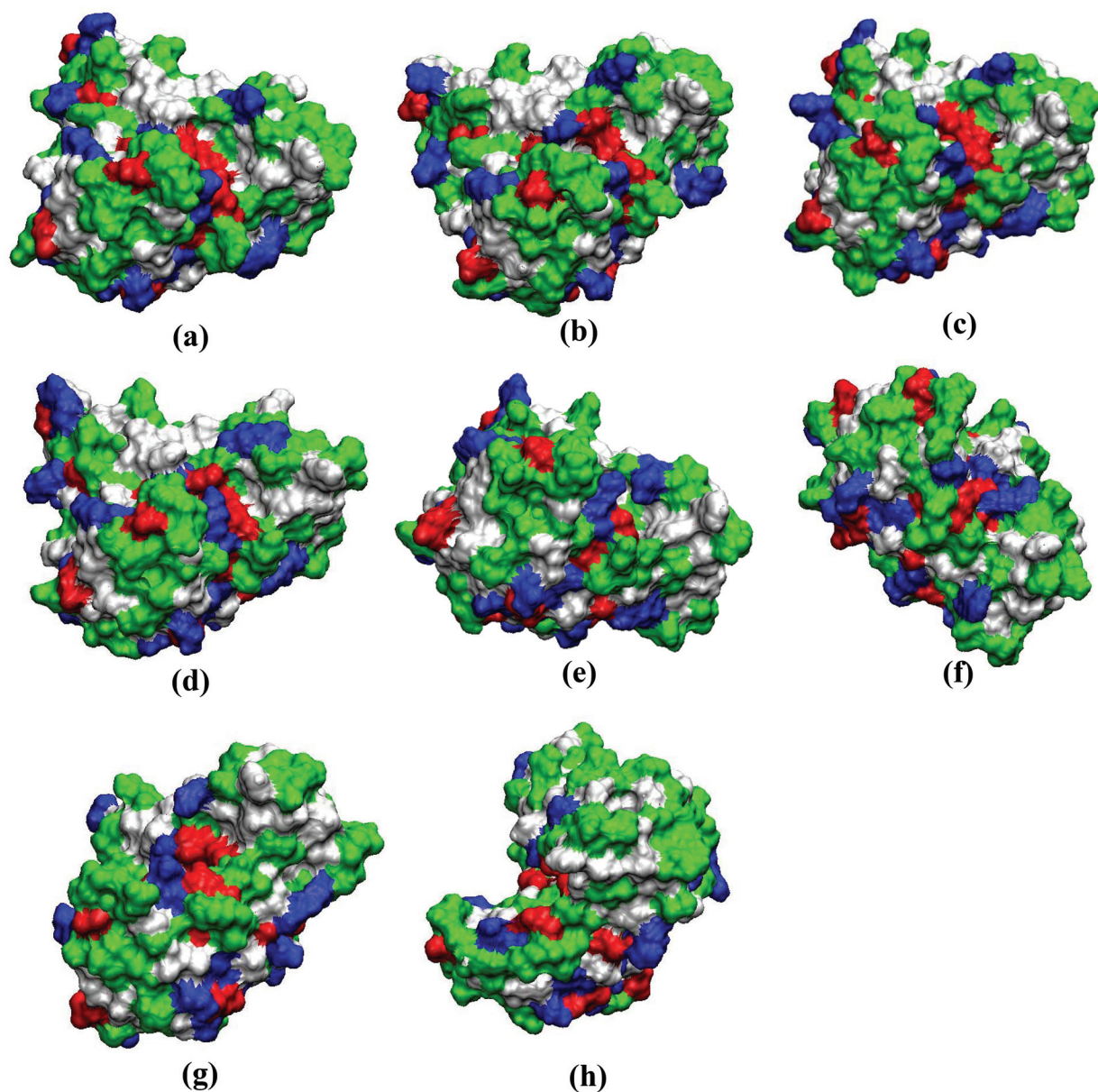


Fig. 6 Snapshots of the surface properties of Act d 2 after thermal simulations: (a) molecule structures at 300 K; (b) 325 K; (c) 350 K; (d) 375 K; and electric field simulation at 2450 MHz (e) molecule structures at 300 K; (f) 325 K; (g) 350 K; (h) 375 K. Note: residue color code: non-polar (white), basic (blue), acidic (red) and polar (green).

there was a slight increase in the Ig-E binding capacity of Act d 2 after treatment at 375 K, no significant differences were observed compared to that treated at 350 K and 325 K. This may be because that the modifying effects on the potential decrease of allergenicity have reached the maximum value. The slight decrease in the binding capacity of Act d 2 can be attributed to its secondary structure changes (turn) under thermal processing.^{19,38} The combination of thermal and electric field treatment significantly decreased the Ig-E binding capacity of Act d 2 (Fig. 7c). The lowest binding capacity of Act d 2 (27.3%) was observed when treated at 375 K, followed by 350 K (49.2%), 325 K (58.8%), and 300 K

(75.3%); this may be due to the decreased amounts of alpha helix and turn structures present in Act d 2 under the combination treatment (Fig. 7b). Thus, the combination of thermal and electric field treatment shows potential applications in decreasing the allergenicity of the kiwifruit allergen Act d 2.

