Computational evaluation of the effect of processing on the trypsin and alpha-amylase inhibitor from Ragi (*Eleusine coracana*) seed

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Ragi, also known as Finger millet, has been promoted as a healthy alternative to major cereals such as rice and wheat. It is a well-known source of various minerals, dietary fiber, and a primary source of carbohydrate in parts of Asia and Africa. Ragi is a drought resistant crop, hence, useful for adapting to the current climate change and depleting water resource conditions in various parts of the world. However, the Ragi is known to have poor digestibility, primarily due to the presence of antinutritional compounds like alpha-amylase and trypsin inhibitor.

We have studied temperature, static electric fields (SEFs) and oscillating electric fields (OEFs) to evaluate the secondary structure changes of alpha-amylase and trypsin inhibitor molecule in Ragi. This was simulated at three temperatures: 300, 343, and 373 K with SEF and OEF at an intensity of 1 V/nm with a frequency of 2.45 GHz (for OEF). STRIDE analysis exhibits various changes in the secondary structure of the protein, especially the loop connecting the alpha helices together. Thermal processing alone has also affected the second alpha helix of the molecule. Overall, the SEFs of 1 V/nm are found to have resulted in the most secondary structure deviations in terms of root mean square deviation and radius of gyration.

**KEYWORDS**

alpha-amylase, antinutrient, Finger millet, Ragi, trypsin inhibitor

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**1 | INTRODUCTION**

Superfoods have become an obsession in western culture, and the term became popular in the 2000s and escalated quickly. “Superfood” refers to food rich in nutrients and extremely beneficial for health. Popular superfoods are quinoa and chia seed. Quinoa is high in anti-inflammatory phytonutrients and fiber, making it beneficial to the heart and preventing/treating disease. Quinoa contains all nine essential amino acids making it a complete protein and is slowly digested, therefore making it an excellent low-GI option. Chia seeds are comprised of all nine essential amino acids and several different phytochemicals with unique benefits containing antioxidant, anti-inflammatory, and anticancer features. They also contain a high concentration of omega-3 essential fatty acids, attributing to heart, artery wall, and cell wall health,
and consist of 37% fiber. Both these superfoods are gaining popularity to a growing health-conscious culture. Under the condition of world’s population growth heading toward 9.5 billion in 2050, superfoods are growing in importance as society demands increasingly healthier diets and the need to feed the undernourished around the world.

Finger millet is a very popular grain that can be considered as an entry superfood due to its nutritional value and health benefits. *Eleusine coracana*, Finger millet, or Ragi is a small, light brown colored (dehusked) seed with a diameter of about 1 to 2 mm. Millets are forage grasses, belonging to the Poaceae family. There are many different types of millets grown around the world, the main ones being pearl millet, Finger millet, proso millet, and foxtail millet. Finger millet is an excellent source of calcium (0.38%), phenolic compounds (0.3%-3%), and dietary fiber (18%). Consumption of Finger millet also has potential health benefits due to its antidiabetic, antitumorigenic, and atherosclerogenic effects. It is also considered to have components that exhibit antioxidant and antimicrobial properties and is gluten free.

Finger millet is primarily grown and consumed in developing countries including India and Africa. Although Finger millet has been a staple ingredient in Indian and East African cuisines for thousands of years, it has not had widespread integration in western cuisines. The potential market for Finger millet is primarily due to its many advantages, including high nutritional value, functional properties, short growing season, and it can tolerate environmental stresses such as hot, arid, and drought-prone temperaments. The California drought from 2011 to 2017 was the most severe in the past century. The warm temperatures with dry soil had a significant impact on the food industry and caused concern for food security. If viable, Finger millet would be an excellent substitution. Another benefit with the cultivation of Finger millet is that it can be stored for longer periods compared to few other grains when appropriate post-harvest measures are taken. The resilient nature and high nutritional content of Finger millet make it a superior grain to grow for combating issues like food security and climate change.

