EFFECT OF MICROWAVE AND ULTRASONIC PROCESSING ON THE CONFORMATION OF EGG WHITE PROTEIN

BY

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ABSTRACT

Eggs are one of the most popular foods due to the abundance of the availability of essential nutrients and hence are consumed widely. Further, consumption of eggs can help prevent disease and maintain health due to the presence of bioactive components. On the other hand, eggs can cause allergic reactions in sensitive patients, and it has now become the second most severe allergy after cow's milk in infants and children. The allergens are present in the egg white proteins and include ovomucoid (Gal d 1), ovalbumin (Gal d 2), conalbumin (Gal d 3), lysozyme (Gal d 4) and α-livetin (Gal d 5). Apart from the allergens, scientists have also been interested in avidin protein present in egg due to its anti-nutritional properties. The presence of avidin in food leads to the formation of avidin-biotin complex making biotin unavailable for absorption in the digestive system.

It is important to note that proteins are sensitive to the external stresses such as heat, electric field, and ultrasonic waves. Various studies have shown that external stresses can cause conformational changes in proteins, further resulting in alteration of their functional properties. Inactivation of allergic and anti-nutritional proteins through the use of processing techniques can be beneficial to humans. It has the potential to reduce the food allergy incidences that have been on the rise in the 21st Century. However, the knowledge and understanding of the mechanism of interactions between various proteins and a wide range of thermal and non-thermal processing conditions are limited. Addressing this problem can help the food industries not only to improve the processing techniques and conditions but also to develop food products suitable for all consumers.

In this study, the effects of thermal processing (Microwave treatment) and non-thermal processing (Ultrasonic processing) on the secondary structures of egg protein have been evaluated using Fourier Transform Infrared Spectroscopy (FTIR) and Circular Dichroism (CD) spectroscopy. The impacts of these two processing techniques on the inactivation of avidin have also been studied. The FTIR studies have shown that egg white protein undergoes conformational changes with an increasing β sheets content and decreasing α-helix secondary structures when subjected to the aforementioned two processing techniques. Microwave treatment is found to be an effective method to reduce the avidin activity. Avidin can also be inactivated by ultrasonic processing. However, the effectiveness is not as significant as in the case of microwave at higher temperatures.

Molecular dynamics (MD) modeling studies have been carried out to visualize the protein behavior under the impact of temperature, time and application of oscillating electric field. Avidin molecule which is an anti-nutrient present in eggs has been used for conducting the MD simulations. Root Mean Square Deviation (RMSD), Number of hydrogen bonds, Radius of Gyration (Rg) and Solvent Accessible Surface Area (SASA) have been calculated to evaluate the changes in the secondary structure during the MD simulations. The results showed that the number of hydrogen bonds altered with the increase of temperature and time. The changes in the number of hydrogen bonds further influence the root mean square deviation and radius of gyration values conforming conformational changes in the avidin protein. Our observations indicate that the effect of oscillating electric field is significant than the conventional thermal application on the structure of avidin, resulting in the protein unfolding during the simulation.

Résumé

Les œufs, en raison de sa disponibilité abondante des nutriments essentiels, sont l'un des aliments les plus populaires et de grande consommation. Les composants bioactifs dans les œufs nous aident à prévenir la maladie et à maintenir la santé. Cependant, les œufs provoqueraient très probablement des réactions allergiques chez les patients particulièrement sensibles aux œufs. Selon les statistiques pertinentes, l'allergie aux œufs est devenue la deuxième allergie. Les allergènes, qui se composent de l'ovomucoïde (Gal d 1), l'ovalbumine (Gal d 2), la conalbumine (Gal d 3), le lysozyme (Gal d 4) et la a-livétine (Gal d 5), s'avèrent présents dans les protéines de blanc d'œuf. Outre ces allergènes qui ont fait l'objet de nombreux travaux, les chercheurs s'intéressent également à la recherche de protéine avidine étant donné ses propriétés anti-nutritionnelles. La présence d'avidine dans les aliments conduit à une formation complexe de l'avidine-biotine, ce qui rend une indisponibilité d'absorption de la biotine dans le système digestif.

Les protéines sont sensibles aux contraintes externes telles que la chaleur, le champ électrique et les ondes ultrasonores. Diverses études montrent que le stress externe provoquerait des changements conformationnels dans les protéines. Grâce aux techniques de traitement modernes, on pourrait maintenant se profiter de l'inactivation des protéines allergiques et anti-nutritionnelles. Cette inactivation des protéines, qui s'accroît depuis le 21^{e} siècle, permettrait de réduire les incidences d'allergies alimentaires. Toutefois, les connaissances et la compréhension du mécanisme des interactions entre diverses protéines et un vaste éventail de de conditions de traitement (thermique et non thermique) sont encore limitées. De ce fait, une étude sur ce sujet aiderait les industries alimentaires non seulement à améliorer les techniques et les conditions de traitement, mais aussi à développer des produits alimentaires adaptés à tous les consommateurs.

Dans cette étude, on utilise les techniques de spectroscopie infrarouge à transformée de Fourier et de spectroscopie de dichroïsme circulaire pour évaluer les effets du traitement thermique et non-thermique sur les structures secondaires de la protéine d'œuf. Les impacts de ces deux techniques sur l'inactivation de l'avidine ont également été étudiés. Les analyses réalisées par la technique FTIR montrent que la protéine de blanc d'œuf subit des changements de conformation avec une croissance des feuillets β plissés et une diminution des structures secondaires en hélice α. Le traitement au micro-ondes s'avère être une méthode efficace pour réduire l'activité de l'avidine. L'avidine peut également être inactivée par le traitement ultrasonique. Néanmoins, l'efficacité n'est pas aussi importante que celle dans le cas des micro-ondes à des températures plus élevées.

Dans ce présent travail, on mène plusieurs études à la modélisation de la dynamique moléculaire pour visualiser le comportement de la protéine sous l'impact de la température, du temps et de l'application d'un champ électrique oscillant. La molécule d'avidine a été utilisée pour réaliser les simulations. L'écart quadratique moyen, le nombre de liaisons hydrogène, le rayon de giration et la surface de surface accessible aux solvants ont été calculés pour évaluer les changements dans la structure secondaire au cours des simulations. Les résultats montrent que le nombre de liaisons hydrogène varie en fonction de l'augmentation de la température et du temps. La fluctuation du nombre de liaisons hydrogène influencent en outre l'écart quadratique moyen et les valeurs du rayon de giration, ce qui se conforme aux changements conformationnels de la protéine avidine. Selon nos observations, vu que l'application thermique conventionnelle sur la structure de l'avidine entraîne le dépliement de la protéine au cours de la simulation, il nous semble que l'effet du champ électrique oscillant serait plus significatif.

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Thesis Format

This thesis is submitted in the format of papers suitable for journal publication. This thesis format has been approved by the Faculty of Graduate and Postdoctoral Studies, McGill University, and follows the conditions outlined in the Guidelines: Concerning Thesis Preparation, which are as follows:

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The thesis must include the following

(a) A table of contents;

(b) An abstract in English and French;

(c) An introduction which clearly states the rational and objectives of the research;

(d) A comprehensive review of the literature (in addition to that covered in the introduction to each paper);

(e) A final conclusion and summary;

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Contribution of Authors

The following are the manuscripts prepared for publication:

1. Zhu, Yao; Vanga, Sai Kranthi Kumar; Wang, Jin and Raghavan, Vijaya. 2018. "Impact of food processing on the structural and allergenic properties of egg white" (Under Review-revision)

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Dr. Raghavan has also provided scientific advice and is directly associated with editing and reviewing the manuscript. Mr. Sai Kranthi Kumar Vanga has provided the technical help of the molecular modeling software GROMACS and participated in reviewing the manuscript. Mr. Jin Wang also participated in reviewing the manuscript.

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Abbreviation

ELISA	Enzyme-linked immunosorbent assay
IgG	Immunoglobulin G
IgE	Immunoglobulin E
UV	Ultraviolet absorption spectroscopy
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ci-ELISA	Competitive indirect enzyme-linked immunosorbent assays
MTS	Manothermosonication
PBS	Phosphate-buffered saline
HABA	2-(4'-hydoxyazobenzene) benzoic acid
FTIR	Fourier transform infrared spectroscopy
CD	Circular dichroism spectroscopy
CHARMM	Chemistry at HARvard Macromolecular Mechanics
PDB	Protein Data Bank
MD	Molecular Dynamics
TIP3P	Transferable Intermolecular Potential With 3-Points
RMSD	Root Mean Square Deviation
Rg	Radius of gyration
SASA	Solvent Accessible Surface Area
NVT	Ensemble in which number of atoms, volume and temperature are constant
NPT	Ensemble in which number of atoms, pressure and temperature are constant
NMR	Nuclear Magnetic Resonance Imaging

CHAPTER I

INTRODUCTION

Eggs are one of the dietary sources to obtain high-quality proteins, various minerals and vitamins. Egg intake can ameliorate nutrient inadequacies and be beneficial to human health. For example, egg lutein is found to help reduce the risks for age-related macular degeneration (McNamara, 2015). Because eggs are readily available and widely used in various food products, they are highly consumed in many countries. For example, the average data from USDA showed that 267 eggs were consumed for each person in U.S.A in 2017.

The egg proteins play a significant role in determining functional properties in various food products. The coagulative properties which lead to solidification upon heating and aeration play a key role in cakes, bread, etc. Eggs also possess emulsification properties which are important in salad dressings and sauces that are widely consumed around the world as part of various cuisines. They also have binding properties and can signigicantly improve texture and acceptability of products like health bars and baked products (Stadelman, 1999). An egg consists of 8-11% shell, 27-32% yolk and 56-61% albumen (Poulsen et al., 2001). Albumen comprises 88.5% water, 10.5% protein, 0.5% carbohydrate and other solutes in very minute quantities (Stevens, 1991). The protein composition of albumen is summarized in Table 1.1.

Nonetheless, the intake of egg is restricted in many countries including the USA because of dietary cholesterol inside in past years (McNamara, 2015). Cholesterol has been considered to increase plasma cholesterol levels, leading to higher risks of cardiovascular diseases (CVD). However, numerous studies during these years showed that there is no significant relation between egg and dietary cholesterol intake and increased CVD risk (McNamara, 2015). Avidin is of great interest to many researchers due to its anti-nutritional properties. It has a high affinity to bind biotin,

hindering the absorption of biotin in the human body. Biotin is a kind of vitamin and can participate in metabolic processes like fatty acid synthesis (Maeda, Kawata, Inui, & Fukuda, 1996).

Table 1.1. Composition of egg white proteins
Source: (Desert et al., 2001; Forsythe & Foster, 1950; Yoshinori Mine, 1995; Stevens, 1991)

Protein	Amount of egg white (%)
Ovalbumin	54 – 66
Ovotransferrin	12 – 13
Ovomucoid	9.5 – 11
Lysozyme	2.3 – 4.5
Ovomucin	1.5 – 3.5
Ovoinhibitor	0.1 - 1.5
Ovoglobulin G2	1.0 - 6
Ovoglobulin G3	1.0 – 6
Riboflavin binding protein	1.0
Avidin	0.05 - 0.5
Ovostatin	0.5

Further, eggs may trigger allergic reactions and are known as the second most common allergy-causing food in the world. Egg allergy belongs to the big 8. Big 8 represents eight foods including milk, egg, peanut, wheat, soybean, tree nuts, fish and shellfish are responsible for triggering almost all the allergic reactions. Around 1-4% of children have been reported to be allergic to eggs around the world (Eigenmann, 2000).

Various studies have shown that the allergenicity of food can be altered using different processing techniques including thermal and non-thermal methods. The effect of specific processing method on a certain food component is being evaluated by many research (Vanga, Singh, & Raghavan, 2017). For example, heat generation in food by microwave treatment results in numerous changes both in conformational structures and physical characteristics like flavor and texture. A study also

reported that immune-reactivity of whey samples descended from the whole milk was treated by microwave heating at 600 or 700 Watt for 10 min, compared to the control (Kaddouri, El Mecherfi, Kheroua, & Saidi, 2006). Ultrasonic processing is a novel technology which can be used to reduce the food allergenicity due to its high-energy (high power, high intensity, low frequency) ultrasonic waves. It can produce immense pressure, shear and temperature gradient, which may be beneficial to alter protein structure and its properties. Studies found that proper concentration of ultrasonic processing not only increased the amount of soluble proteins, but also reduced two major peanut allergens Ara h 1 and Ara h 2 levels in peanuts and resulted in a certain inhibition on IgE-binding (Li, Yu, Ahmedna, & Goktepe, 2013).

1.1 Hypothesis and Implications

Thermal, microwave and ultrasonic processing have been widely used in food industry. Extensive work has been done to evaluate the nutritional values of processed products. However, there is limited information on the impact of these processing techniques on the structure as well as functional properties of egg white proteins. Studies about inactivating avidin in egg white are also limited. In this project, the influence of microwave and ultrasonic processing methods on the digestibility and secondary structure of the egg white protein was studied. The avidin activity was also determined and the changes in the secondary structure and surface properties of avidin was visualized using Molecular Dynamics (MD) simulations.

1.2 Objectives

1.2.1 Overall Objective:

The overall objective of the study is to evaluate the changes in the secondary structure conformations of the egg white protein during microwave and ultrasonic processing and its effect

on the protein digestibility and the inactivation of avidin. Besides, to study the changes in the protein structure under different external stress using molecular dynamics simulation.

1.2.2 Specific Objectives:

1) To investigate the effect of microwave and ultrasonic processing on the secondary structure of egg white protein using Fourier transform infrared spectroscopy (FT-IR) and Circular dichroism (CD) spectroscopy.

2) To study the impact of microwave and ultrasonic processing on the inactivation of avidin and egg white protein digestibility

3) To evaluate the applicability of molecular dynamic simulation for studying the effect of thermal and oscillating field on avidin.

CHAPTER II

Review of Literature

2.1 Abstract

Egg is one of the most nutritious foods that is easily available and has become a favorite source of major nutrients like lipids and proteins around the world. However, eggs can trigger severe allergenic reactions, especially in infants and children. The reactions are mostly IgE-mediated with a range of symptoms related to nose and throat and further can lead to life-threatening anaphylaxis. Major egg allergens are abundant in egg white which include ovalbumin (OA), ovomucoid (OVM), ovotransferrin (OVT) and lysozyme (Lys). A total of ten allergens have been recognized to date and researchers are actively working on understanding their structure-function relationship which could help reduce the allergy incidences. In addition to allergens, avidin present in egg white is also extensively studied due to its anti-nutritional properties. Avidin is known to form a complex with biotin which makes it unavailable for absorption.

This review focuses on the effects of thermal and non-thermal processing methods on the structure and functional properties of various egg proteins including a wide range of allergens and anti-nutrients.

Novel processing techniques including various non-thermal techniques show promising results in reducing the allergic reactions of egg. Anti-nutrients like avidin can be partly inactivated under combined high-pressure processing and heat treatment.

Keywords: Egg protein; avidin; thermal processing; non-thermal processing; novel processing; egg allergy

2.2 Introduction

Eggs are abundant in nutrients which are beneficial to human health. The nutritional composition not only includes proteins and lipids but also contains various vitamins like thiamin, riboflavin, vitamin A, B, D, E and minerals like Ca, P, K, Na, Mg, Fe, Zn. Recent research has also shown that eggs contain significant quantities of carotenoids like lutein and zeaxanthin that could play an important role both in well-being and in prevention of various diseases. The egg yolk contains high antioxidants which could provide further health benefits. They are also considered to be an important source of anti-bacterial and bioactive compounds which find their application in various industries including food, pharma and biotechnology (Lesnierowski & Stangierski, 2018). The egg is also a vital source of choline nutrient (680 mg/100 g) which plays a major role in memory development and brain health (Applegate, 2000; Handelman, Nightingale, Lichtenstein, Schaefer, & Blumberg, 1999; Mellott, Williams, Meck, & Blusztajn, 2004). For example, research conducted on rat pups showed that the group treated with choline developed abilities in spatial memory three days sooner compared to control group. The choline was applied to rat dams in their embryonic days of 12-17 (Mellott et al., 2004).

Despite their health and nutritional profile, eggs contain few compounds that could cause adverse reactions in human body especially in form of allergic reactions. Eggs are one of the most common allergy-causing foods, especially in children and infants. Egg is found to be the second most common trigger for allergy in children after cow's milk (Gray et al., 2015; Savage, Matsui, Skripak, & Wood, 2007). Exposure to egg in sensitive children can result in hives, rashes leading to anaphylaxis. Most of egg allergens are present in egg white, and they include ovomucoid (Gal d 1), ovalbumin (Gal d 2), conalbumin (Gal d 3) and lysozyme (Gal d 4) (Poulsen et al., 2001). The best way to manage egg allergy is complete abstinence from any food products containing eggs.

However, since egg has plenty of applications in the food industry, complete avoidance is not easy (Yoshinori Mine & Yang, 2008). In this review, we have outlined the components of the egg proteins and effects of various processing methods on their structure-functional relationship which could be of great interest to researchers and the industry.

2.3 Major Egg White Proteins

2.3.1 Ovalbumin

Ovalbumin is a phospho-glycoprotein composed of 385 amino acids whose molecular weight is estimated to be 45 kDa (Abeyrathne, Lee, & Ahn, 2014). Further, the ovalbumin molecular sequence contains a total of four thiol groups and one disulphide bond formed between the cystine residues 87-133. This molecule is generally linked with a carbohydrate chain at the amide nitrogen belonging to Asparagine 298 residue (Stein, Leslie, Finch, & Carrell, 1991). The heavy glycosylation leads to the allergenicity of ovalbumin (Aalberse, 2000). Ovalbumin is an acidic molecule with an isoelectric point (pI) of 4.5. Half of the residues present in the molecule are hydrophobic in nature and one-third of them are charged amino acid residues (Huopalahti, Anton, López-Fandiño, & Schade, 2007). Although ovalbumin has certain attributes that make it heat stable, it can be denatured or aggregated by proteolysis due to exposure of the air-water interfaces during thermal processing (Huopalahti et al., 2007; Stevens, 1991). According to its structure, ovalbumin shows certain homology with serpin family proteins, but does not exhibit any protease inhibitory activity (Huntington & Stein, 2001). Furthermore, Ovalbumin plays a major role in immunological and nutritional studies. Ovalbumin acts as a standard protein in several experiments including protein assay, structural properties, animal testing on inhalant and dietary allergies (Abeyrathne et al., 2014; Yoshinori Mine & Yang, 2008).