4. Conclusions

In the study, results obtained from thermal and electric field stress simulations showed that kiwifruit allergen Act d 2 is a

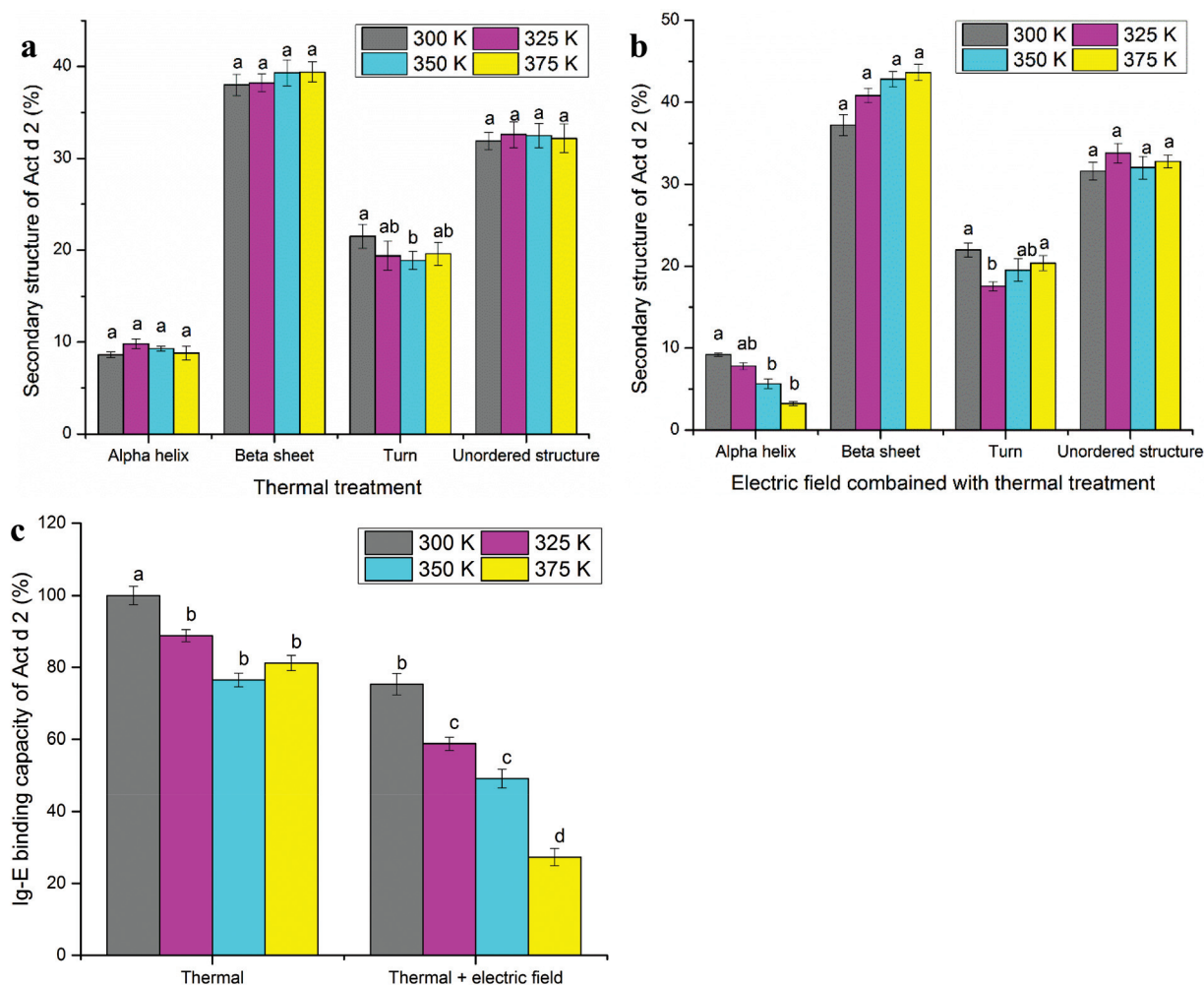


Fig. 7 Experimental analyses of the secondary structures of Act d 2 under thermal (a) and electric field (b) treatment and allergenicity changes of Act d 2 (c). Note: values with different letters in various columns are significantly different ($p < 0.05$) from each other.

thermal-stable protein. There were no significant changes in the secondary structure under thermal processing, even when the processing temperature was increased to 375 K. However, the secondary structure of Act d 2 started to change under the combination of thermal and electric field treatment at 300 K and showed significant changes when the temperature increased to 375 K. The results obtained from the experimental study were in accordance with the simulations. After thermal processing, no significant differences were observed in the secondary structure of Act d 2 using CD spectroscopy except for a slight decrease in the turn structures. Similarly, the allergenicity of Act d 2 remained at the initial level. However, the combination of thermal and electric field treatment significantly decreased the Ig-E binding capacity of Act d 2 by 75.3% when treated at 375 K, accompanied by significant decreases in the alpha helix and turn structures of Act d 2. This suggests that the decreased allergenicity of Act d 2 is due to the loss of alpha helix and turn structures under these external stresses. The combination of thermal and electric field treatment can be considered as an alternative processing method to decrease

the allergenicity of kiwifruit. However, the immune responses of kiwifruit-allergic individuals to these processed proteins are unknown, and further clinical studies are needed. Further, in subsequent research, the simulation duration should be increased from 10 to 50 ns to further analyze the protein structure changes under external conditions.

Abbreviations

MD	Molecular dynamics
OAS	Oral allergy syndrome
GROMACS	Groningen machine for chemical simulations
PDB	Protein Data Bank
NPT	Constant pressure, constant temperature
NVT	Constant volume, constant temperature
VMD	Visual molecular dynamics
RMSD	Root mean square deviation
RMSF	Root mean square fluctuations
SASA	Solvent accessible surface area

CD	Circular dichroism
OD	Optical density
ANOVA	One-way analysis of variance
TPLs	Thaumatococcus-like proteins
HHP	High hydrostatic pressure.

Conflicts of interest

The authors declare no competing financial interest.

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