However, one major disadvantage with Finger millet is its poor digestibility, associated with the presence of antinutritional factors such as trypsin and alpha-amylase inhibitors, which inhibit the actions of trypsin, alpha-amylase, and other proteases in the gut, preventing the digestion and subsequent absorption of protein. Digestibility can be increased with food processing techniques such as drying, freezing, filtration, heating, baking, and popping because these techniques can significantly disable the enzyme inhibitors, which, in turn, increases the digestibility. Increased digestibility can be attributed to the change in protein structure affecting the protein quality and quantity. A protein’s function changes under external stresses such as heat, pH, chemical, and electromagnetic fields, leading to denaturation and loss of function. These processing techniques can alter the structure of the protein found in Finger millet in both a positive and negative way. The digestibility can be improved with processing methods, but the protein structure can be altered, which decreases the amount of protein in the millet, an undesirable outcome.

In this study, the effects of temperature, static electric fields (SEFs), and oscillating electric fields (OEFs) on the conformation of bifunctional trypsin and alpha-amylase protein of Finger millet were analyzed by using molecular dynamic (MD) simulation to determine the best approach to altering the protein structure to increase digestion. MD simulation models structural changes that occur in the Finger millet protein during processing. Specifically, it allows visualization of the atomic and molecular interactions taking place within the system. Typical processing techniques involve thermal, mechanical, and soaking, which all affect the protein found in the millet. Application of the MD simulation technique provides a theoretical approach to understand the impact of various processing techniques without physical experimentation. In this study, the visualization output demonstrates the changes in protein structure and functionality that occur after varying temperatures and electric fields applied at various simulation steps. The changes in structure and functionality can also be evaluated to gain insights into the impact of processing techniques on the functional properties of the protein. To further quantify the results of applying high electric fields and thermal processing on protein structure in Finger millet, the following analyses were performed: STRIDE analysis, root mean square deviation (RMSD), solvent accessible surface area (SASA), radius of gyration (Rg), and dipole moment effect on surface hydrophobicity and hydrophilicity.

## 2 MATERIALS AND METHODS

### 2.1 Molecular modeling

In this study, all the MD simulations were performed using the implemented classical MD algorithm (classical) in Groningen machine for chemical simulations (GROMACS) software package, version 5.1.2 from the Stockholm Center
The PDB accession code 1B1U (Bifunctional inhibitor of trypsin and alpha-amylase from Ragi [Indian Finger millet, *E. coracana* seed]) was downloaded from Protein Data Bank (http://www.pdb.org). Figures 1A and 1B show a cartoon and surface representation of the RATI protein structure. The inhibitor protein RATI belongs to the plant alpha-amylase/trypsin inhibitor family and consists of 122 amino acids with five disulfide bridges and is the only inhibitor that can simultaneously inhibit trypsin and alpha-amylase by forming a ternary complex with alpha-amylase and trypsin.

The CHARMM27 force-field and spc216 generic three-point water model were adopted for protein and solvent, respectively. The RATI protein was placed in a periodic cubic water box of dimensions $6.546 \times 6.546 \times 6.546$ nm to satisfy the minimum image convention. Subsequently, 8471 water molecules were filled in the box and the final volume and density of the system were estimated to be $280.475$ nm$^3$ and $993.26$ (g/L), respectively (Figure 1C). This entire system was neutralized by adding three chlorine ($\text{Cl}^-$) atoms. The assembled solvated, electroneutral system was then subjected to energy minimization in order to ensure that it contains no steric clashes or inappropriate geometry. During energy minimization, the structure was relaxed using the steepest descent minimization algorithm with minimization stopping when the maximum force value of 1000 kJ/nm/mol was reached. After energy minimization, two 100 ps equilibrations were carried out at the constant temperature plus constant volume (NVT) ensemble and at the constant temperature plus constant pressure (NPT) ensemble, successively. Equilibration step was necessary in order to avoid any distortion of the protein when MD simulation was started. A total of nine MD simulations were run at 1 ns to evaluate the effect of temperature (300, 343, and 373 K) and SEF and OEF at an intensity of 1 V/nm with a frequency of 2.45 GHz. For comparison, MD simulations in the absence of an external electric field at the temperatures were also carried out. The external electric fields were applied along the $x$-axis of the equilibrated solvated protein system. During the MD simulation, the temperature was maintained using a Berendsen thermostat and pressure was set to 1 bar using the Parrinello-Rahman barostat. The MD simulation data were later analyzed by using built-in GROMACS analyzing tools and Visual Molecular Dynamics (VMD) software.
3 | RESULTS AND DISCUSSION