2.3.2 Ovomucoid

Ovomucoid is a heat-stable glycoprotein containing 186 residues and a molecular weight of 28 kDa with an isoelectric point (pI) of 4.1 (Julià et al., 2007; Winiarska-Mieczan & Kwiecień, 2007). The structure of ovomucoid consists of three domains: domain I and domain II (defined as α-type) and domain III (β-type). It contains both sulfated oligosaccharides and sialyloligosaccharides and a total of nine disulfide bonds (Huopalahti et al., 2007). Further, intra-domain disulfide bonds exist in each domain that contributes to its stability (Stevens, 1991). Domain III is different from domain I and domain II because it has shorter disulfide bonds between the first and second cysteine residues. The disulfide bonds not only contribute to the higher heat stability, but also are responsible for increasing difficulty in digestion and hence it is classified as a protease inhibitor which is also observed in other molecular like soy inhibitors (Vagadia, Vanga, & Raghavan, 2017; Vagadia, Vanga, Singh, & Raghavan, 2016; Winiarska-Mieczan & Kwiecień, 2007). Hen egg ovomucoid can only bind to one molecule of protease every time, which is different from ovomucoid in duck and turkey egg (Huopalahti et al., 2007). This is due to the flexible enzyme-inhibitor contact position resulting in varying inhibitory activity of ovomucoid present in different species. This phenomenon is beneficial to prevent a wide range of bacterial proteinases (Stevens, 1991).

Researchers also found that ovomucoid is the most allergic protein in egg (Dhanapala, Withanage-Dona, Tang, Doran, & Suphioglu, 2017). Minute amounts of ovomucoid can trigger allergy, and this ability does not disappear even after thermal treatment or enzymatic hydrolysis due to its higher stability (Julià et al., 2007). However, this unique property can be used as a tool for detection of egg in a food products even after thermal processing.

2.3.3 Ovotransferrin/Conalbumin

Conalbumin, also known as ovotransferrin, is a monomeric glycoprotein and one of the most allergic proteins found not only in egg white but also in the yolk. Its molecular weight is found to be 76 kDa, with a total of 686 amino acids and 15 disulfide bonds (Abeyrathne et al., 2014). Since each molecule of ovotransferrin can bind to two Fe^{3+} ions, they play a key role in iron transportation from the hen oviduct to the developing embryo (Huopalahti et al., 2007; Stevens, 1991). This property contributes to its applications in iron-fortified products like iron-fortified beverages and iron supplements. It has two different forms: apo-ovotransferrin (iron free) and holo-ovotransferrin (iron bound). After binding to iron, the stability of ovotransferrin was enhanced due to its increased resistance to physical and chemical stresses. Further, researchers also found that ovotransferrin has antimicrobial, antiviral and antioxidant properties (Abeyrathne et al., 2014). The iron-transferrin complex can effectively prevent both gram-positive or gram-negative bacteria from growing in different foods. Moreover, apart from Fe ion, bicarbonate ions can also enhance the antimicrobial activity (Huopalahti et al., 2007). At the same time, bacteriostatic ability due to the bicarbonate ion can be reversed by adding other Fe^{3+}. Other studies proposed different mechanisms on the origin of antimicrobial activity of ovotransferrin. They suggested ovotransferrin might have the ability to bind with bacterial cell membrane which in turn would affect the functional property (Huopalahti et al., 2007). The two opinions are both reasonable and can contribute to the strong antimicrobial activity of ovotransferrin as observed in various food products.

2.3.4 Ovomucin

Ovomucin is a sulfated glycoprotein which is responsible for the thick gel texture of egg whites (Abeyrathne, Lee, Jo, Suh, & Ahn, 2016). It is considered as one of minor allergens present in egg

(Bush & Hefle, 1996; Walsh et al., 1988). The molecular weight of ovomucin is 1.8-8.3 $\times 10^3$ kDa (Abeyrathne et al., 2014; Baumgartner & Schubert-Ullrich, 2010). Subunits in the structure of ovomucin can be divided into two forms based on their composition and properties: α - and β – ovomucin (Huopalahti et al., 2007). Furthermore, albumen contains both soluble and insoluble ovomucin. However, the primary difference is that the soluble ovomucin exists in both thick and thin albumen, but insoluble ovomucin occurs only in thick egg white portion (Huopalahti et al., 2007). Research showed that the physical stability and foaming properties of egg white are highly dependent on ovomucin and degradation of ovomucin can lead to changes in its functional properties (Stevens, 1991). Concerning its functions or applications, several studies suggested an antitumor activity in ovomucin which is found to inhibit growth of cancer cells (Huopalahti et al., 2007). In recent years, the biological activities of ovomucin seem to catch more and more researchers' attention and interests, especially in the food industry. The development of functional food containing ovomucin is becoming an important aspect of research and food product development.

2.3.5 Lysozyme

Lysozyme is a kind of muramidase or hydrolase. It widely exists in nature, found in tissues of some animal or human organisms, secretions, and body fluids. It also occurs in bacteria and in some plants (Huopalahti et al., 2007). It should be noted that the role of lysozyme in egg allergy has been in controversy due to the conflicting findings from different studies (Anet et al., 1985; Langeland, 1983; Miller & Campbell, 1950). Further studies are required to assess and understand its role in egg allergy. The molecular weight of lysozyme is 14.4 kDa, and it has 129 amino acids in a single polypeptide chain (Abeyrathne et al., 2014). Lysozyme is a single polypeptide chain comprising two domains connected with an α-helix and is cross-linked by four disulfide brides

(Young, Tilton, & Dewan, 1994). Two of the four disulfide bridges in its structure contribute to extremely high heat stability and the other two are responsible for its enzymatic activity (Abeyrathne et al., 2014; Huopalahti et al., 2007). Its activity is unaltered under high temperature (100℃) or in acidic solutions (pH 3.0-4.0) (Huopalahti et al., 2007). However, it can be easily and rapidly inactivated upon reacting with thiol substances. Further, Lysozyme also forms aggregates under 80℃ and neutral pH effects their functional properties (Iwashita, Handa, & Shiraki, 2017). Lysozyme can protect the host from invasion of foreign bacteria, especially gram-negative bacteria (Stevens, 1991). Thus, it is widely used in food, pharmaceutical, and drug industries. Addition of lysozyme can avoid spoilage of food by preventing the growth of pathogens, which in turn can extend their shelf life. Furthermore, lysozyme can serve as an antimicrobial when combined into packaging polymers which finds its application in food packaging industry (Appendini & Hotchkiss, 2002; Huopalahti et al., 2007). In pharmaceutical and medical industries, lysozyme has been used to prevent infections or to serve as a stimulant in immunological studies to help treatment of bronchopulmonary diseases (Huopalahti et al., 2007).

2.3.6 Avidin

Avidin is a water-soluble glycoprotein which binds with biotin (Green, 1963; Yossa, Sarker, Karanth, Ekker, & Vandenberg, 2011). It is present in the oviducts or oviductal secretory tissues like egg white of birds, reptile, lizards, and amphibians (Elo, Räisänen, & Tuohimaa, 1980; Strzelczyk, Bujacz, Plażuk, Zakrzewski, & Bujacz, 2013). Avidin can be found in hen egg and it can make up from 0.05 – 0.5 % of the protein of egg white (Green, 1975). However, the maturity of laying hen influences the content of avidin in egg white (Durance, 1991). Some findings showed that when a hen begins laying eggs, avidin content increases from the first to the 15^{th} egg and then becomes constant (Bush & White, 1989; Wet & Hsu, 1970).

Each monomer contains 128 amino acids residues (Yossa et al., 2011). Theoretical molecular weight of 15 kDa with corresponding theoretical combined molecular weight of 57 kDa (Malaikaew, Svasti, Kumar, & Buranaprapuk, 2011; Rothemund, Thomas, & Rylatt, 2002). Avidin is found to contain eight antiparallel successive beta-strands associated with its three-dimensional crystal structure (Spolaore, Damiano, Raboni, & Fontana, 2014). This structure is the reason for high stability of avidin. A carbohydrate side chain and a disulfide bridge are also identified in avidin's structure that contribute to its stability (Laitinen et al., 2002). Avidin is an alkaline (Laitinen et al., 2002). Glycosylation and high pI results in avidin accumulation in liver and kidneys which may trigger a tumor if avidin is not purged by the body (Hytönen et al., 2003; Yao, Zhang, Sakahara, & Nakamoto, 1999). Further, avidin can bind to a material based on the charge of the material because of its high pI. Avidin aggregates usually when mixed at ambient temperatures with anionic detergents, such as sodium dodecyl sulfate (SDS). The resulting aggregates fail to penetrate the stacking gel during polyacrylamide gel electrophoresis (PAGE). To avoid the formation of such aggregates, avidin can be acetylated and the pI will be reduced (Bayer, Ehrlich‐Rogozinski, & Wilchek, 1996).

Avidin is known to bind with biotin, resulting in vitamin H or B_7 deficiency (Green, 1975). Biotin is one of the water-soluble vitamins (Yossa et al., 2011). Up to 4 molecules of biotin can bind with one avidin molecule. Further, it suggests that the affinities are characterized by the dissociation constant which is around 10^{-15} M between them; it is remarkably higher than other antigen-antibody complexes making this complex very stable (Strzelczyk et al., 2013; Yossa et al., 2011). Biotin plays a significant role in humans because it affects metabolic processes such as fatty acid synthesis and amino acid catabolism (Maeda et al., 1996). Their experiments showed that biotin-deficient rats had remarkably higher plasma ammonia concentration than biotin-supplemented

ones. Therefore, biotin deficiency is proved to be related to 'hyperammonemia'. But the mechanisms that triggers this condition is not clear and warrants further investigation.

Biotin plays an important role in the growth of microorganisms such as fungi and bacteria. Due to the stable complex compound between avidin and biotin, the growth of these microorganisms is efficiently inhibited (Elo et al., 1980; Green, 1990). However, the avidin-biotin complex has so many applications that it is, to some degree, advantageous to the biotechnology industry. Its applications in affinity chromatography, fluorescence-activated cell sorting, western blotting, ELISA and tumor targeting have been explored by scientific community (Strzelczyk et al., 2013). In addition, it can also be used for protein detection (Spolaore et al., 2014). However, the use of avidin-biotin complex applications is limited because its binding property with biotin is not specific (Huopalahti et al., 2007). Avidin has been found to help in several anticancer treatments (Anton, Nau, & Nys, 2006). With its increasing applications in pharmaceutical industries, some beneficial outcome of avidin is noted to be the relatively low organ retention than initially expected. The research suggested that there are antibodies against avidin in human serum and hence humans can tolerate avidin to a certain extent (Bubb et al., 1993).

In 2007, a 2-year-old boy was found to be allergic to avidin (Enriquez-Matas et al., 2007). It is the first case reported regarding the egg allergy related to avidin. He was diagnosed to be hypersensitive to egg proteins. In addition, when he was 13-month-old, he got an acute and severe urticaria after contacting with raw egg incidentally. However, specific IgE to several major allergens in egg showed negative. Therefore, experts assumed the boy was allergic to avidin rather than the other proteins in eggs. However, there were no previous reports regarding avidin allergy, and the reported phenomenon itself should be further verified (Enriquez-Matas et al., 2007).

In recent years, avidin-like proteins were discovered from a wide range of sources. *Bradyrhizobium japonicum* is a symbiotic bacterium which can fix nitrogen on the root nodules of soybean plants (Nordlund, Hytönen, Laitinen, & Kulomaa, 2005). When its gene encoding is examined, it was found that the protein is homologous with avidin. Rhizavidin from *Rhizohium etli* is also one of the avidin-like proteins (Helppolainen et al., 2007). In 2008, a novel avidin-like biotin-binding protein known as Bradavidin 2 (Helppolainen et al., 2008) was discovered. It is from *Bradyrhizobium japonicum* which finds its application in improving crop yields when added to legume seeds (Slaton, Roberts, & Ross, 2011). Tamavidins which originated from Tamogitake mushroom was reported to have a kind of avidin-like proteins (Takakura et al., 2009). Taskinen et al. (2013) found a new protein like avidin from zebrafish then called it zabavidin. Most recently, a novel avidin-like protein called lentiavidins was characterized from shiitake mushroom (Takakura, Sofuku, Tsunashima, & Kuwata, 2016). This has renewed the interest of various researchers into the class of avidin-like proteins and their properties, biochemistry and applications in various industries.

2.4 Processing Methods

The number of allergic reactions triggered by various foods have increased drastically in recent years. Numerous scientists and experts believe that the change in the trends of diet, especially in the developed nations over the past forty years could be responsible for these allergies. Hence their efforts in understanding the processing effects on allergies and further the structure-function relationship of proteins have drastically increased (Vanga, Jain, & Raghavan, 2016; Vanga & Raghavan, 2016). This review outlines the effects of thermal processing and non-thermal processing methods on various egg allergens and anti-nutritional compounds.

2.4.1 Thermal processing

2.4.1.1 Traditional heat treatment

Heat treatment is a traditional processing method aimed to increase microbial safety, eliminating toxins or improve texture and palatability of food. The structures of allergenic components tend to change after heat treatment, which explains why thermal processing can minimize allergenicity. The tertiary structures of proteins begin to change approximately at 50-60℃ according to different molecular weight, stability, and size followed by secondary structure changes as temperature increases beyond 65℃ (Vanga et al., 2017).

Ovalbumin was treated at different temperatures (25-100℃) and pHs (4.5, 7.0, 9.5) and their effects on structure and antigenicity were assessed by fluorescence spectroscopy, in silico prediction and enzyme-linked immunosorbent assay (ELISA) (Stănciuc, Banu, Turturică, & Aprodu, 2016). The antigenicity of ovalbumin reduced by 82% after thermal treatment at over 80℃ with pH 9.5 when compared to untreated control. This study further showed that pH plays a vital role in denaturation as only 5.5% reduction was found when the same thermal treatment was performed at pH 7.0. The results proved the significance of temperatures and pH on structure and antigenic properties of a protein (Stănciuc et al., 2016). Claude et al. (2016) evaluated the effects of thermal processing on ovalbumin and its ability to cause an allergic reaction in both sensitization and elicitation stages using sera from patients' sensitive to ovalbumin and mouse model. They found that aggregation as a result of thermal treatments reduces the allergenicity. Though there is an increased production of IgG, the IgE-binding is reduced both in human and mice samples.

In another study, ovomucoid was first isolated and dissolved in a basic PBS buffer. Then it was processed by heat from 85 to 98℃ in basic, neutral buffer and egg white. Ovomucoid in the basic

buffer had more resistance to heat. Ovomucoid in egg white shared no similar denaturation patterns with the ovomucoid in the former two buffers (Julià et al., 2007).

Tong et al. (2012) studied changes in allergenicity and conformational structure of ovotransferrin after thermal processing. Circular dichroism spectroscopy (CD), ultraviolet absorption spectroscopy (UV) and fluorescence spectroscopy were used to analyze the alteration of conformational structure. ELISA was applied to examine changes in the allergenicity of ovotransferrin. The IgG and IgE binding to ovotransferrin changed as the conformational structure altered. They found that allergenicity can change in case of unfolding or changes to disulfide bonds in the structure.

In the early investigations, there was a belief that common cooking methods like boiling, frying could destroy avidin (Durance, 1991), but studies showed that not all parts of egg white were heated to target temperature within standard cooking times resulting in residual activity of avidin. However, due to very low concentration of avidin in egg, the possibility of humans suffering from biotin deficiency is rare. In 1992, Durance et al. conducted an experiment to study the inactivation kinetics during thermal processing of avidin (Durance & Wong, 1992). Temperature ranged from 73 ℃ to 93 ℃ and the HABA-standard curve method was used to measure avidin activity. Inactivation kinetics was assumed to follow the first-order reaction kinetics. Their results showed that the inactivation of 90% avidin activity was reached at 121℃ for 20 min.

2.4.1.2 Microwave heating

Microwave has an electromagnetic frequency ranges of 300 MHz to 300 GHz (Vanga et al., 2017). Despite a wide range of applications in medical and telecommunication fields, microwave can also be used in food processing for applications such as pasteurization, drying, and defrosting (Richardson, 2001). Two frequencies of 915 MHz and 2.45 GHz can be used for industrial and

food-related applications (Doona, 2010; Tewari & Juneja, 2008). Heat generation in food by microwave treatment results in numerous changes both in conformational structures and physical characteristics like flavor and texture (Vanga et al., 2017).

A molecular dynamics study reported that lysozyme was denatured in electromagnetic fields (English & Mooney, 2007). The nonequilibrium canonical ensemble (NNVT) simulations with the frequency in areas ranged from 50 to 500 GHz, and intensity of 0.1-0.5 V/Å were performed at 298 K. In their study, the secondary structure of lysozyme changed significantly, and the speed and extent of onset of denaturation were influenced largely by field intensity. Table 2.1 outlines the thermal processing effects on various egg proteins.

Table 2.1. Thermal processing methods of egg white proteins

Protein	Processing method	Effects and outcome	Reference
Ovalbumin	Heat treatment at 25-100℃ for 20 min at pH 4.5, 7.0 or 9.0	Over 82% antigenicity was reduced and structure changed in 80℃ at pH 9.5	(Stănciuc et al., 2016)
Ovomucoid	Heat treatment (85 to 98℃, pH 7.4, 9.1, 9.6)	Ovomucoid in the basic buffer showed more resistance than in neutral solutions	(Julià et al., 2007)
Ovotransferrin	Heat treatment	Changed IgG and IgE binding capacity and conformational structures	(Tong et al., 2012)
Avidin	Thermal processing (73 to 93℃) with different time	Inactivation of 90% avidin activity (25 min at 121℃)	(Durance & Wong, 1992)
Lysozyme	NNVT simulations with the frequency from 50 to 500 GHz at 298K for 0.5 ns	Significantly changed secondary structures	(English & Mooney, 2007)

2.4.2 Nonthermal processing

2.4.2.1 High-pressure processing

High-pressure processing was first employed to eliminate microorganisms for improving the shelf-life of food products (Hite, Weakley, & Giddings, 1914). It is also known as high hydrostatic pressure processing. Its regular working pressure varies from 100 to 800 MPa (D. F. Farkas & Hoover, 2000). It is found to be effective on conformational alteration of food components, resulting in structural and functional property changes of proteins including reduction in allergenicity (Rivalain, Roquain, & Demazeau, 2010). The conformational changes in proteins are due to the impact of pressure on regions stabilized by hydrophobic and electrostatic interactions which are pressure liable. Hydrogen bonds also play an important role in stabilizing protein structures, but are almost pressure insensitive (Ledward, 2000).

Ovalbumin was subjected to high pressure in the range of 0.1-600 MPa at 10℃ for 10 min to investigate the changes in its allergenicity and structure. A reduction in allergenicity was observed and the maximum inhibition rate was reported to be 89.7% at 500 MPa. A change in tertiary structures was detected by fluorometric measurement but no difference in secondary structures occurred in CD spectroscopy (Odani, Kanda, Hara, Matuno, & Suzuki, 2007). The effects of high pressure on enzymatic hydrolysis were studied in 2008 (López-Expósito et al., 2008). Ovalbumin was hydrolyzed by pepsin under 400 MPa at 37℃ for 10, 60, 120 min. The native structure broke down in minutes and produced lower allergenic hydrolysates. The result demonstrated high pressure could accelerate the proteolysis with reduced IgG and IgE binding. A recent study revealed that this novel food processing method can inactivate avidin (Ajaypal Singh & Ramaswamy, 2014). The processing impact on avidin was compared in high-pressure processing (HPP) with moderate temperatures and traditional thermal processing. Thermal processing was

performed at different temperatures for pure avidin, avidin in albumen and avidin in the whole egg. The pure avidin was treated at 80, 90, 100℃ for 45 min while avidin in egg or egg white was treated at 30, 40, 50℃ for 10 min (Ajaypal Singh & Ramaswamy, 2014). Avidin was completely inactivated at 100℃ for 10 min. In terms of High-pressure processing the processing pressures of 500, 600, 700 MPa were applied combined with temperatures 30, 40, 50℃ to avidin in egg and albumen. The optimal condition for inactivation was 50℃ with a pressure of 700 MPa. The outcome showed that HPP is effective in inactivation of avidin. The investigation on the effects of different forms of the samples also showed avidin in the egg has a low resistance either to thermal or pressure processing compared to pure avidin.

2.4.2.2 Ultraviolet radiation

UV radiations with a wavelength of 100 to 400 nm are widely used in the food industry for pasteurization and post-harvest processing (Vanga et al., 2017). Ultraviolet radiation was applied to egg white and several physical and functional properties such as size, immunoreactivity, foaming properties were measured (Manzocco, Panozzo, & Nicoli, 2012). Samples of egg white were exposed to UV-C light (15W) with an irradiance frequency of 35.4 W/m^2 for 5 and 30 min at 8℃. For 5 and 30 min, the dose used was 10.6 and 63.7 kJ/m^2, respectively. Ultraviolet processing caused backbone cleavage and aggregation of egg white but no alteration was made in immunoreactivity and gelling properties. However, foaming properties were enhanced (Manzocco et al., 2012).

2.4.2.3 Ultrasound

Ultrasound is another novel non-thermal food processing method that employs sound waves with a wide frequency range (Feng, Barbosa-Cánovas, & Weiss, 2011). According to the frequency, three types of ultrasound are generally applied: power ultrasound (20-100 kHz), high frequency

ultrasound (100 kHz-1 MHz), and diagnostic ultrasound (1-500 MHz) (Wu, Guo, Teh, & Hay, 2012). It can cause vibration of particles in the medium, resulting in occurrence of a cyclic succession of expansion and compression. The expansion and compression cycles change the surrounding pressure and cavitation bubbles may be generated. When these bubbles collapse, they will generate energy which leads to increase in temperatures and pressures (Wu et al., 2012). Studies reported that ultrasound can raise the temperature to 5000 K and pressures to 10-100 MPa (Weiss, Gulseren, & Kjartansson, 2011). These conditions in the solvent results in formation of free radicals like H^+ because of sonochemical reactions (Valdramidis, Cullen, Tiwari, & O'Donnell, 2010). Thus, ultrasound can lead to both the conformational alterations and changes in physical properties of proteins (Shriver & Yang, 2011).

Manas et al. (2006) investigated the inactivation kinetics of lysozyme using ultrasound under pressure. The inactivation reaction was conducted at different temperatures using phosphate buffer (pH 6.2) with heat (90-130℃), ultrasound (117 μm, 20 kHz) and manothermosonication (MTS, 117 μm, 20 kHz, 200 kPa). MTS is a method in which a combination of heat, ultrasound, and pressure are applied. They concluded that ultrasound performed at room temperature with any pressure was unable to inactivate lysozyme. However, MTS at 70℃ for 3.5 min decreased lysozyme activity by 10-fold (Manas et al., 2006). Further, after introducing the step to pasteurize, MTS was able to inactivate 90% of lysozyme in 10 min.

2.4.2.4 Gamma irradiation

Gamma irradiation is a processing method which exposes food directly to an ionizing wave. Gamma irradiation was first developed to extend shelf life and to destroy microorganisms or food pathogens (Farkas, 2006). Radiation treatment at doses of 2-7 kGy can effectively eliminate

potentially pathogenic non-spore-forming bacteria without negatively affecting the sensory and nutritional aspects of foods (József Farkas, 1998; Prakash, Guner, Caporaso, & Foley, 2000).

Kim et al. (2002) reported a novel allergenicity-reducing method which is the combination of gamma irradiation with heat treatment. Ovalbumin was tested under three different conditions: heat, irradiation after heat treatment, heat after irradiation. Heat treatment was conducted at various temperatures 37, 60, 70, 75, 80, 85, and 90℃ for 15 min. Gamma irradiation was operated at a dose of 10 kGy with the dose rate of 10 kGy/h. Denaturation of ovalbumin began at temperatures around 70℃, and the most denaturation happened between 80 and 85℃ in a neutral solution. The study concluded that gamma irradiation, regardless of the temperature employed for heat treatment could not reduce the allergenicity of ovalbumin. Furthermore, a significant increase in the antigenicity of one of the samples processed by heat after irradiation was observed. However, the results observed by Lee et al., (2007) on the application of electron beam and gamma radiation were different from the previous study. Ovalbumin was irradiated by gamma or an electron beam under the same conditions (3, 5, 7, 10 kGy). The study showed that both types of irradiation can reduce the antigenicity and IgE binding ability of ovalbumin. Contrary to Lee at al. (2007), other researchers observed no reduction in the allergic properties of ovalbumin under gamma radiation (Kim et al., 2002). Though the difference can be attributed to various reasons ranging from buffer concentrations, testing procedures and testing efficacy, further studies are needed to understand changes in the structure of ovalbumin protein under radiation and its effect on the allergic properties of protein. These studies should concentrate on the difference in the secondary structure changes in ovalbumin under thermal and non-thermal processing conditions and their combination.

Table 2.2. Non-thermal processing methods of egg white proteins

Protein	Processing method	Effects and outcome	Reference
Avidin	High-pressure processing (500, 600, 700 MPa) with heat treatment	Complete inactivation of avidin at 100℃ for 10 min or at 50℃ with a high-pressure treatment at 700 MPa.	(Ajaypal Singh & Ramaswamy, 2014)
Lysozyme	Ultrasound (117 μm, 20 kHz) and manothermosonication (MTS, 117 μm, 20 kHz, 200 kPa)	No effect of ultrasound on inactivating lysozyme but MTS can inactivate 10-fold the activity within 3.5 min at 70℃.	(Manas et al., 2006)
Egg white	Ultraviolet with an irradiance frequency of 35.4 W/m^2 for 5 and 30 min at 8℃. The dose for 5 and 30 min was 10.6 and 63.7 kJ/m^2	1. Backbone cleavage and aggregation of egg white 2. No effects on immunoreactivity and gelling properties but enhanced foaming properties	(Manzocco et al., 2012)
Ovalbumin	Gamma irradiation (10 kGy) combines with heat treatment (37-90℃)	Highest denaturation at 80-85℃ Reduced allergenicity	(Kim et al., 2002)
	Gamma and an electron beam irradiation at 3, 5, 7, 10 kGy	Reuduced IgG and IgE binding ability and allergenicity	(J.-W. Lee et al., 2007)
	High pressure in the range of 0.1-600 MPa at 10℃ for 10 min	1. Reduced allergenicity (maximum inhibition rate of 89.7% at 500 MPa). 2. Tertiary structures changed but secondary structure did not change	(Odani et al., 2007)
Ovomucoid	Gamma irradiation (10 kGy) with heat treatment (100℃, 15 min) at different pH	Reduced allergenicity	(Lee et al., 2002)

Gomaa et al., (2015) evaluated the changes in the allergenic and structural properties of various egg proteins using a combination of gamma radiation and thermal processing. They reported that no significant changes in the allergenicity as well as the secondary structures were observed due to gamma radiation. However, they reported that changes in food matrix plays a vital role in allergy detection.

The allergenicity of ovomucoid was also evaluated (Lee et al., 2002) after the processing with gamma irradiation (10 kGy) combined with heat treatment (100℃, 15 min) in different pH solutions (pH 7.0, 9.0 and 10.0). The study indicated that the concentration of ovomucoid could be effectively reduced by the combination of gamma irradiation and thermal processing. Table 2.2 outlines the effects of various non-thermal processing methods on egg white proteins.

2.4.3 Chemical treatments

2.4.3.1 Enzymatic hydrolysis

Enzymatic hydrolysis is one of the primary and widely used methods to reduce allergenicity. Hydrolysis can alter not only the structure of proteins including the conformational or linear epitopes present in the molecule, but also can influence their functional characteristics (Vanga et al., 2017). However, the unspecific proteolysis nature of the enzyme on these proteins may instigate new challenges. New epitope centers or unexpected changes in the functional properties that can occur after enzymatic hydrolysis could risk food safety (Sathe, Teuber, & Roux, 2005; Vanga et al., 2017).

Yang et al. (2017) evaluated the effects of alcalase hydrolysis on allergenicity and antigenicity of albumen. The hydrolysis was conducted at different temperatures (30-80℃ with 10℃ intervals) and pH 6 to pH 12. The optimal pH and temperature for both free and immobilized alcalase was found to be 8.0 and 60℃, respectively. The calibration curve method was used to calculate the

degree of hydrolysis and hydrolysates were characterized by SDS-PAGE. After 3-hour hydrolysis, the degree of hydrolysis of free alcalase (25%) is more than that of immobilized one (20%). Both were beneficial to reduce the IgG and IgE binding activity. However, immobilized alcalase can be cyclically used as it is more stable compared to free alcalase.

Enzymatic hydrolysis method was also applied to enhance the functional properties of ovomucin (Abeyrathne et al., 2016). Pepsin, trypsin, papain, and alcalase were used for hydrolysis in this study. Each enzyme reacted with ovomucin at 37℃ for up to 24 h and ended with enzyme inactivation at 100℃ for 15 min. The optimal condition for inactivating ovomucin with an enzyme was 37℃ for three hours. Findings revealed that pepsin, papain, and alcalase could hydrolyze ovomucin. The hydrolysates of ovomucin had an antioxidant activity and ACE-inhibitory activity (Abeyrathne et al., 2016). In another study, hydrolysis using trypsin and pronase showed no significant effect on avidin (Green, 1975).

2.4.3.2 Acid treatment

To date, many studies found that acidic conditions are helpful in improving food digestibility and in reducing the activity of allergic proteins. Armentia et al. (2010) reported that addition of vinegar to some food proteins (e.g. chicken, lentils) improved their digestive function in the stomach, leading to reduction in the symptoms pertaining to food allergy. Specifically, it revealed that the patients showed a negative result through the test of double-blind placebo controlled challenge when they consumed 10g of chicken with vinegar. However, the results were positive after taking the same quantity of chicken without vinegar. Similarly, this study reported that the IgE-binding activity was reduced when tested in vinegar treated lentils compared to the untreated ones. Adition of vinegar provides a more acidic condition for the stomach which will enable it to fully activate the digestive pepsin moieties. This might result in the protonation of acidic amino acid residues in

allergens which can potentially reduce the risk of food allergy. A study found that acetic acid treatment of egg white is effective in reducing the allergenicity (Lee et al., 2017). Eggs were boiled for 10 min, and then were treated with acetic acid solutions (pH 2, 3 or 4) for 24 h. The results showed that pH 2.0 or pH 3.0 acetic acid treatment significantly reduced the allergenicity of ovalbumin. Reduction of allergenicity under acidic treatment can be primarily attributed to the protein cleavage and the polarity changes on the protein surface. These changes can lead to conformational change that will not trigger an allergic reaction in the patient. Further studies are required to evaluate the effect of acidic conditions on the protein structures and their functional properties including their allergenicity and digestibility.

2.4.3.3 Protein glycation

Protein glycation involves a reaction between monosaccharides and amino acids present in proteins which may result in structural deviations and functional property changes in proteins. A Schiff base is formed after the reaction of carbonyl group of glucose with the amine group of protein during the initial process (Van Buren & LeWinter, 2011). Although this primary reaction is reversible, the carbonyl-protein adduct is eventually converted to a stable product, Amadori. Finally, it is able to form an advanced glycation end product (AGE), a stable protein which is widely used in the food industry (Van Buren & LeWinter, 2011). Thus, protein glycation has the potential to be reduce food allergenicity as it involves chemical reactions that can lead to permanent changes in the protein structure. Ma et al. (2013) investigated the effects of glycation with the moist-heat processing of ovalbumin on the antigenicity, allergenicity, and conformations of ovalbumin. The experiments were conducted in different concentrations of sodium carbonate-bicarbonate buffer (final concentration of 0.2, 0.05 and 0.02 mol/L at pH 9.6). The reaction was performed in a water bath at 60℃ for 30 min followed by an immediate cooling in ice water. They

concluded that glycation would increase the allergenicity as IgE binding capacity increased with an increasing concentration of sodium carbonate-bicarbonate buffer.

Many studies found that *N-acetylglucosaminidase* can hydrolyze N-terminal to reduce the allergenicity of an allergic protein. Hwang et al. (2016) reported the allergenicity of ovalbumin was reduced through a natural *N-acetylglucosaminidase* which was isolated from *ascidian viscer*. Park et al. (2017) also found that ovalbumin allergenicity was decreased by 1000-fold by *N-acetylglucosaminidase* treatment after measurement of OVA-specific IgE binding and histamine levels which were then analyzed by Ci-ELISA. Table 2.3 outlines chemical modifications techniques that can change functional properties of egg white proteins.

Table 2.3. Chemical modification techniques and changes on functional properties of egg white proteins

Protein	Processing method	Effects and outcome	Reference
Egg white	Acetic acid treatment	Partly decreased allergenicity in egg white but little effects on ovomucoid	(Lee et al., 2017)
Ovalbumin	N-acetylglucosaminidase	Reduced allergenicity and antigenicity	(Hwang et al., 2016; Park et al., 2017)
	Glycation with moist-heat processing (a water bath at 60℃ for 30 min with different concentration of sodium carbonate-bicarbonate buffer at pH 9.6)	1. Increased allergenicity 2. IgE binding capacity increases with the concentration of sodium carbonate-bicarbonate buffer increases	(Ma et al., 2013)

Ovomucoid	Deglycosylated processing	Unaltered IgE binding capacity	(Besler et al., 1997)
Avidin	Enzymatic Hydrolysis (trypsin and pronase)	No effects	(Green, 1975)
Albumen	Enzymatic hydrolysis (free and immobilized alcalase) Conducted at 30-80℃ with 10 intervals and pH 6-12 with 1 interval.	1. Optimal condition: 8.0 and 60℃ for 3 h. Degree of hydrolysis of free Alcalase was 25% while 20% of immobilized alcalase. 2. Reduced IgG and IgE binding	(Yang et al., 2017)
Ovomucin	Enzymatic hydrolysis at 37℃ with 24 h (Pepsin, trypsin, papain and alcalase)	1. The optimal inactivation condition was 37℃ for 3 h. 2. The hydrolysates of ovomucin displayed an antioxidant activity and ACE-inhibitory activity.	(Abeyrathne et al., 2016)

2.5 Conclusion

The current understanding of the scientific community in relating various structural aspects of egg proteins with their functional properties including their allergenicity is limited. Hence, considerable resources are being spent in exploring innovative techniques for studying the changes in various egg proteins due to numerous thermal and non-thermal processing techniques. Novel processing techniques including various non-thermal techniques show promising results in

reducing the allergic reactions without compromising the nutritional quality and safety of the products. Ultrasound and UV applications have greatly reduced the allergic activity in egg proteins like lysozyme, but at the same time improved other functional properties like foaming stability which might be desirable in few food products. The activity of anti-nutritional components like avidin was also reduced by high pressure processing and their combination with heat treatment.

However, further research is required in understanding the conformational changes in proteins due to various processing methods. Further, innovative techniques like molecular modeling (Ashutosh Singh, Vanga, Orsat, & Raghavan, 2017; Vanga, Singh, & Raghavan, 2015), proteomics (Di Girolamo, Muraca, Mazzina, Lante, & Dahdah, 2015) and genome associated studies must be undertaken to evaluate these allergens (Hong et al., 2015).

CONNECTING TEXT

In the review, we have seen that allergens and anti-nutrient compound avidin present in egg white can be inactivated using various processing techniques. The conformational changes in the secondary structures of protein were also observed in different studies outlined in the review that have directly influenced the functional properties. In the next part of the thesis, an experimental study on the effect of microwave and ultrasonic processing on conformation of egg protein was conducted using Fourier Transform Infrared Spectroscopy (FT-IR) and Circular Dichroism (CD) Spectroscopy. Inactivation of avidin is of interest in this study due to its anti-nutritional properties.

CHAPTER III

Effects of Ultrasonic and Microwave Processing on Avidin assay and Secondary Structures of Egg White Protein

3.1 Abstract

The effect of microwave and ultrasonic processing on the secondary structure of egg white protein was evaluated using FTIR and CD spectroscopy. The influence of these methods on avidin activity was also evaluated. Microwave treatment was performed at 60ºC, 70ºC and 80ºC for 1, 3 and 5 min. Avidin activity was found to decrease in all the processed samples. However, the reduction in its activity was higher when treated at 80ºC. The secondary structure analysis revealed that the β-sheets increased at temperatures 60ºC and 70ºC as the processing time rose while became almost constant at 80ºC. α-helices were found to decrease with time at each of the temperatures at which microwave was applied. Turns and unordered structures remained constant. Ultrasonic processing was carried out for 1, 4, 8, 12 and 16 min. α-helices declined significantly with remarkable increase of β-sheets when samples were treated for 1 min compared to control. However, the secondary structure content did not change significantly when the duration of ultrasonication was increased, showing minimal processing capabilities of ultrasound.

Key words: Avidin, Egg white, Secondary structure, FTIR, CD spectroscopy

3.2 Introduction

Eggs have been a part of human diet for centuries and are known for their superior nutritional qualities (McGee, 2007). As technology improved, more and more egg related products emerged and products with extended shelf-life became popular (Stadelman, Newkirk, & Newby, 2017). Eggs are one of the most versatile ingredients used in cooking around the world. In 2013, global hen egg production reached 65.5 million tonnes with 59% being produced only from Asia (Kheiralla, Yousif, Hassan, Khiery, & Ghanim, 2016). Eggs are one of the most nutritious foods on the planet due to the presence of various bioavailable components and other essential nutrients (e.g., omega three fatty acids) in higher quantities.

In 2016, United States Department of Agriculture (USDA) reported 100g of raw whole egg to contain 12.56 g of protein and 9.2 g of fatty acids based on the national nutrient database (USDA, 2015). It also contains a decent amount of vitamins (vitamin A, vitamin B6 and vitamin B12) and minerals. Each 100 g raw whole egg contains 56 mg calcium, 12 mg magnesium, 198 mg phosphorus, 138 mg potassium and 142 mg sodium. Furthermore, many studies found that lutein and zeaxanthin, i.e., the antioxidants present in eggs are beneficial to ocular health (Abdel-Aal, Akhtar, Zaheer, & Ali, 2013; Hester, 2016). For example, consuming 1.3 egg yolks per day for 4.5 weeks can significantly increase the lutein concentration in blood by 28-50% and zeaxanthin by 114-142% (Handelman et al., 1999) and these nutrients are beneficial to reduce the risk of two very common eye disorders: cataracts and macular degeneration (Delcourt, Carriere, Delage, Barberger-Gateau, & Schalch, 2006; Gale, Hall, Phillips, & Martyn, 2003). Moreover, eggs from hens fed with omega-3 enriched feeds can contain higher omega-3 fatty acids compared to the regular ones. In medical and nutritious researches, omega-3 fatty acids are considered to be one of best substance in reducing triglyceride level in blood which may cause heart disease (Balk et al.,

2006). Study found that consuming one omega-3 enriched egg per day for three weeks can reduce triglycerides by 16-18% (Bovet, Faeh, Madeleine, Viswanathan, & Paccaud, 2007).