3.1 | Secondary structure analysis

VMD STRIDE algorithm was used to study the effects of temperature, external SEF, and OEF on the secondary structure of 1B1U. The algorithm uses torsional angle information along with hydrogen bond energies that are generated to map and assign the most probable secondary structure to the protein.\textsuperscript{24} RATI whose structure was determined from Ragi seeds was used in the simulations.\textsuperscript{15} RATI is 38% helical with four alpha helices and one 3/10 helix, in total containing 47 residues. Further, it contains 4% beta sheet with four strands containing six residues.\textsuperscript{15} The five alpha helices in the native state are formed between residues 20-29 (CYS-CYS), 37-50 (THR-ALA), 53-64 (ALA-MET), 87-93 (ARG-PHE), and 93-96 (PHE-LYS), which is the 3/10 helix.\textsuperscript{15} The molecule is highly stable due to its five salt bridges, also called the disulfide bonds. These can be observed between CYS residues: 6-55, 20-44, 29-85, 45-103, and 57-114. Researchers have shown that the primary trypsin-binding site is the turn between the first two alpha helices, ie, between the residues 31-38.\textsuperscript{15} Further, the conformations (torsion angles) loop was also reported to be similar to other trypsin-binding proteins.\textsuperscript{25} The alpha-amylase binding properties were found to be influenced by the first few residues of the molecule where the first Serine residue plays a primary role due to its free $\text{N}$ terminus.\textsuperscript{26,27}

STRIDE analysis is a very useful tool for identifying changes in secondary structures during simulations under different external conditions such as temperature or electric fields. It gives an output with residues on the $y$-axis and time/frames on the $x$-axis as shown in Figures 2 to 4. Each band of color corresponds to the residues present in the secondary structure during simulation with respect to the duration. The color changes represent the changes in the structure, allowing an exact time picture when the change occurs. A final comparison can be made between the initial structure and the final structure to assess the overall change in the secondary structure and determine the residues responsible for the changes.

Figure 2A shows the secondary structure of the protein at 300 K experiencing alterations over time; however, the final structure only differs with a 3-10 helix and at a turn interchange region. Comparing this to the final structure of the protein at 343 K in Figure 2B and at 373 K in Figure 2C, the residues between 31-35 have an increasing tendency of becoming turns.

**FIGURE 2** STRIDE analysis of 1B1U at (A) 300 K, (B) 343 K, and (C) 373 K
FIGURE 3    STRIDE analysis of 1B1U under static electric field of 1 V/nm at (A) 300 K, (B) 343 K, and (C) 373 K

FIGURE 4    STRIDE analysis of 1B1U with oscillating electric field of 1 V/nm at (A) 300 K, (B) 343 K, and (C) 373 K
from being coils in the native state. The beta sheet between the residues 51-55 is also under increased stress as it changes into turns and alpha helix. The other section where a significant interchange between turns and coils was observed in between the residues 75-79 when the protein was at temperatures 300 and 343 K. However, when the temperature was increased to 373 K this region was stable having coil orientations. The 3-10 helix between 95-99 also was under significant stress as it increasingly showed changes in coils with increasing temperature. Analyzing the intermediate changes in structure over time, Figures 2B and 2C develop much more turns instead of coils and fewer 3-10 helix. Interestingly, one of the alpha helices in the 343-K structure disappears at two instances, and the protein at 373 K maintains two steady beta sheets throughout time along with more turns instead of coils.