However, eggs are becoming one of the most common allergy-causing foods especially in infants and children. These allergic reactions can result in mild symptoms like skin rashes, hives, nasal congestion, and vomiting or other digestive problems and rare severe reactions leading to anaphylaxis. Experts estimate that approximately 1-4% of children are allergic to eggs around the world and about 80% of them outgrow the allergy by the age 16 (Eigenmann, 2000; Gray et al., 2015).

Eggs also contain avidin anti-nutrient which binds with biotin making it unavailable biologically. The reaction forms an avidin-biotin complex which is found to be highly stable. The first case that avidin can cause allergy was reported in 2007 (Enriquez-Matas et al., 2007). As the 2-year-old boy was hypersensitive to eggs but not allergic to existing egg allergens, the experts suspected that it was avidin to cause his allergic reactions and suggested that further studies about allergenicity of avidin should be further conducted.

Various studies have been carried out to reduce the avidin activity. Avidin is stable because it cannot be reduced using normal cooking method like boiling (Durance & Wong, 1992). Besides, it is difficult to inactivate avidin using proteolysis (pronase and trypsin) (Green, 1975). Avidin lost almost all the activity when processed at 121℃ for 25 min (Durance & Wong, 1992). When processing time exceeded to 75℃, avidin began to unfold itself and lose ordered secondary structures (Swamy, Heimburg, & Marsh, 1996). A complete denaturation of avidin took place when processed at 100℃ for 10 min or subjected to a high-pressure processing at 700 MPa at 50℃ (Ajaypal Singh & Ramaswamy, 2014).

Apart from avidin in the egg, it contains various other proteins like ovalbumin (OA), ovomucoid (OVM), ovotransferrin (OVT) and lysozyme (Lys). Many studies reported that functional properties of foods can be modified through food processing. Thermal and non-thermal processing are effective in changing various functional properties in food due to denaturation leading to loss in tertiary and/or secondary interactions, formation of new intra- or inter-molecular bonds, aggregation, and/or rearrangements of disulfide bonds, as well as other conformational modifications (Mondoulet et al., 2005; Vanga et al., 2017). Various researchers have been trying to assess the changes in the secondary structure due to a wide variety of thermal processing methods, including microwave technology (Vanga, Singh, et al., 2016). Various studies have provided crucial insights into the secondary structure deviations of proteins that can directly influence their physico-chemical properties (Beck, Knoerzer, & Arcot, 2017; Gomaa & Boye, 2015; Vagadia, Vanga, Singh, Gariepy, & Raghavan, 2018; Vanga et al., 2015).

In comparation to thermal processing, non-thermal processing was found to maintain most of the desirable characteristics and often maintain more nutritional benefits (Raso & Barbosa-Cánovas, 2003). Currently, ultrasonic processing is a novel technology which can be used to modify various structural properties in proteins due to its high energy (high power, high intensity, low frequency) ultrasonic waves. It can produce high pressure, shear and temperature gradient, which may be beneficial to alter protein structure and its properties (Jiang et al., 2014). Studies found that proper concentration of ultrasonic processing not only increased the amount of soluble proteins, but also reduced two major peanut allergens Ara h 1 and Ara h 2 levels in peanuts and resulted in a certain inhibition on IgE-binding (Li et al., 2013). Similar results were found on the shrimp, the allergenicity of boiled shrimp treated with ultrasonic treatment (30 kHz, 80W, 10 min) decreased by 50%, compared with the control (raw shrimp) (Li et al., 2013). In this study, we have evaluated

the changes in secondary structure of egg white after microwave and ultrasonic processing using FTIR and CD spectroscopy. Inactivation of anti-nutrient avidin was also performed.

3.3 Materials and Methods

3.3.1 Chemicals

d-biotin, 2-(4'-hydoxyazobenzene) benzoic acid (HABA), sodium phosphate monobasic and dibasic, pepsin and pancreatin were bought from Sigma-Aldrich, ON, Canada. Hydrochloric acid, sodium hydrate and sodium carbonate were bought from Thermo Fisher Scientific, Canada.

3.3.2 Sample preparation

Fresh unpasteurized eggs were bought from the local grocery store. Eggs were broken manually to separate egg white from yolk. The egg whites collected were mixed well and packed in centrifuge tubes (50 ml) for further studies. Fresh mixture was prepared before each run.

3.3.3 Microwave treatment

Microwave processing of egg white was conducted in Mini WAVE Digestion Module (SCP Science, Canada) that operates at a frequency of 2.45 GHz. 6 ml of egg white mixture was mixed with 14 ml of distilled water in cylindrical quartz reactor vessel by vortex mixer to avoid aggregation after processing. Each experiment had 6 replicates (equal to the number of chambers in the machine). Then all the vessels were assembled and put into the chamber for microwave processing at combinations of 60°C, 70°C and 80°C for 1, 3, 5 minutes. There are IR sensors located on the sidewalls along with a single magnetron located at the bottom of the treatment chamber which monitors the sample temperatures. All the samples were immediately frozen at -20°C after processing for freeze drying.

3.3.4 Ultrasonic processing

Ultrasonic processing was carried out using Branson Sonifier 400 (Branson Ultrasonic Corp., Danbury, CT, USA) and all the experiments were carried out in triplicates. A sonication horn was immersed in the small glass beaker with 15 ml of liquid egg white samples for 1, 4, 8, 12 and 16 minutes. All the samples were processed with a power output of 400 W. The ultrasonic processing was conducted in an ice bath to keep the samples at room temperature (around 25°C). All the processed samples were frozen immediately in -20°C for freeze drying.

3.3.5 Extraction of protein

Extraction of the protein is carried out using the procedure suggested by Durance (1991). 3 g of samples powder were dissolved in 27 g of 0.2M phosphate buffer (pH 7.9) and vortexed for 40 s. Sample pH is adjusted below 7.9. Milky suspensions were centrifuged at 15,000 × g for 30 min and clear supernatant was used for further analysis.

3.3.6 Avidin Assay

The avidin activity is assayed in triplicate for each experimental condition using the procedure suggested by Durance and Singh (Durance, 1991; Durance & Nakai, 1988; Ajaypal Singh & Ramaswamy, 2014) with some modifications. 2 mL of sample solution and 0.1 mL of HABA stock were mixed in a cuvette (1 cm path length) and absorbance was read at 500 nm (A_1). If A_1 is greater than 0.600, the sample was further diluted by using 0.1 M sodium phosphate buffer (pH 7.8). Then, 0.05 mL biotin stock was added and A_{500} (A_2) was read again. Change in absorbance with addition of d-biotin is calculated ($A_1 - A_2 = A_3$)

Finally, avidin activity is calculated using the following equation (Durance, 1991; Durance & Nakai, 1988; Ajaypal Singh & Ramaswamy, 2014):

$$Avidin\ activity\ \left(\frac{\mu g}{mL}\right) = \frac{Vol\ Assay \times 68000 \times A_S \times 1000}{Vol\ Sample \times 34000 \times 4} \qquad (3.1)$$

where 68,000 is the molecular weight of avidin (Durance & Nakai, 1988); 34,000 is the difference between the molar extinction coefficient of HABA-avidin complex and HABA alone at 500 nm and 4 is the number of avidin subunits per native avidin molecule (Ajaypal Singh & Ramaswamy, 2014). The specific gravity is assumed to be 1.0 for the purpose of calculations here.

3.3.7 Circular Dichroism Spectroscopy

Circular dichroism (CD) spectroscopy was used to examine the secondary structures of egg white protein. Circular dichroic measurements were determined by Jasco 810 spectropolarimeter (JASCO, Tokyo, Japan) with thermostat set at 20°C. Samples were scanned from 260 nm to 195 nm with a bandwidth of 1nm and pitch of 0.2 nm. The final spectrogram was averaged by five spectra accumulated using a cuvette with a path length of 0.01 cm. Each sample was dissolved in phosphate buffer (pH 7.9) to reach the final concentration of 1 mg/mL in all the measurements and the high voltage never exceeded 700 volts. Data were then analyzed by CDPro software (Sreerama & Woody, 2000).

3.3.8 FT-IR Spectroscopy

The Fourier transform infrared spectroscopy (FTIR) technique was used to study the secondary structures of egg white proteins. The sample powder was put on the diamond crystal under normal air. The data were collected by Windows-based OMNIC software (Version 8, Thermo Nicolet Co., Madison, WI) connected to the FTIR (Nicolet Magna 158 750 FTIR, Nicolet Instrument Corp., Madison, WI). 32 scan spectra at a spectral resolution of 4 cm^{-1} were recorded and averaged in the mid-infrared region (4000 - 500 cm^{-1}). A background spectrum without the sample was collected under the same conditions and subtracted from each sample sample spectra.

A curve-fitting procedure was applied using OriginPro (Version 9, Origin Lab Corporation, Northampton, MA, USA) to quantify the conformational changes of egg white protein in the amide

I band. The sum of squares of the differences was minimized between experimental spectra and the computed spectra developed by the summation of the component curves. The component bands were determined using the second derivative spectrum generated by the software, and the results were given in terms of the peak areas at corresponding wavelengths.

3.3.9 *In-vitro* protein digestibility

The *in-vitro* protein digestibility of egg white protein was conducted following the procedure suggested by Hejazi et al. and Vilela et al. (Hejazi & Orsat, 2016; Vilela, Lands, Chan, Azadi, & Kubow, 2006) with some small modifications. The sample powder was dissolved in double distilled water in 50-mL Erlenmeyer flasks to give a final protein concentration of 3 mg/mL and the pH of the sample solution was adjusted to 1.5 using 0.1 N HCl. All the flasks were put in water bath at 37°C and digestion was started by adding freshly prepared pepsin solution (5 mg pepsin/mL in 0.01 M HCl) to reach an enzyme to substrate ratio of 1: 100. The first digestion took 30 min and was interrupted by adding 1.0 M NaOH solution. Then the pH of solution was adjusted to 7.8 using 1.0 M NaOH. The second digestion was started by adding freshly prepared pancreatin stock solution (5 mg/ mL in sodium phosphate buffer, pH 7.0) to reach an enzyme to substrate ratio of 1: 30. 150 mM Na_2CO_3 solution was used to stop the reaction after 60 min of digestion at 40°C. After the digestion, the protein content of samples before digestion, after first digestion and after second digestion was determined using BCA method. The *in vitro* protein digestibility was calculated using the following equation:

$$IVPD\% = \frac{initial\ protein - final\ undigested\ protein}{initial\ protein} \times 100 \qquad (3.2)$$

3.3.10 Statistical analysis

The experimental data were fitted by analyses of variance (ANOVA) using SPSS software (SPSS Inc., Chicago, IL, USA). Duncan multiple range test was applied to separate the means and significance was accepted at $p \leq 0.05$.

3.4 Results and Discussions

3.4.1 Residual Avidin activity

The impact of microwave treatment and ultrasonic processing are summarized in Fig 3.1(a) and (b), respectively. In the case of microwave treatment, the original avidin activity of samples was 38.36 ± 1.48μg/mL, which is highest compared to other processed samples.

For each temperature, the avidin activity declined with increased processing time. As expected, higher inactivation of avidin was observed at higher temperatures. There was a significant decrease in avidin activity in all the processed samples. When egg white samples were processed at 60°C or 80°C, there is no significant difference between 1 min and 3 min. However, as the processing time increased to 5 min, a significant reduction in avidin activity was observed. No significant difference was noticed among samples processed at 70°C with increased processing time. When the samples were subjected to 1-minute treatment, a significant decrease was noticed between 60°C and 70°C. For 3-minute processing, avidin activity decreased significantly at 70°C and 80°C when compared to 60°C. In the case of 5-minute treatment, it is noticeable that a significant decline in avidin assay occurred at 80°C in comparison to the values at 60°C or 70°C. It can be found that temperature can significantly influence the avidin activity. Vagadia et al. (2018) suggest that microwave processing is an effective method to inactivate soybean trypsin inhibitor and can be a potential alternative approach to eliminate the anti-nutrients in foods. Hefnawy reported that microwave processing is beneficial to reduce anti-nutritional factors like tannins and restrain the

nutrients in food (Hefnawy, 2011). Another study also confirmed that microwave treatment can significantly decrease the activity of some anti-nutrients like tannin and saponin in buckwheat which increased the bioavailability of mineral and other nutrients (Deng, Padilla-Zakour, Zhao, & Tao, 2015).

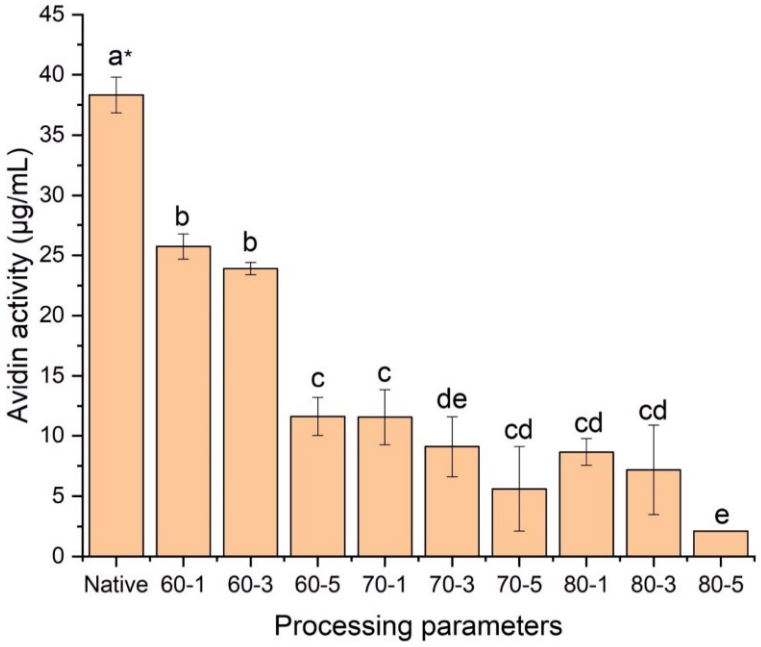


Figure 3.1(a). Avidin assay of microwave processed samples (*Common letters indicate lack of significant difference (P > 0.05) based on ANOVA)

The effects of ultrasound on avidin assay are presented in Fig 3.1(b). The avidin activity of unprocessed sample was found to be 24.28±0.52 µg/mL, which is higher than all the processed egg white samples. In general, the avidin activity decreased as the processing time increased. However, the significant inactivation of avidin was only observed when the egg white samples were processed for 16 min.

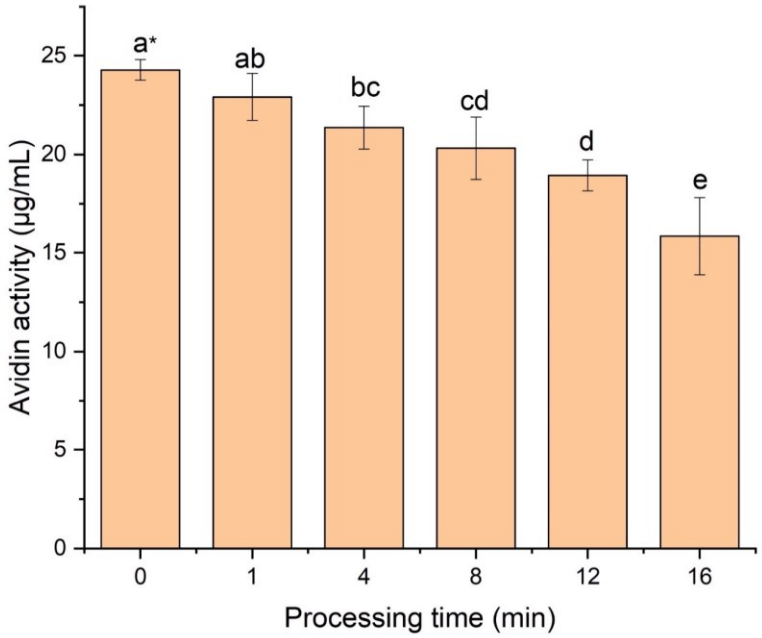


Figure 3.1(b). Avidin assay of ultrasound processed samples (*Common letters indicate lack of significant difference (P > 0.05) based on ANOVA)

A study worked on soybean seeds showed that ultrasonic processing can inactivate anti-nutritional factors including lipoxygenase isozyme and isoflavone, especially trypsin inhibitor (Yang, Gao, Yang, & Chen, 2015). The phenomenon was observed possibly because ultrasound may cause particle vibration, bubble generation and collapse, and shear stresses, resulting in change of activity of anti-nutrients (Yang et al., 2015). Ertas (2013) reported that ultrasonic processing can partly reduce the anti-nutrient phytic acid content, but it is less effective than microwave processing. In the present study, there is a similar trend that microwave treatment is more effective in decreasing avidin activity compared to ultrasonication. The reduction of avidin activity also suggests that the secondary structure of avidin may change, but further analysis on pure avidin should be done to verify if the structure changes. Besides, the effects of structural changes on allergenicity and functional properties of avidin should also be evaluated.

3.4.2 CD analysis of changes in the secondary structure of egg white protein

The CD spectra of egg white protein after ultrasound and microwave treatment are shown in Fig. 3.2(a) and (b) respectively.

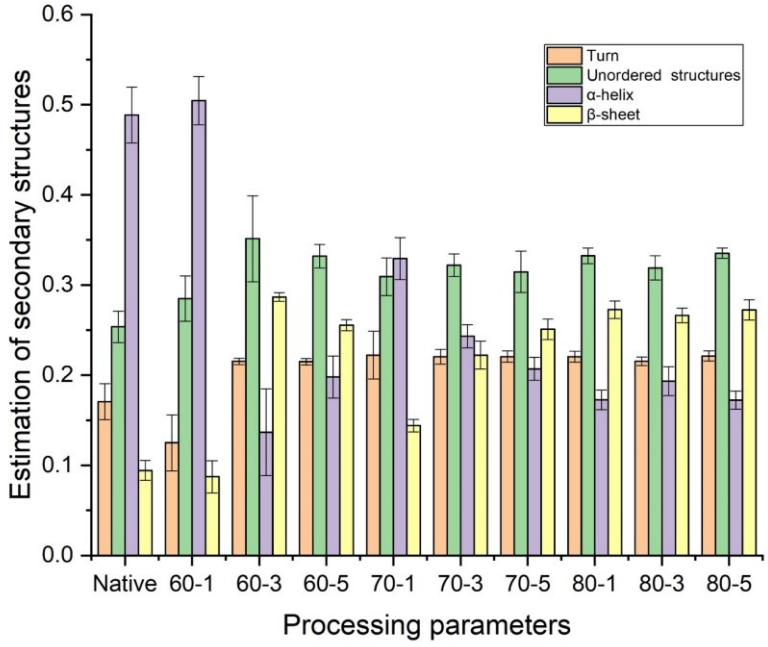


Figure 3.2(a). Variations in estimation of secondary structures of microwave processed egg white in the CD spectrum

From the spectrum of native egg white (Fig. 3.3), a minimum and a shoulder were observed at 220 nm and 208 nm. The typical negative peaks at around 208 nm showed the possible existence of α-helix component of proteins and positive peaks at 195nm demonstrated the presence of β-sheet (Han et al., 2015). With the application of microwave, it is shown that egg white protein begins to denature at 60°C for 3 min. α- helix content decreased with time for each temperature while β-sheets increased from 1 minute to 3 minutes and became almost constant at 60 and 70°C. For 80°C, the β-sheet content is constant regardless of varied time. However, it increased more than 2 folds

compared to the control. There is a little increase in unordered and turn structures of all the processed samples in contrast to the native one.

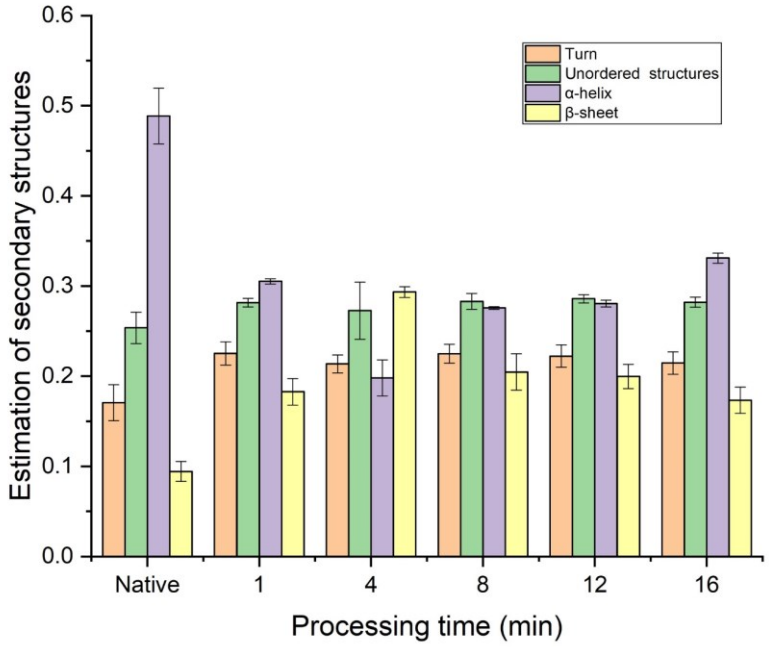


Figure 3.2(b). Variations in estimation of secondary structures of ultrasound processed egg white in the CD spectrum

When egg white is subjected to ultrasonic treatment, similar results were obtained. There is an obvious reduction of α- helix when egg white was processed for 1 minute while β-sheet content increased remarkably. With the processing time increased, all the content became almost constant. Turn and unordered structures remained almost the same when ultrasound was applied. In terms of protein, ultrasonic processing of eggs meets the requirements for minimally processed products but studies on microbiology can be further conducted to understand the effect of ultrasound on pasteurization.

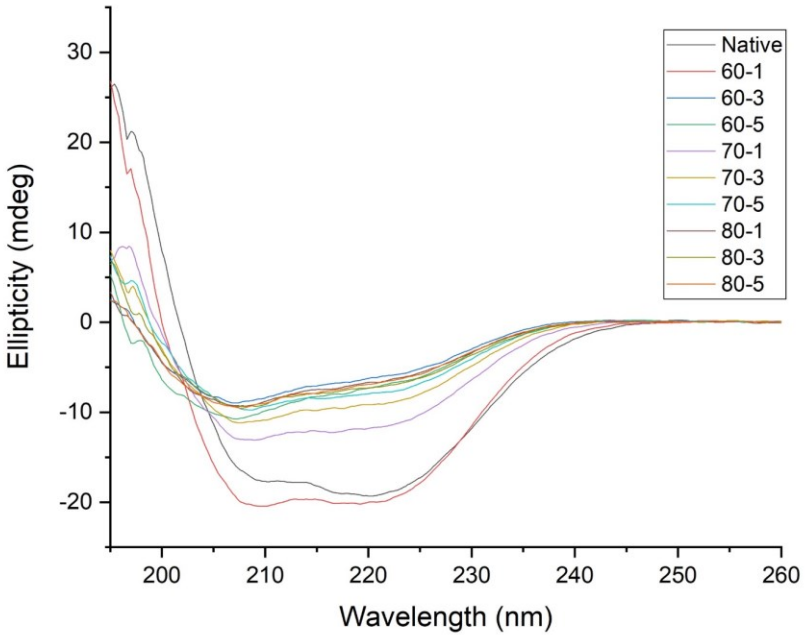


Figure 3.3. CD spectrum of raw and microwave processed egg white samples

3.4.3 FTIR analysis of conformational changes in egg white protein

FTIR was utilized to investigate the effects of microwave and ultrasonic processing on the secondary structures of egg white protein. In FTIR spectra, the amide I (1700– 1600 cm^{-1}) region is one of the most valuable and sensitive area and is primarily used to study the secondary structure deviations in proteins (Vanga, Singh, et al., 2016), including overlapping bands of β-sheets, α-helices, turns and random coils. The amide I region involves major contributions from C=O stretching vibrations coupled with the minor contributions from C-H stretching vibration. The strength of the hydrogen bond formed between C=O and N-H moieties can influence the exact frequency of this region, and each type of secondary structure is related to a characteristic hydrogen bond pattern. In that case, secondary structures of protein can be determined by separating amide I absorption (Jackson & Mantsch, 1995; Ashutosh Singh et al., 2016; Vanga, Singh, et al., 2016). The typical FTIR spectra of raw and microwave processed egg white samples are shown Fig. 3.4.

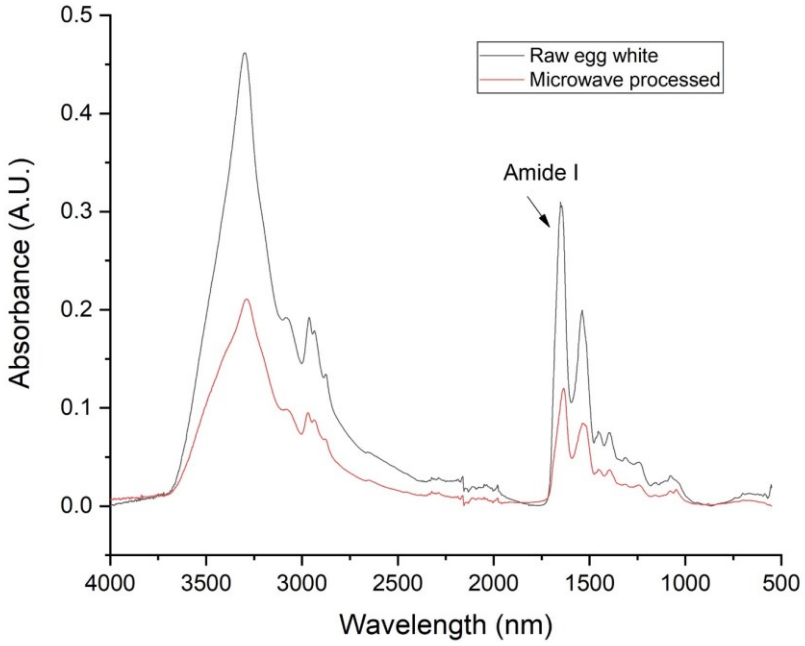


Figure 3.4. FTIR spectrum of raw and microwave processed (80-5 min) egg white samples

In the amide I region, peaks observed from 1613-1637 cm^{-1} are considered as the presence of β-sheets. For native samples, major peaks at 1645-1662 cm^{-1} are found to be a great portion of α-helix present in egg white. The correlations between the amide I frequencies and secondary structures are summarized in Table 3.1.

Table 3.1. Amide I band frequencies and assignment to protein secondary structure (Grewal et al., 2017; Qian, Ma, Wang, & Jiang, 2016; Ashutosh Singh et al., 2016; Swamy et al., 1996; Vanga, Singh, et al., 2016)

Secondary structure	Frequencies(cm^{-1})
β-sheet	1613-1637 1682-1696
Unordered	1637-1645
α-helix	1645-1662
Turns	1662-1682 1630

The peak areas for microwave processing at 60°C, 70°C and 80°C for 1, 3, and 5 min are summarized in Fig. 3.5(a). The changes in the peak areas as the ultrasonic processing time from 0, 1, 4, 8, 12 to16 min are also shown in Fig. 3.5(b).

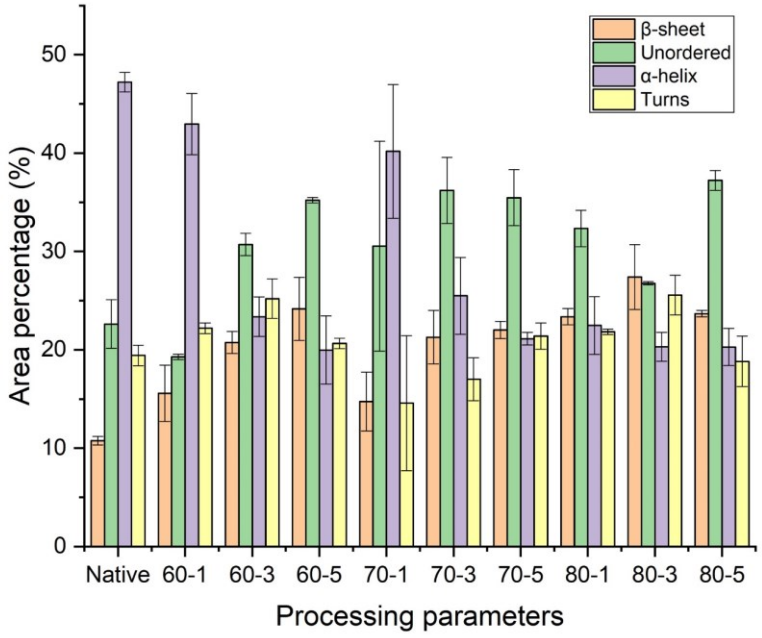


Figure 3.5(a). Variations in relative areas of the fitted to the normalized FTIR spectra of the Amide I region of microwave processed egg white

It was noticed that α- helix was the major secondary structure present in raw egg white, representing about 40-50% of the total secondary structures. In all the microwave processing experiments with an exception of native and processed samples at 60°C for 1 min, unordered structures were found to be dominant accounting for 27-37% of the total protein secondary structures which are converted from the helices present in native state.

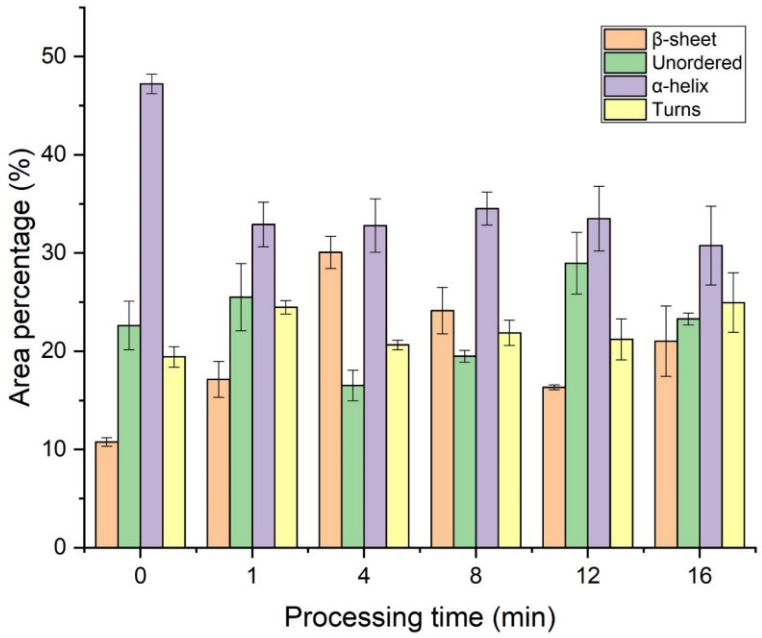


Figure 3.5(b). Variations in relative areas of the fitted to the normalized FTIR spectra of the Amide I region of ultrasound processed egg white

The effects of microwave treatment on the secondary structure of egg white protein are presented in Fig. 3.5(a). There was an obvious rise in the β-sheets and a remarkable decline in the α- helices of all the processed samples in comparison to the raw sample. β-sheets are found to increase at elevated processing time when the samples were treated at 60°C and 70°C, while α- helices were decreasing. However, when egg white was subjected to 80°C, the contents of β-sheets and α-helices remained constant despite increasing the processing time. These finding corresponds to the secondary structure data obtained from circular dichroism spectroscopy. Turn structures remained constant throughout the thermal application except for 70°C where an increase in turns was noticed as the processing time rose.

There are some variations between FTIR and CD results, especially the contents of α- helices and unordered structures. The difference between FTIR and CD results could be attributed to the status

of the sample. In FTIR analysis, lyophilized powder was used while aqueous solution was utilized for CD analysis (Feng, Cai, Wang, Li, & Liu, 2018). Besides, different definitions for different types of secondary structure may be applied to different techniques, resulting in difference between FTIR and CD (Byler & Susi, 1986). Another reason for difference is that in some calculations, empirical basis sets are adopted as standards (Byler & Susi, 1986). The unordered structures were found to increase after microwave processing and remained almost constant when increasing temperatures or time.

Figure 3.5(b) shows the peak areas for ultrasonic samples processed for 0, 1, 4, 8, 12 and 16 min. From the figure, there was a reduction in the area of peaks assigned to α- helix component between raw and processed samples. However, as the processing time increased, there was no significant difference in the content of α- helices, which matched with the CD results. β-sheet contents of processed samples were found to increase from 0 min to 4 min then decreased slightly from 4 min to 16 min. When compared to raw samples, a rise in β-sheets of all the processed ones was observed. In the case of turn structures, they remained almost constant as the processing time increased, which showed a similar trend as that of CD results. Some fluctuations in content of unordered structures were observed. However, CD results showed turn content was steady, accounting for around 28% of the total protein secondary structures, from 0 min to 16 min.

The significant observation of the changes in CD spectra during microwave processing and ultrasonic treatment could be considered that β-sheet increased with the sacrifice of α- helix. The results correspond to the finding of Mine et al. (1990). The structural transformation can be considered that egg white is partially unfolded or loosened and the structure became more flexible and extended. It is further confirmed by Bohr et al. (Bohr & Bohr, 2000), suggesting that microwave has a non-thermal effect which could speed up the rates of the denaturation and folding

process. The external stresses may disrupt the hydrogen bond and the conformational changes may also lead to exposure of hydrophobic regions and further enhance the structure of β-sheet (Han et al., 2015; Yoshinori Mine et al., 1990; Van Kleef, 1986).

Our study confirmed the finding that exposure to ultrasound can cause re-folding of protein that could result in a different secondary structure composition (Stanic ‐ Vucinic et al., 2012). Although the study showed ultrasound to have minimal effects on secondary and tertiary structures of protein (Paulsson & Dejmek, 1990), there are several findings that contradict these results. Stathpulos et al. (2004) reported sonication-induced aggregates to have higher β-sheet content. For proteins with great native α- helices, a reduction in α- helix with a concomitant increase in β-sheet are shown in the aggregates. Gülseren et al. considered that ultrasound can lead to irreversible changes in secondary structures of bovine serum albumen and found that there was a slight increase in ordered structures of ultrasonic treated samples (Gülseren, Güzey, Bruce, & Weiss, 2007).

3.4.4 Effect of processing on the protein digestibility

The effect of microwave and ultrasonic processing methods on egg white protein digestibility is summarized in the Tables 3.2 and 3.3. The digestibility of the raw egg white protein was found to be around $73.44 \pm 0.0426\%$. In the case of microwave processing, most of the processing combinations revealed an improvement in protein digestibility of egg white. When processed at 60°C, a significant increase of the *in vitro* protein digestibility (IVPD) was observed in comparison to control (unprocessed samples) for one-minute and three-minute processing. However, the value decreased when processed up to 5 min at 60°C. As the temperature increased to 70°C and 80°C for one-minute processing, the protein digestibility has significantly increased when compared to control. Then with the increase of processing time at 70°C and 80°C, the IVPD was found to decline. The value decreased significantly when processed at 70°C for 5 min. For microwave

processing at 80°C, there was a significant reduction in protein digestibility with increased processing time, but the values are higher in comparison to control. When egg white protein was processed for one minute, a significant rise in protein digestibility was noticed when increasing the temperature from 60°C to 80°C. But for other processing time, protein digestibility did not show significant improvement with the increase of temperature.

Table 3.2. Effect of microwave processing on protein digestibility (IVPD %) of egg white protein. Common letters indicate lack of significant difference (P > 0.05) based on ANOVA and Duncan.

Time (min)	**Temperature (°C)**		
	60	**70**	**80**
1	83.58±0.0023% bc	87.31±0.0012% ab	89.26±0.0185% a
3	85.78±0.0002% ab	81.66±0.0030% bc	82.36±0.0021% bc
5	78.94±0.0286% cd	71.15±0.0032% e	75.97±0.0495% de

Table 3.3. Effect of ultrasonic processing on protein digestibility (IVPD %) of egg white protein. Common letters indicate lack of significant difference (P > 0.05) based on ANOVA and Duncan.

Time (min)	**IPVD**
0	73.44±0.0426% bc
1	72.54±0.0050% c
4	75.34±0.0018% abc
8	72.24±0.0005% c
12	78.16±0.0137% ab
16	79.41±0.0153% a

Vanga et al. (2016) reported that the digestibility of peanut protein increased significantly with the rise of temperature and time of using microwave treatment. The increasing protein digestibility is related to the conformational changes in protein structure, which is in accordance with CD and FTIR results in this study. Inactivation of anti-nutritional factors in food can also be part of the reason that leads to higher protein digestibility (Rehman & Shah, 2005). Thus, the decrease of anti-nutrient avidin activity in this study may also attribute to the increased protein digestibility. The increase of protein digestibility also suggests the reduction of allergenicity. A study conducted on soybean trypsin inhibitor showed that microwave processing may cause the conformational changes of soymilk protein. The structural alteration leading to formation of protein aggregates may reduce the susceptibility to the digestive enzymes, resulting in lower protein digestibility (Vagadia et al., 2018).

Vanga et al. (2017) suggested that the effect of thermal processing could be enhanced with the increased temperature, resulting in a complete denaturation of protein. The denaturation of protein can form aggregates which can lead to reduction in its susceptibility to digestive enzymes. Chemical reactions between proteins and other components in the food like carbohydrates can also take place under thermal stress and thus leading to the undesirable component formation and discoloration. Another finding showed a decrease in protein digestibility of food legumes when cooked at 121°C from 10 to 90 min and 128°C for 20 min (Rehman & Shah, 2005). They suggested that the reduction in the availability of amino acids with the increased time and temperature may be the reason for the decrease of IVPD values.