The structure in Figure 3A at 300 K with a SEF of 1 V/nm establishes a turn that is maintained and consequently one of the alpha helices is shortened by converting into a coil structure. Additionally, the beta sheets are eliminated and replaced by coils showing major disruptions in the secondary structure of the molecule with the application of SEF between residues 31-43. The 3-10 helix (51-55) is increasingly disrupted upon the application of SEF electric fields in combination with temperature as shown in Figures 3A, 3B, and 3C. The SEF has also resulted in significant disruptions between the residues 107-117 by eliminating isolated bridges and forming turns. It is interesting to note that these disruptions were more prominent when 1 V/nm static field was applied in combination with 343 K (Figure 3B) compared to 373 K (Figure 3C). Other major differences between Figures 3A and 3B is that a significant amount of coil and isolated bridge are replaced with a turn and a π-helix appears briefly in the intermediate stage between residues 111-115. Similar changes were observed in Ara h 6 peanut protein, where π-helix appeared in the simulation, but only in the case of oscillating fields.24 Figure 3C is very similar to Figure 3A, the beta sheet is diminished and coil section replaces alpha helix again. However, there is less 3-10 helix over time. The STRIDE analysis on the proteins that underwent a SEF revealed the most dramatic changes in secondary structure compared to the other simulations, ie, simulations with no electric field (Figure 2) and oscillating field (Figure 4).

The protein at 300 K with an OEF of 1 V/nm as shown in Figure 4A has a steady secondary structure throughout the simulation period. The beta sheets are less disturbed over time, and the 3-10 helix is broken less often compared to the application of static field in Figure 3. The only differences between initial structure and final structure consist of turns changing into coils and isolated bridges between the residues 107-115, and a 3-10 helix between residues 50-55 into turns. The structure is similar to Figure 4B, except the 3-10 helix is diminished over time and the beta sheets are briefly replaced with coils and isolated bridges twice in the intermediate stage. The structure in Figure 4C differentiates with the beta sheet changing more often, more 3-10 helix appearing over time and coils replacing turns over time.

The STRIDE analysis has shown that the loop holding the alpha helix I and alpha helix II are particularly vulnerable to external stresses, which is the primary trypsin-binding site. Especially, the application of SEF showed significant changes in the secondary structure of this region along with the two alpha helices stabilizing the loop.

### 3.2 RMSD analysis

Root mean square deviation provides an arithmetic value of deviations in the structure of a molecule. It is calculated by comparing changes in a molecule’s structure to control or reference molecule not under any stress.26 The RMSD of a protein can be represented by the following equation:

\[
\text{RMSD} = \sqrt{\frac{1}{N} \sum_{i=1}^{N} \left| r_{\text{final}}(i) - r_{\text{initial}}(i) \right|^2},
\]

where \( r_{\text{final}}(I) \) is the final coordinate for an atom \( I \), and \( r_{\text{initial}}(i) \) is the initial coordinate of atom \( i \), and \( N \) is the number of atoms. The average values for RMSD for the 1-ns simulation can be found in Table 1. The values in Table 1 show that the highest average RMSD value can be achieved with a SEF at 373 K. Further, the RMSD of the protein has increased significantly with an increase in temperature under all electric field conditions. When the temperature was increased without the application of any electric field the RMSD increased by about 12% for 343 K and by 42% for 373 K. The RMSD increased by about 2% when the temperature was increased from 300 to 343 K in combination of SEF and by 18% when increased to 373 from 300 K. However, it should be noted that the RMSD of protein molecule at 300 K under SEF of 1 V/nm was 1.867 ± 0.272 nm, which is the highest compared to the proteins at 300 and 300 K with OEF. The application of OEF with raising temperature resulted in an increase by 28% between 300 and 343 K and 36% between 300 and 373 K.
| Temperature (K) | No electric field (nm) | Static electric field (1 V/nm) (nm) | Oscillating electric field (1 V/nm) (nm) |
|----------------|------------------------|--------------------------------------|------------------------------------------|
| 300            | 0.897 ± 0.107          | 1.867 ± 0.272                        | 1.237 ± 0.176                            |
| 343            | 1.027 ± 0.125          | 1.905 ± 0.382                        | 1.721 ± 0.356                            |
| 373            | 1.544 ± 0.216          | 2.264 ± 0.363                        | 1.937 ± 0.638                            |