Regarding ultrasonic processing, protein digestibility has risen with the increase of processing time. A significant increase of protein digestibility can be observed until the samples were processed for 16 min. Han et al. (Han, Swanson, & Baik, 2007) reported that the effect of soaking under

ultrasonic processing on IVPD of legumes is inconsistent and insignificant, which was also observed in this study. The results reinforced that the minimal processing effects can be reached using ultrasound.

3.5 Conclusion

This study showed that the anti-nutrient avidin in egg can be inactivated considerably using microwave treatment. The maximum inactivation rate can reach 95% at 80°C for 5 min. Ultrasonic processing can also be one of effective processing methods to reduce avidin activity. In this study, no more than 45% of avidin reduction was achieved. The egg white protein digestibility can be significantly enhanced when subjected to one-minute microwave processing. Besides, the IVPD values increased with the temperature rose for one-minute processing. However, the values decreased as the processing time further increased in comparison to those of one-minute processing. When the samples were subjected to ultrasound, the IVPD did not increase significantly until 16 min, reinforcing that ultrasonic processing showed the minimal effect on food. Microwave and ultrasonic processing are effective on altering the conformational structures of egg white protein. In CD analysis, 48.87% of α- helices declined to 17.23% with the increase of β-sheets from 9.43% to 27.23%. Similar trend was observed in FTIR analysis. However, the mechanism inside is still unknown. Further studies need to be conducted for better understanding the impacts of these two processing methods on proteins.

CONNECTING TEXT

Since the experimental study shows that avidin can be greatly inactivated using microwave processing, it is important to study the interactions between the protein and microwave treatment. Therefore, molecular dynamic (MD) simulation technique is performed to evaluate the effect of external stresses including thermal and electric field (2450 MHz) on the conformation and surface properties of avidin in the following chapter. The effect of the thermal stress and the oscillating electric field using MD on avidin molecule could help understand the implications of changes in functional properties.

CHAPTER IV

Effects of Thermal and Electric Fields on Avidin Conformation

4.1 Abstract

Though eggs are known for their superior nutritive values and ease in availability in most countries, they are also responsible for causing egg allergies. Further, eggs also have an anti-nutrient known as Avidin which forms an Avidin-Biotin complex and makes biotin unavailable in the body for absorption. In this study, the molecular dynamic (MD) simulation was used to evaluate the effect of external stresses including thermal and oscillating electric field (0.05 V/nm, 2450 MHz) at 300K, 333 K, 343 K and 353 K on anti-nutrient Avidin. The modeling was carried out using GROMACS software. The alteration in conformation was evaluated in terms of number of hydrogen bonds, root mean square deviation, radius of gyration and solvent accessible surface area. The application of thermal stress and electric field resulted in conformational changes in the avidin protein. The results also showed that hydrogen bonds have a significant influence on the stability of alpha-helices which are responsible for the compactness of the molecule. Change in the hydrogen bonds can increase the surface area and hence the surface properties of avidin significantly.

Keywords: molecular dynamic modeling; avidin; electromagnetic fields; GROMACS

Abbreviation

CHARMM	Chemistry at HARvard Macromolecular Mechanics
PDB	Protein Data Bank
MD	Molecular Dynamics
TIP3P	Transferable Intermolecular Potential With 3-Points
RMSD	Root Mean Square Deviation
Rg	Radius of gyration
SASA	Solvent Accessible Surface Area
NVT	Ensemble in which number of atoms, volume and temperature are constant
NPT	Ensemble in which number of atoms, pressure and temperature are constant
CD	Circular Dichroism spectroscopy
FTIR	Fourier Transformation Infrared Spectroscopy
NMR	Nuclear Magnetic Resonance Imaging

4.2 Introduction

Eggs are a good dietary source of proteins, lipids, essential vitamins and minerals. Nutritional values aside, there are various bioactive components in eggs, exhibiting diverse biological activities such as antimicrobial activities, anticancer, antioxidant properties, and antihypertensive activities, making egg and egg components a valuable asset in health or disease prevention and treatment for human (Kovacs-Nolan, Phillips, & Mine, 2005; Lesnierowski & Stangierski, 2018). For example, lecithin found in eggs delay aging processes, protect the walls of the stomach and liver, facilitate the utilization of fat-soluble vitamins and ameliorate blood circulation. Further, lecithin is also an indispensable component promoting healthy brain and nerve tissue function (Lesnierowski & Stangierski, 2018). Besides, the antibacterial activity of lysozyme makes it valuable in the medical, pharmaceutical and veterinary industries (Lesnierowski & Stangierski, 2018).

Eggs are highly accessible and largely consumed around the world; however, an increasing number of cases related to egg allergy have been reported, especially in children. Eggs are considered to be the second most common food to trigger an allergy (Gray et al., 2015; Savage et al., 2007). The major egg allergens are found in egg white, which include ovomucoid (Gal d 1), ovalbumin (Gal d 2), conalbumin (Gal d 3) and lysozyme (Gal d 4) (Poulsen et al., 2001). Besides the major allergens, other proteins present in eggs such as α-livetin (Gal d 5), apovitellenins I, apovitellenins VI, and phosvitin may be allergic for some people (Jacobsen et al., 2008; Y Mine & Rupa, 2004). Avidin as an anti-nutrient in eggs, is of interest for many researchers. Furthermore, it is suspected to be an allergen due to a specific case in 2007 (Enriquez-Matas et al., 2007). A two-year-old boy was found to have hypersensitivity to egg but results of specific IgE showed no correlation with

any of the major allergens. In this study, the experts assumed that avidin in egg caused the allergenicity and recommended further investigation.

Avidin can be found in hen egg and the proportion is around 0.05 – 0.5 % of total protein in egg white (Green, 1975). Avidin is a water-soluble glycoprotein which has a strong binding capacity with biotin (Green, 1963; Yossa et al., 2011). Avidin is a tetramer comprised of monomers containing 128 amino acid residues (Yossa et al., 2011). In its primary structure, a single disulfide bond and a carbohydrate side chain are found to be linked to the residue Asn (17) (Green, 1975; Laitinen et al., 2002). Eight orthogonal antiparallel β-strands are identified in secondary structures (Spolaore et al., 2014). The unique organization in both primary and secondary structures is responsible for the high stability of avidin. The high binding capacity between avidin and biotin can cause vitamin B_7 deficiency (Green, 1975). Biotin plays a significant role in metabolic processes of humans and the growth of microorganisms (Elo et al., 1980; Green, 1990; Maeda et al., 1996). For example, biotin deficiency in human may increase plasma ammonia concentration which causes 'hyperammonemia' (Maeda et al., 1996). Lack of biotin can remarkably inhibit the growth of microorganisms (Elo et al., 1980).

Studies on the inactivation of avidin have been found in the literature primarily exploring ways for improving the absorption and digestion of biotin in the body. The majority of avidin is found to be inactivated by thermal processing at 121℃ for 25 min (Durance & Wong, 1992). Another finding showed avidin can be completely denatured when it was subjected to heat treatment at 100℃ for 10 min or a high-pressure processing at 700 MPa at 50℃ (Ajaypal Singh & Ramaswamy, 2014). However, it is hard to remove avidin when egg is normally cooked like frying and boiling (Durance & Wong, 1992). Besides, avidin is difficult to be inactivated by enzymatic hydrolysis such as pronase and trypsin (Green, 1975). In terms of secondary structure, a study carried out using FTIR

showed that avidin highly transited to an unfolded state with the loss of ordered secondary structures over 75℃ (Swamy et al., 1996).

The relation between conformation and functional properties of protein is important in food industry. There are various techniques that can get conformational information of proteins such as Circular Dichroism (CD) spectroscopy, Fourier Transformation Infrared Spectroscopy (FTIR), Nuclear Magnetic Resonance Imaging (NMR). Nonetheless, in order to study how the proteins behave during food processing, molecular dynamics (MD) simulation is now used in food process engineering. It can provide information about the molecular interactions that take place in a physical system (Ashutosh Singh et al., 2017). MD was first developed to study structural and dynamic properties of biomolecules and finds its application in pharmaceutical industry such as drug delivery. In recent years, MD began to be applied for the study of food proteins.

In this study, the changes of secondary structures in avidin after the application of thermal stresses and oscillating electromagnetic field (microwave processing - 2450 MHz) were evaluated. The quantification of conformational changes in avidin were done by evaluating root mean square deviation, radius of gyration, and dipole moment. Apart from this, the surface properties have also been examined.

4.3 Materials and Methods

MD simulations were carried out by the classical MD algorithm implemented in GROningen MAchine for Chemical Simulations (GROMACS) software package, version 5.0.4 from the Stockholm Center for Biomembrane Research, Stockholm, Sweden (Van Der Spoel et al., 2005). Avidin, an anti-nutrient in egg white, are comprised of 128 amino acid residues per monomer, where 49% of secondary structures contains beta sheet and there are only 4% helical structures inside. Avidin molecule contains an eight-stranded antiparallel β-sheet connected by loops

numbered according to the N–C direction: L1-2, L2-3, L3-4, L4-5, L5-6, L6-7, and L7-8. Avidin classified as glycoprotein with PDB code 5CHK was used in this study (Strzelczyk & Bujacz, 2016). The CHARMM27 was selected for protein and the TIP3P water model was used for solvent (Astrakas, Gousias, & Tzaphlidou, 2012).

Avidin was put in a periodic water box of dimensions 7.799 × 7.799 × 7.799 containing 15089 water molecules to satisfy the minimum image convention. After the system was neutralized, it was minimized for energy with converging criterion of maximum force value of 10 kJ/nm/mol using steepest descent for 20000 steps followed by 200 ps equilibrations to the constant temperature and constant volume (NVT) and the constant temperature and constant pressure (NPT). During the MD simulation, the Berendsen thermostat was used to maintain the desired temperatures and the Parrinello-Rahman barostat was applied to set the pressure at 1 bar. Eight MD simulations were run for 2 ns to evaluate the effects of temperature (300K, 333K, 343K and 353K) and external oscillating electric field with the intensity of 0.05 V/nm and frequency of 2450 MHz. All the electric fields were applied at the X axis of the equilibrated solvent protein system. The electric field conditions were set for simulations of microwave processing. VMD software was employed to obtain structural diagrams and STRIDE algorithm implemented inside was utilized to characterize the changes after thermal processing and microwave treatment (Humphrey, Dalke, & Schulten, 1996). Several parameters such as root mean square deviation (RMSD) of backbone atoms, radius of gyration (Rg) and surface hydrophobicity and hydrophilicity were studied using GROMACS analyzing tools. VMD was also utilized to take snapshots of protein conformational changes.

4.4 Results and Discussions

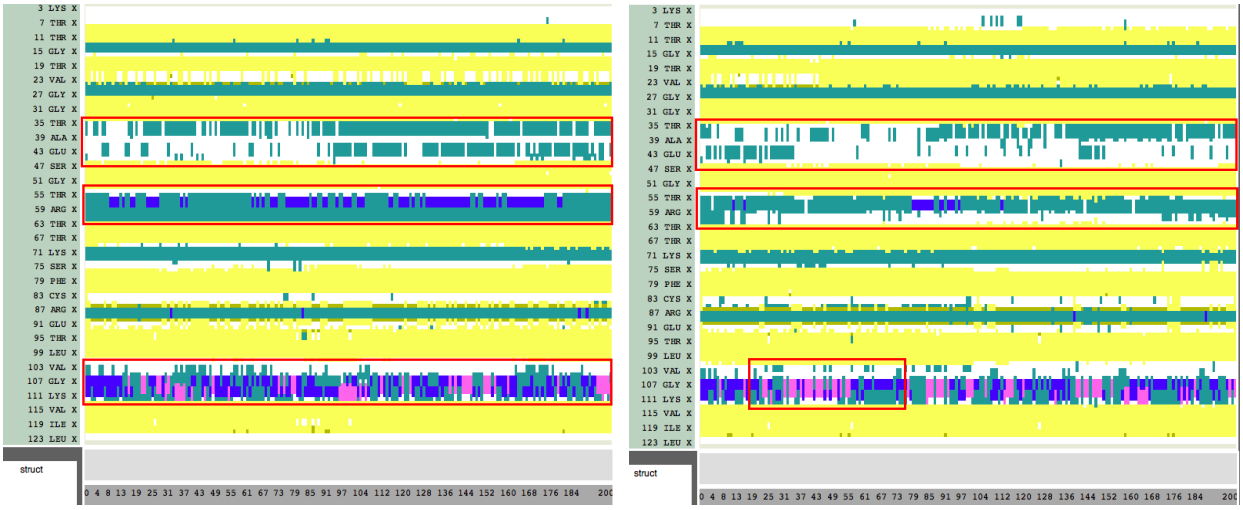
4.4.1 Secondary structure analysis

The effects of temperatures and external electric fields on the conformational properties were analyzed by STRIDE algorithm implemented in VMD software (Version 1.9.3). This analysis can simulate the secondary structural changes occurred corresponding to the time when avidin was subjected to thermal stresses or external electric fields.

The changes that take place within the protein structure under thermal stress is presented in Fig. 4.1 and under oscillating electric field as shown in Fig. 4.2. It was observed that significant disruptions between residue 39-43 were caused by external thermal stresses as temperature increased from 300 K to 333 K. Besides, turns tend to convert into coils in this region except for 343 K. Between residue 55 and 59, 3/10 helix was reducing as elevated temperatures caused turns and 3/10 helix converted into a coil at 353 K. Turns were observed between residue 59-63 at 300 K, 343 K and 353 K while there were some disruptions at 353 K. Besides, it is interesting to find that these residues exhibited coil conformation at 333 K, which was not observed at other temperatures. Coils began to convert into extended configuration at 353 K, which is a β-sheet between residue 84-87. As the temperature increased, a significant change was noticed between residue 103 and residue 111. There was a transformation from 3/10 helix and turns into α-helix. As processing time increased, it tended to exhibit helical structures. However, in the case of external electric field, our observations were significantly different. Between residue 103-111, α-helix was found to transform to turns and 3/10 helix at elevated temperatures. There was a conversion from turns to coils between residue 35-43 as temperature increased 300 K to 353 K.

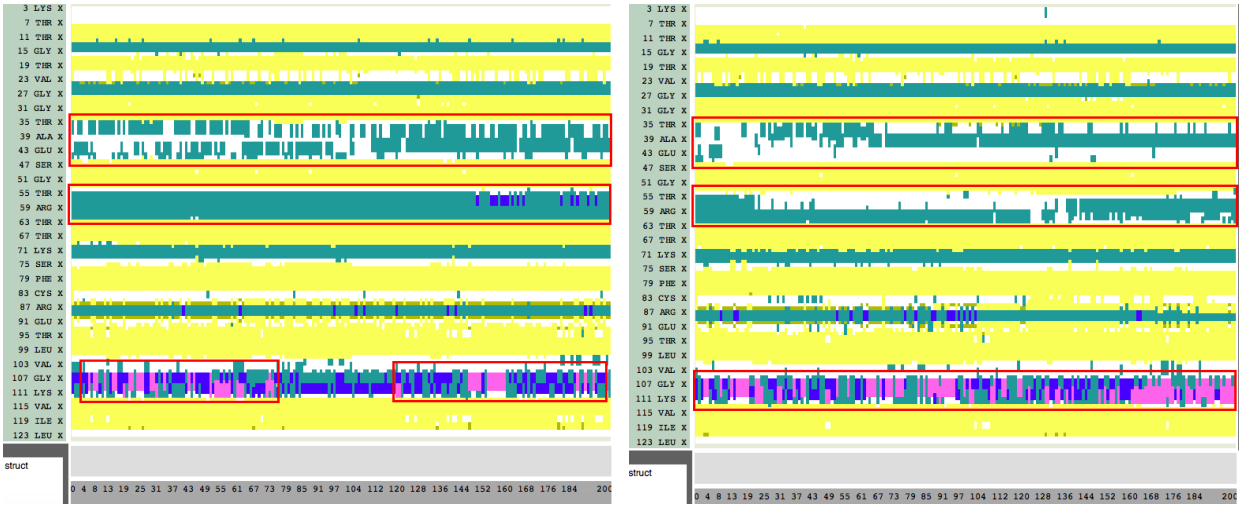
(a) 300K

(b) 333K



(c) 343K

(d) 353K



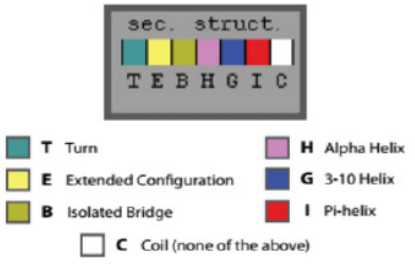


Figure 4.1. STRIDE analysis showing the evolution of 5CHK without electric field (a) 300 K, (b) 333K, (c) 343K, (d) 353K.

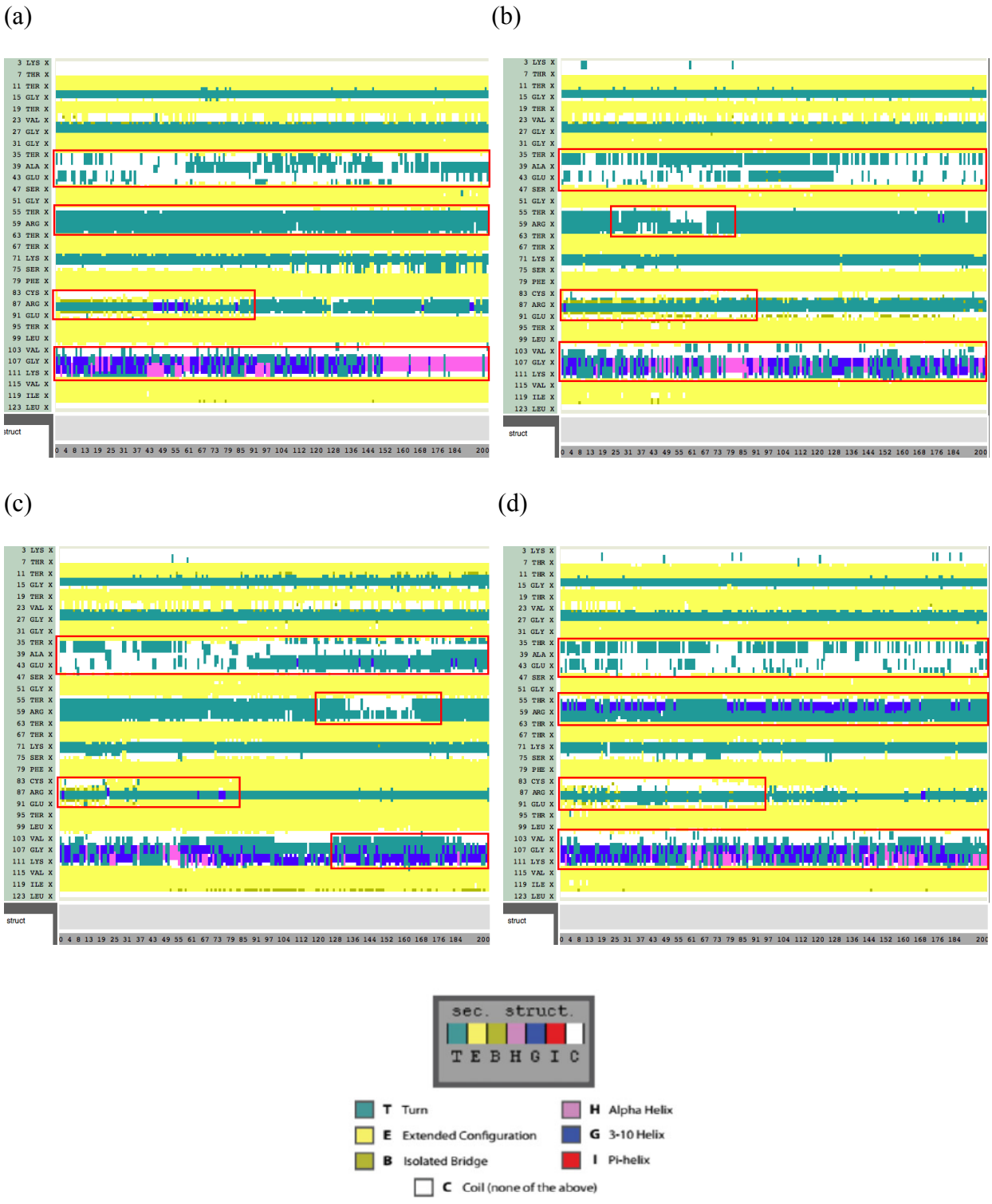


Figure 4.2. STRIDE analysis showing the evolution of 5CHK with oscillating electric field of 0.05 V/nm, 2450 MHz (a) 300 K, (b) 333K, (c) 343K, (d) 353K.