**Figure 5** Root mean square deviation (RMSD) analysis, (A) at 300, 343, and 373 K; (B) Applied electric field of 1 V/nm at 300, 343, and 373 K; (C) Applied oscillating field of 1 V/nm at 300, 343, and 373 K; (D) Effects of temperature, applied electric field, and oscillating electric field (OEF) at 300 K; (E) Effects of temperature, applied electric field, and OEF at 343 K; (F) Effects of temperature, applied electric field, and OEF at 373 K

Figure 5A demonstrates the significance that temperature plays on RMSD of the protein. At 300 and 343 K, the change over time is very similar; however, at 373 K, there is a notable difference in the structure of the protein, resulting in the RMSD values raising significantly. Figure 5B displays the changes that occur with an applied SEF and temperature and Figure 5C shows that of an OEF and temperature. In case of both SEF and OEF, maximum distortion on the RMSD was found when the electric field was applied in combination with the highest temperature values. Figures 5D, 5E, and 5F compare temperature change to SEF and OEF at 300, 343, and 373 K. For each temperature, the SEF caused the most significant change, followed by the oscillating field. The temperature alone has little effect compared to the other two. These results are in line with studies done on Ara h 6 protein where SEF and OEF in the order of 0.05 V/nm were applied. It was reported that the RMSD change in the case of SEF to be higher compared to that of the oscillating field.24
3.3 Solvent accessible surface area

Surface area properties are fundamental when determining the various functional characteristics of a protein. It can evaluate interactions between various molecules and solvents and is highly dependent on secondary structural changes. Thus, changes to the secondary structure can lead to the functional characteristics of proteins. SASA indicates the area available on the surface of a protein for interactions with solvents and other molecules. When proteins are exposed to thermal and electric field stresses, they undergo conformational changes, which can lead to increased exposure of hydrophobic residues to water and solvents. In this study, SASA was estimated using GROMACS software. Simulation times of 1 ns were used due to hardware limitations (future wet lab studies are recommended for further insight). It can be calculated using the following equation:

\[
\text{SASA} = A = \sum \left( \frac{R}{\sqrt{R^2 - Z^2}} \times D \times L \right),
\]

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 of \( i \) from the center of the sphere.

Figure 6A shows SASA staying almost constant at different temperatures. When an SEF of 1 V/nm is introduced in Figure 6B, the accessible surface area increases at all temperatures of 300, 343, and 373 K. An oscillating field of 1 V/nm

![Figure 6](image-url)
kept the accessible surface area constant for each temperature as shown in Figure 6C. Comparing the different effects at 300 K shows that there is some variation in temperature, SEF, and OEF. These differences are more distinct at 343 K, where oscillating and SEF caused more variation in the SASA as seen in Figures 6D and 6E. At 373 K, there was almost no difference in the different effects as seen in Figure 6F. Table 2 demonstrates that the highest SASA values correlate to an OEF at 373 K. This occurrence presents itself because when the protein undergoes both temperature increase and an OEF, its tertiary structure is denatured, therefore unfolding and creating more surface area. Simulations were only run for 1 ns due to software limitations. There is an effect on the surface area of the molecule, although rather limited. Further simulations running for a more extended period are suggested, such as 10-100 ns.

### 3.4 | Radius of gyration

Radius of gyration of a protein quantifies the distribution of atoms relative to their center of mass. This gives an understanding of the changes in shape and size of a protein as it experiences external stresses\(^{30}\)

\[
R_g = \sqrt{\frac{1}{N} \sum_{i=1}^{N} |r(i) - r_{center}|^2},
\]

where \(r(i)\) is the coordinate of an atom \(i\), \(r_{center}\) is the coordinate of the protein’s center of mass, and \(N\) is the number of atoms. The \(R_g\) for a protein is an indicator of how compact the atom is packed. The lower the number of gyration, the more dense the molecule’s structure is packed.\(^{31}\)

As shown in Figure 7A, the temperatures of 300 and 343 K did not change the \(R_g\). However, at 373 K, there is a notable increase in variation in the sample. Figure 7B shows the variation in \(R_g\) under a SEF strength of 1 V/nm. There is no significant change in the \(R_g\) depending on the temperature when the protein is under external forces of 1 V/nm, but over time there is a slight increase in the \(R_g\). Figure 7C shows the variation in \(R_g\) for protein undergoing oscillation of 1 V/nm. There is a bigger difference in variation with the protein undergoing oscillation at 373 K than at 300 and 343 K. Once again, as time progresses, the \(R_g\) increases over time. Figure 7D depicts the protein at 300 K under normal, static, and oscillating electric fields of 1 V/nm. There are no significant differences between the three different conditions at this temperature, each is relatively similar. Figure 7E shows the protein at 343 K under normal, static and oscillating electric fields. Under just an external force of temperature, the protein has a low \(R_g\). When the temperature is added to a SEF of 1 V/nm, the radius begins to increase. The biggest variation can be seen in the OEF of 1 V/nm. There is more variation over time and there is a bigger increase in \(R_g\) when compared to the other experiments at the same temperature. Figure 7F shows the protein at 373 K under normal, static, and oscillating fields at 1 V/nm. There is a steady increase in \(R_g\) in each experiment as time progresses at this 373 K. There is little variation in the normal condition and that under a static field, but the oscillating field has more variation over time and therefore a larger radius. Table 3 shows the largest \(R_g\) to have been achieved at 373 K with an OEF.

### 3.5 | Dipole moment distribution

Proteins experience a difference in charge called a dipole moment due to their secondary structure conformation containing things like helices, sheets, turns, coils, etc. The presence of an electric field can change the alignment of dipoles with respect to the direction of the applied field.\(^{32}\) The equation for calculating the electric dipole of a protein is given\(^{12}\)

\[
d = \sum_{i=1}^{N} q_i \cdot \hat{r}_i,
\]

where \(\vec{d}\) is the dipole, \(q_i\) is the charge of the atom, \(\hat{r}_i\) is the directional vector of each atom, and \(N\) is the number of atoms. In this study, the electric field was applied. This causes the protein to orientate itself in this direction. The strength of the field determines if the protein unfolds or re-orientates itself.
FIGURE 7  Radius of gyration (Rg) analysis. (A) Effects of temperature on Rg at 300, 343, and 373 K; (B) Effects of applied electric field of 1 V/nm on Rg at 300, 343, and 373 K; (C) Effects of applied oscillating electric field (OEF) of 1 V/nm on Rg at 300, 343, and 373 K; (D) Effects of temperature, applied electric field, and OEF of 1 V/nm on Rg at 300 K; (E) Effects of temperature, applied electric field, and OEF of 1 V/nm on Rg at 343 K; (F) Effects of temperature, applied electric field, and OEF of 1 V/nm on Rg at 373 K

TABLE 3  Average radius of gyration for 1-ns simulation

| Temperature (K) | No electric field (nm) | Static electric field (1 V/nm) (nm) | Oscillating electric field (1 V/nm) (nm) |
|-----------------|------------------------|-------------------------------------|------------------------------------------|
| 300             | 1.347 ± 0.005          | 1.349 ± 0.009                       | 1.355 ± 0.007                           |
| 343             | 1.349 ± 0.005          | 1.358 ± 0.010                       | 1.373 ± 0.013                           |
| 373             | 1.359 ± 0.009          | 1.357 ± 0.010                       | 1.376 ± 0.017                           |