It tended to exhibit coil properties between residue 55-63 at 333K and 343 K but exhibit 3/10 helix conformation at 353 K with electric fields. In this region, some disruptions in turns were also observed at 333 K and 343 K. When avidin was subjected to the external electric fields, 3/10 helix was present at 353K between residue 55 and 59 but was absent at other temperatures. Between residue 83-91, coils converted into β-sheet as the processing time raised which was similar to the deviations observed during thermal processing.

Comparing Fig. 4.1(a) to Fig. 4.2(a), the application of external electric fields at 300 K lead to a conversion of turns and 3/10 helix into α-helix between residue 103 and 111, but reverse transformation occurred at 353 K. Between residue 83-87, coils existed at 300 K, 333 K and 343 K. However, coils were disappeared to transform into β-sheets at 353 K as time increased. Similar observation was noted with external electric fields. Our finding corresponds to the study that disruptions in region of turns or coils were observed in egg white lysozyme under electromagnetic fields (Frishman & Argos, 1995; Solomentsev, English, & Mooney, 2010). The loss of helical structure was also observed in insulin with the application of oscillating electric field of intensity lower than 10^8 V/m (Akin Budi, Legge, Treutlein, & Yarovsky, 2005). Movement of turns between residue 35-47 have accordance with the study of Legge et al (2006). When the temperature increased, turns were found to shift in this region, reinforcing the flexible position of turns (Legge, Budi, Treutlein, & Yarovsky, 2006).

4.4.2 Root mean square deviation (RMSD)

By calculating the root mean square deviation, the arithmetical value of the deviations in the protein structures compared to the control (without any external stress) can be obtained (Vagadia et al., 2016; Vanga et al., 2015). The RMSD is determined by using the following formula (Ashutosh Singh, Orsat, & Raghavan, 2013):

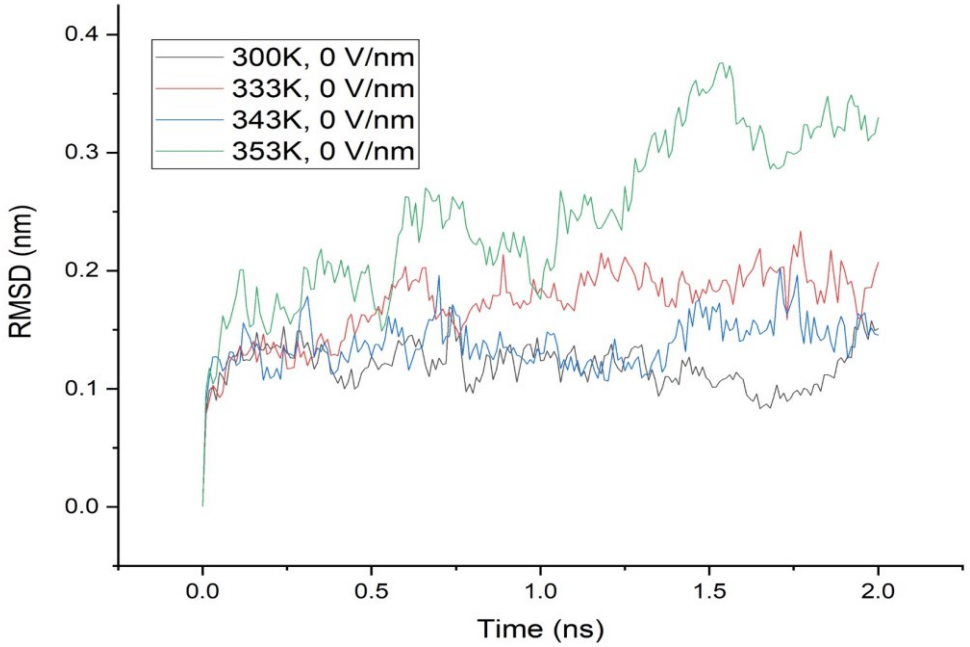
$$RMSD = \sqrt{\frac{1}{N}\sum_{i=1}^{N}\left|r_{final}(i) - r_{initial}(i)\right|^2} \quad (4.1)$$

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

The average RMSD values for 2 ns simulation are presented in Table 4.1 and the variations can be observed in Fig. 4.3. It can be noted that temperature plays an important role in the RMSD values. With respect to reference (300 K), all the RMSD values increased at other temperatures, especially at 353 K. The increase of RMSD values is caused by the loss of secondary structures of protein, further resulting in protein unfolding. For oscillating electric field, there were no significant effects on the RMSD levels at 333 K but a remarkable increase at 343 and 353 K was observed in comparison to the reference (300 K). Similar results were observed by Vanga et al. (2015) and Vagadia et al. (2016) where Ara h 6 and soybean trypsin inhibitor were used for study, respectively. There are significant fluctuations in RMSD values both with or without external electric field at higher temperatures which may be caused by the alteration in the orientation of the protein. Amino acids form turns via hydrogen bonds which may also contribute to fluctuation in values (Ashutosh Singh, Munshi, & Raghavan, 2013). These observations confirm that temperature and external electric field can influence the secondary structures of avidin. Chignolin's structure is found to be destabilized with the introduction of oscillating field due to its increased RMSD values (Astrakas et al., 2012). Regarding to lysozyme, the RMSD values significantly increased when electric field with the intensity of 0.05 V $Å^{-1}$ (0.5 V/nm) was applied (English, Solomentsev, & O'Brien, 2009). A study showed that RMSD values fluctuating around a plateau indicated that the protein oscillates within a free energy basin. The fluctuation of RMSD values can also be observed in samples treated with electric field in our study (Su, Xu, Li, Chen, & Wang, 2011). The increase of RMSD

values also indicated that deviations in the backbone changed the structure and resulted in relaxed configurations, primarily caused by the rearrangement of hydrogen bonds (English et al., 2009).

(a)



(b)

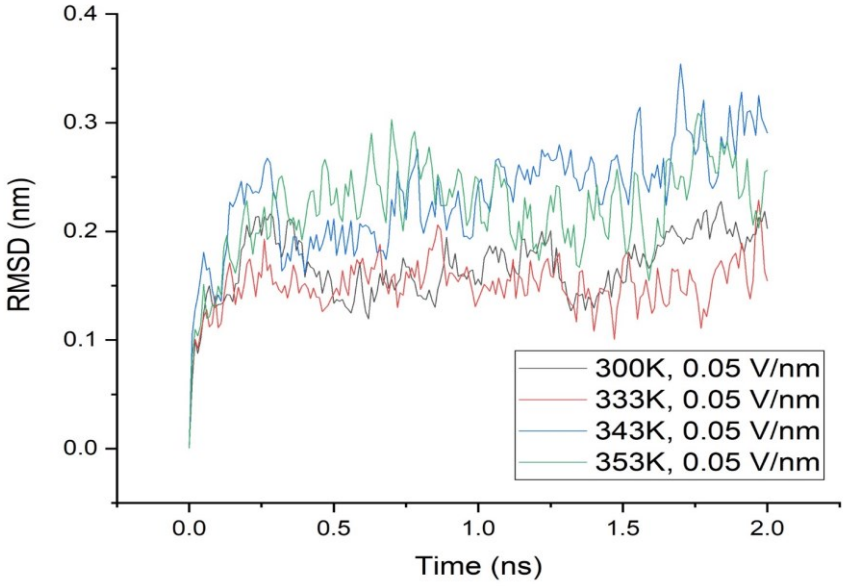


Figure 4.3. RMSD variations observed in avidin (a) thermal processing (b) thermal processing + oscillating electric field

Table 4.1. RMSD values of avidin

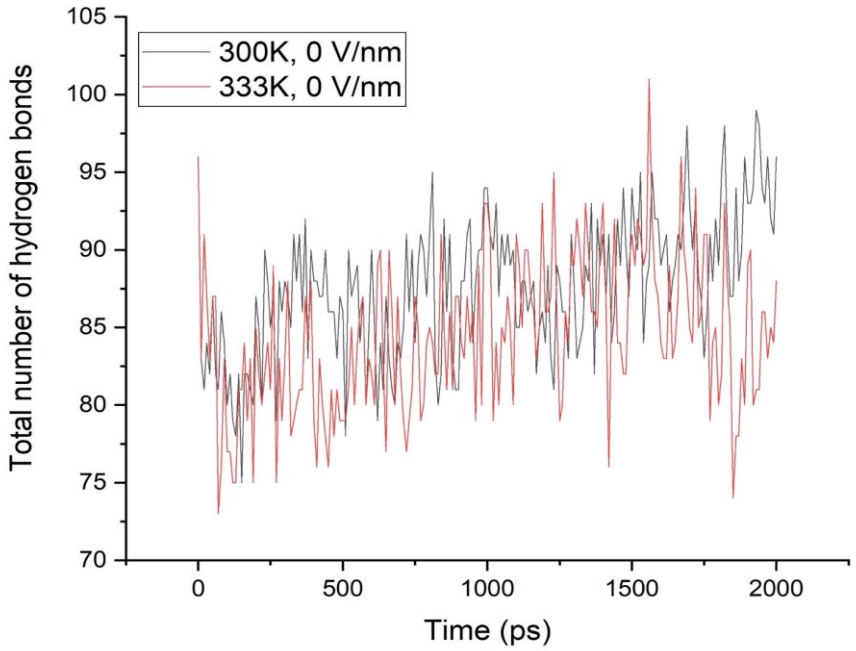
Temperature (K)	No electric field (nm)	Oscillating electric field (0.05 V/nm, 2450 MHz) (nm)
300	0.119±0.019	0.168±0.031
333	0.171±0.031	0.151±0.023
343	0.138±0.021	0.234±0.047
353	0.249±0.068	0.221±0.042

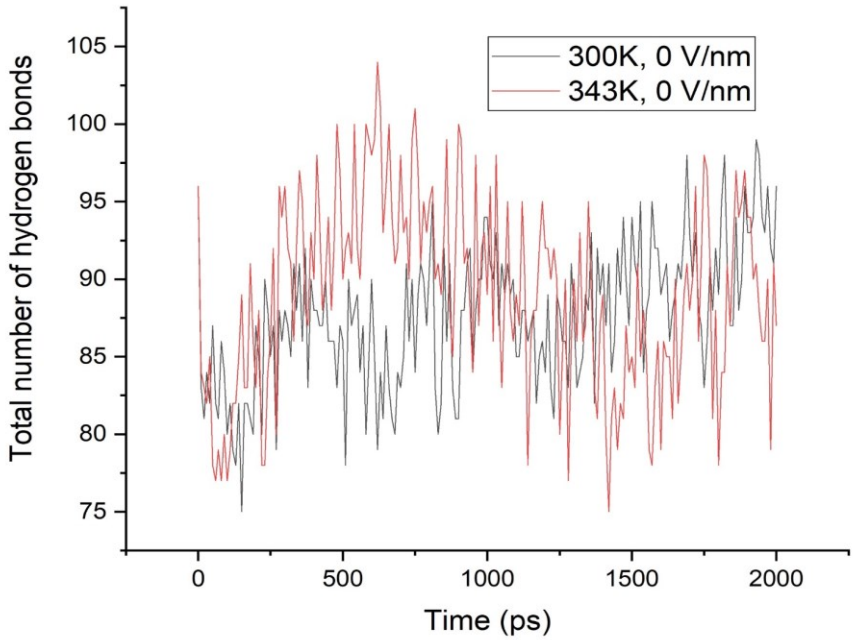
4.4.3 Hydrogen bonds

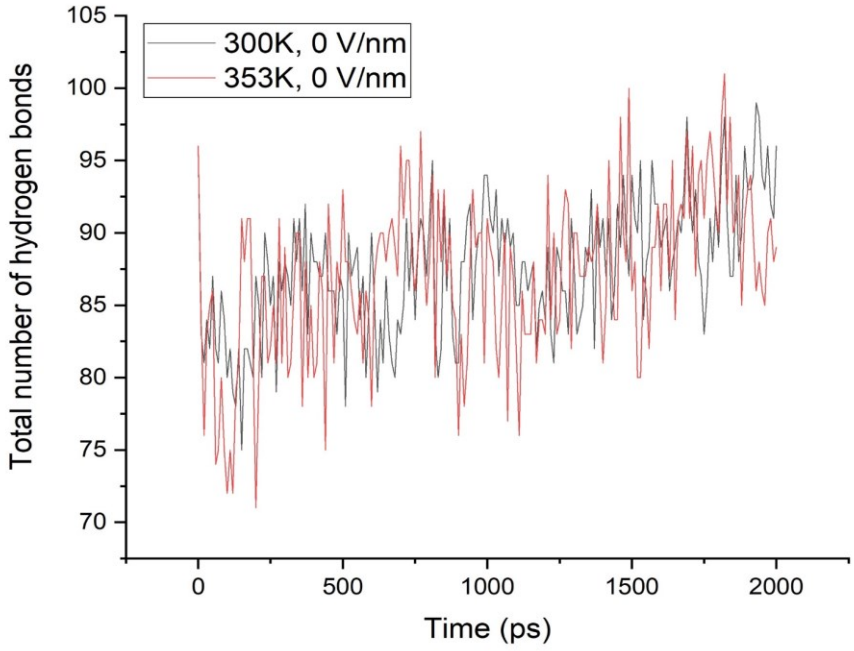
Hydrogen bonds play a key role in structural and functional properties of proteins such as folding, molecular dynamics and enzymatic activity. The reduction of number of hydrogen bonds due to external stress can lead to destabilization of secondary structures that could lead to protein unfolding (Budi, Legge, Treutlein, & Yarovsky, 2004; Ashutosh Singh, Munshi, et al., 2013). The number of hydrogen bonds in proteins and helical structures formed during 2 ns simulation with or without external electric field stress at three temperatures are presented in Fig. 4.4 and 4.5, respectively and the averages are summarized in Table 4.2 and 4.3, respectively. It is interesting to note that significant variations were observed between the number of hydrogen bonds with or without external electric field. However, the number of hydrogen bonds in helical structures was remarkably reduced at elevated temperature both with and without electric fields, which is in a good agreement that α-helix was reduced with the rise of temperature. This corresponds to the finding of Budi et al. that hydrogen bonds become unstable under the thermal stress (Budi et al., 2004). The numbers of hydrogen bonds in protein and helical structures under electric field are both less than those which were subjected to thermal processing only. This shows that oscillating

electric field has more significant effects on changing secondary structures of avidin protein. Astrakas et al. (2011) observed the destruction of hydrogen bonds with the application of high electric field, suggesting the protein was unfolding. English & Mooney reported that the secondary structures of lysozyme changed remarkably when it was subjected to the electric field and suggested that the denaturation of protein is related to the breakage of intra-protein hydrogen bonds and alignment of total dipole moment (English & Mooney, 2007). Another study conducted on lysozyme showed that the hydrogen bonds in core undergo reversible perturbation like breakage and reformation under electromagnetic fields while those exposed to solvent were perturbed irreversibly and experienced dipole alignment (Solomentsev et al., 2010). Legge et al., reported the transition of α- helix into π- helix in the structure of insulin B chain due to loss of hydrogen bonds in helical structure during the simulation which was also observed in our study (Legge et al., 2006).