An applied voltage of 1 V/nm was run during simulation along with an oscillating voltage of 1 V/nm. In Figure 8A, the temperatures of 300 and 343 K did not change the dipole moment distribution; however, at 373 K, there is a noticeable decrease in values. Figure 8B shows the effect of an applied electric field of 1 V/nm on the dipole moments. At 300 and 343 K, there is a similarly steady increase as time progresses and 373 K has drastically larger values but still increasing. Figure 8C shows the effect of an OEF on dipole moment, with 343 and 373 K displaying similar extreme values. However, at 300 K, the dipole moments maintain a much more moderate level. OEFs cause the dipole moments to consistently alternate extreme high and low values causing more heat generation, this, in turn, could denature the protein further. The results in Table 4 show that the largest dipole moment occurs at 373 K with a SEF of 1 V/nm. However, studies have reported that alpha-amylase and trypsin inhibitors are considered as potential antiobesity molecules.33,34 Thus, the functional properties of RATI after related processing methods, especially high temperature treatments, are needed to be evaluated in the future.
4 | CONCLUSION

In the present study, we evaluated protein structure changes of RATI, belonging to alpha-amylase/trypsin inhibitor family, present in Ragi seed when treated with thermal (300-373 K) and electric fields (static and oscillating electric fields) with an intensity of 1 V/nm. No significant differences in the secondary structures, RMSD, SASA, and total dipole moment of RATI were observed when treated with various temperatures (eg, 300, 343, and 373 K), which indicates RATI is a heat-stable protein attributed to the presence of four alpha helices and five salt bridges in protein. In comparison, apparent changes in the secondary structures of RATI including the translations between 3-10 helix structures, turn structures, and coil structures, were shown when the electric fields, especially SEFs (1 V/nm) were applied. Further, the highest RMSD and total dipole moment of RATI were observed under the simulation of 373 K combined with 1 V/nm of SEFs, followed by simulation of 373 K combined OEFs, and simulation of 373 K. These changes in the structural properties of RATI might be contributed to the protein quality changes of Ragi seed. Therefore, the SEFs with an intensity of 1 V/nm combined with temperature showed a potential application in improving the protein quality of Ragi seed, while the related real experiments are in need to prove this hypothesis. Moreover, the Rg and SASA of RATI were no significant changes under the different conditions. Thus, performing simulation experiments with higher electric field intensity (>1 V/nm) and longer simulation durations (10-20 ns) to further analyze the protein structure changes are still needed in the future.

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TABLE 4 Average dipole moments for 1-ns simulation

| Temperature (K) | No electric field (nm) | Static electric field (1 V/nm) (nm) | Oscillating electric field (1 V/nm) (nm) |
|-----------------|------------------------|-------------------------------------|----------------------------------------|
| 300             | 158.685 ± 22.461       | 343.131 ± 62.877                    | 137.916 ± 52.947                      |
| 343             | 153.071 ± 23.082       | 333.075 ± 59.648                    | 160.295 ± 75.278                      |
| 373             | 130.416 ± 26.452       | 477.055 ± 92.109                    | 171.231 ± 87.560                      |

FIGURE 8 Dipole moment analysis. (A) Effects of temperature on dipole moment at 300, 343, and 373 K; (B) Effects of applied electric field of 1 V/nm on dipole moment at 300, 343, and 373 K; (C) Effects of applied OEF of 1 V/nm on dipole moment at 300, 343, and 373 K.
CONFLICT OF INTEREST
The authors report no conflict of interest.

AUTHOR CONTRIBUTIONS
Shannon Fenwick, Data curation-Lead, Investigation-Lead, Methodology-Lead, Software-Lead, Writing-original draft-Equal; Sai Kranthi Vanga, Formal analysis-Lead, Software-Lead, Writing-review & editing-Lead; Andrea DiNardo, Data curation-Supporting, Formal analysis-Supporting, Software-Supporting, Writing-original draft-Supporting; Jin Wang, Data curation-Supporting, Software-Supporting, Writing-review & editing-Lead; Vijaya Raghavan, Supervision-Equal, Writing-review & editing-Equal; Ashutosh Singh, Project administration-Equal, Resources-Equal, Software-Equal, Supervision-Equal, Writing-review & editing-Equal.

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