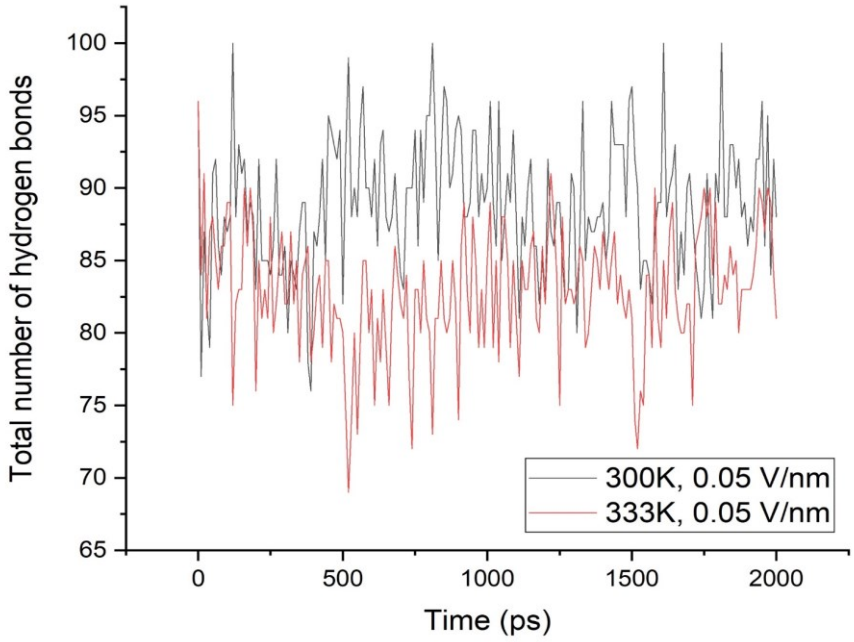
(a)

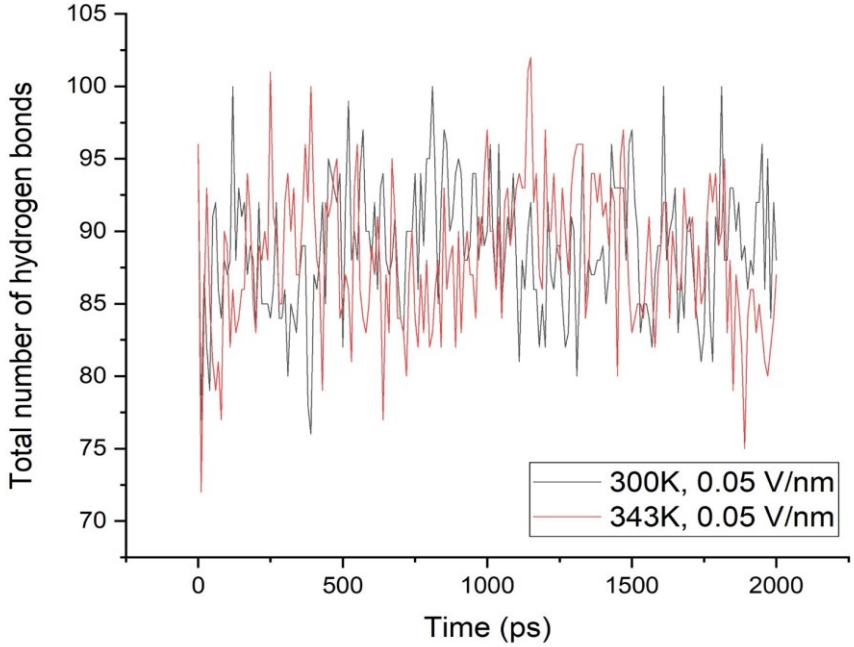



300K, 0 V/nm
343K, 0 V/nm
Total number of hydrogen bonds
105
100
95
90
85
80
75
0
500
1000
1500
2000
Time (ps)


300K, 0 V/nm
353K, 0 V/nm
Total number of hydrogen bonds
105
100
95
90
85
80
75
70
0
500
1000
1500
2000
Time (ps)

(b)


Total number of hydrogen bonds
100
95
90
85
80
75
70
65
0
500
1000
1500
2000
Time (ps)
300K, 0.05 V/nm
333K, 0.05 V/nm


Total number of hydrogen bonds
105
100
95
90
85
80
75
70
0
500
1000
1500
2000
Time (ps)
300K, 0.05 V/nm
343K, 0.05 V/nm

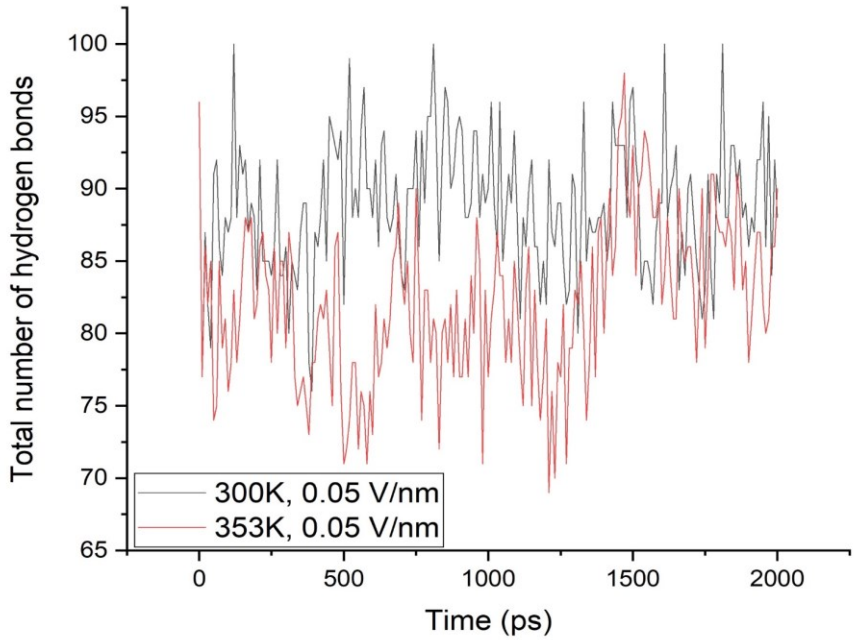


Figure 4.4. Total number variations of hydrogen bonds observed in avidin (a) under thermal processing (b) under external electric field.

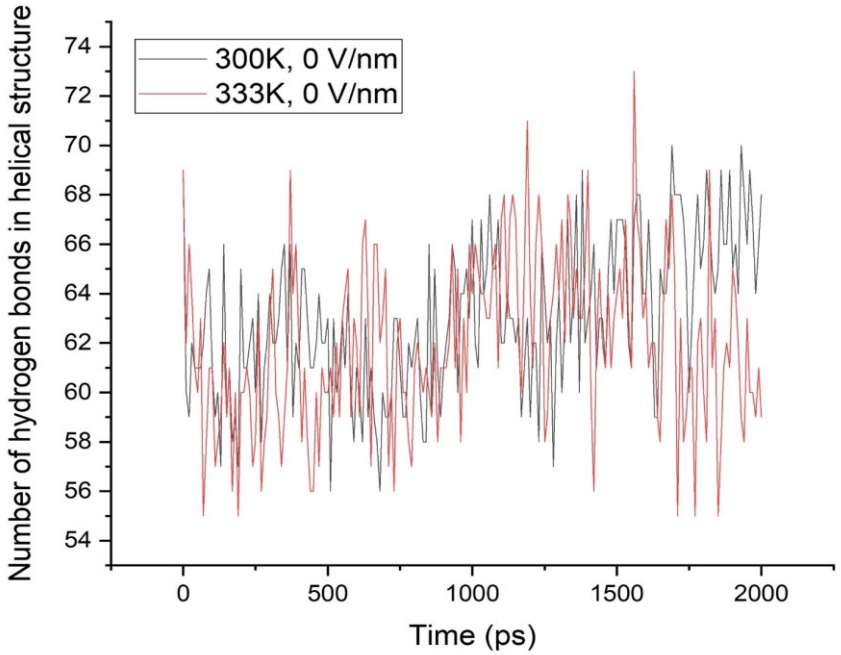
Table 4.2. Total number of hydrogen bonds

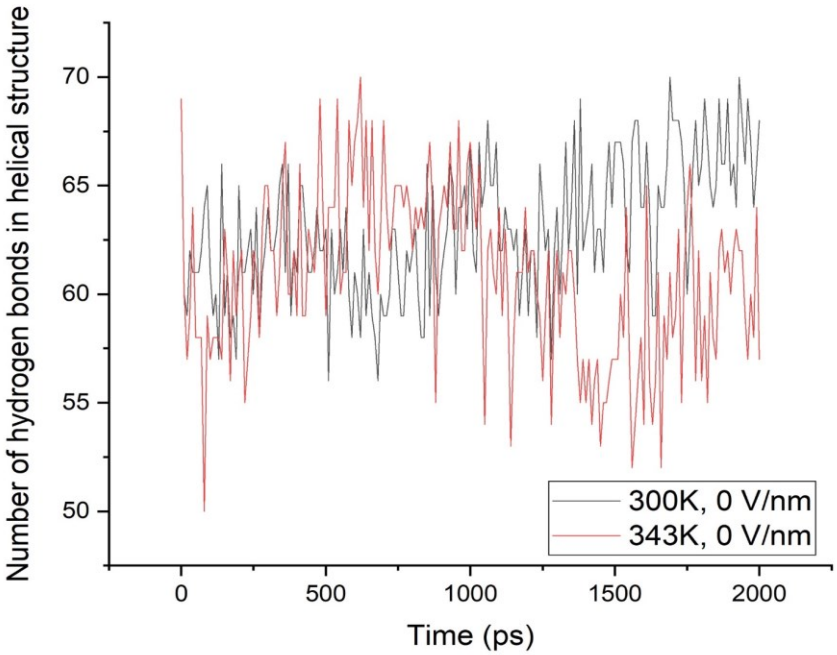
Temperature (K)	No electric field (nm)	Oscillating electric field (0.05 V/nm, 2450 MHz) (nm)
300	87.632±4.488	88.841±4.557
333	84.448±4.898	82.930±4.221
343	89.075±6.116	88.234±4.999
353	86.975±5.655	82.164±5.509

Table 4.3. Number of hydrogen bonds in helical structure

Temperature (K)	No electric field (nm)	Oscillating electric field (0.05 V/nm, 2450 MHz) (nm)
300	63.025±3.106	63.139±3.105
333	61.930±3.421	59.269±3.438
343	60.796±3.857	61.612±3.779
353	60.597±3.365	58.841±3.888

(a)




Number of hydrogen bonds in helical structure
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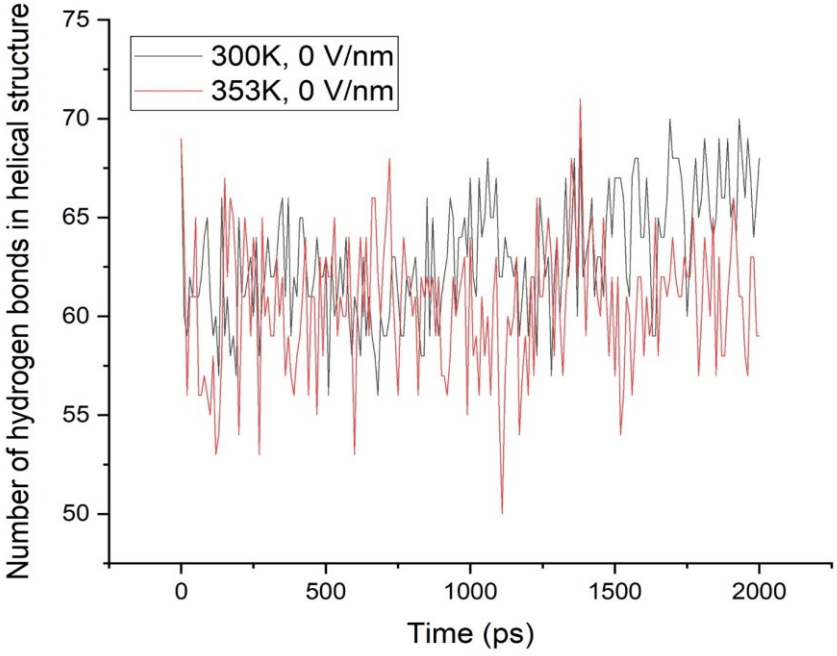
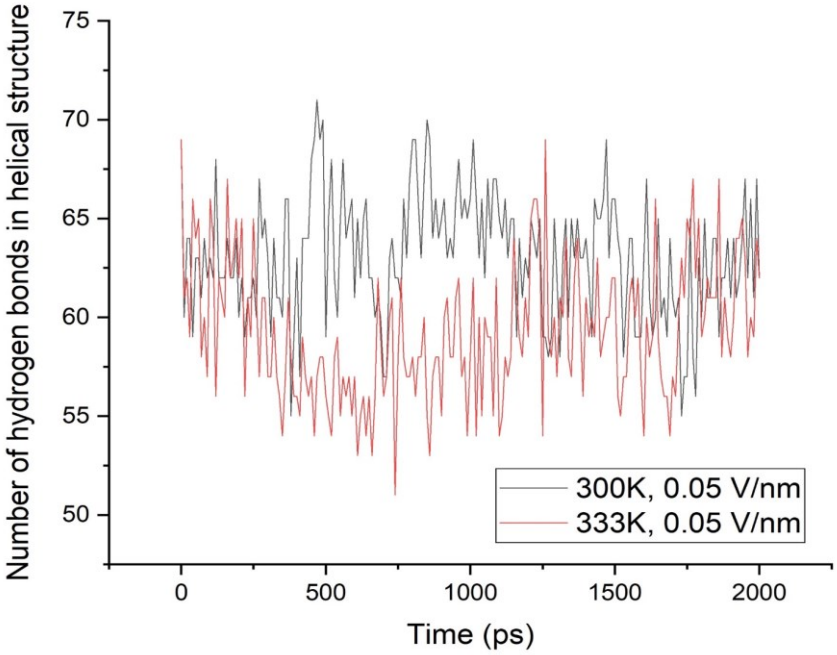
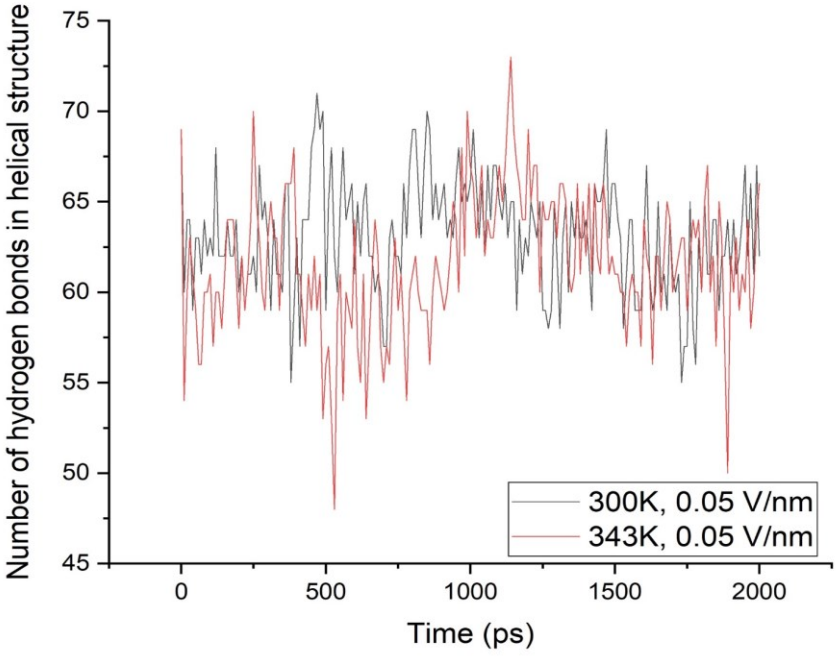
65
60
55
50
0
500
1000
1500
2000
Time (ps)
300K, 0 V/nm
343K, 0 V/nm


Number of hydrogen bonds in helical structure
75
70
65
60
55
50
0
500
1000
1500
2000
Time (ps)
300K, 0 V/nm
353K, 0 V/nm

(b)


Number of hydrogen bonds in helical structure
75
70
65
60
55
50
0
500
1000
1500
2000
Time (ps)
300K, 0.05 V/nm
333K, 0.05 V/nm


Number of hydrogen bonds in helical structure
75
70
65
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55
50
45
0
500
1000
1500
2000
Time (ps)
300K, 0.05 V/nm
343K, 0.05 V/nm

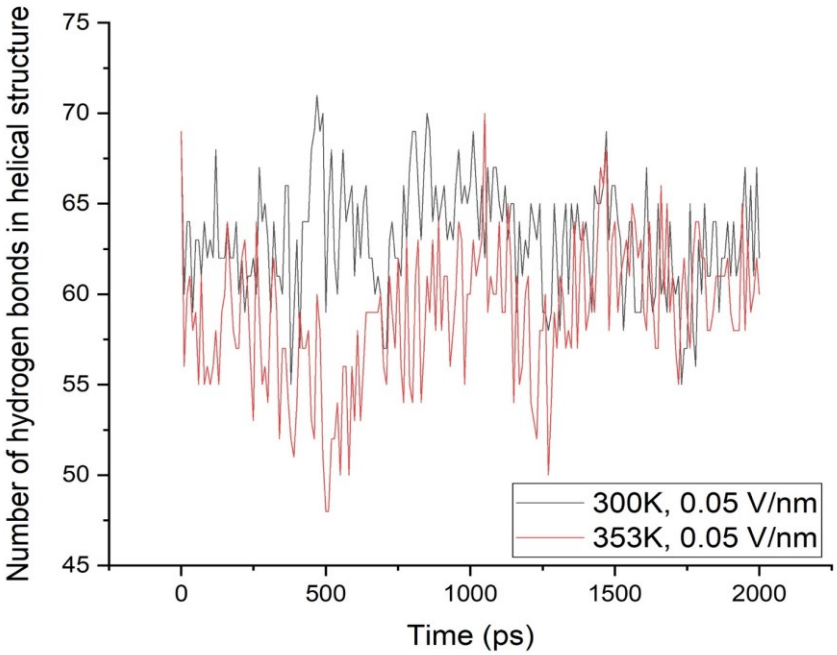


Figure 4.5. Number variations of hydrogen bonds in helical structure observed in avidin (a) under thermal processing (b) under external electric field.

4.4.4 Radius of gyration (Rg)

The Radius of gyration measures the distribution of the atoms according to their center of mass (Budi et al., 2004; Vanga et al., 2015). The changes in shape and size of the protein under external stresses can be evaluated by calculating Rg. It can be represented as (Ashutosh Singh, Orsat & Raghavan, 2013):

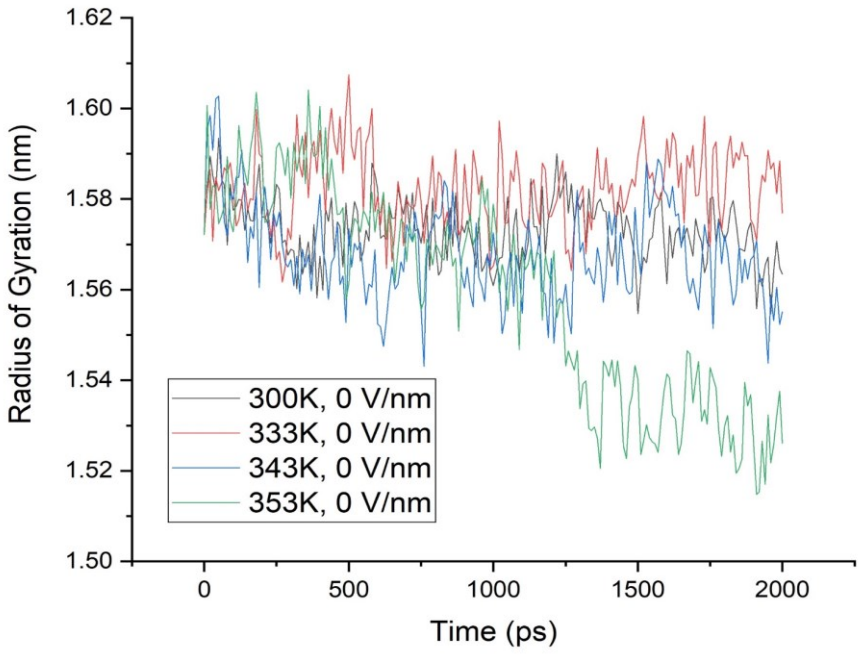
$$Rg = \sqrt{\frac{1}{N}\sum_{i=1}^{N}|r(i) - r_{center}|^2} \qquad (4.2)$$

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

According to Fig. 4.6, there was an obvious trend that the Rg values of avidin declined with increased temperatures. Radius of gyration is reported to have correlation with the compactness of atomic packing. The reducing Rg values (Table 4.4) observed in this study means the structure is

more packed at higher temperatures. However, Rg values of the protein under external electric field showed a slight increase at 343 K and 353 K compared to the control (300 K). The phenomenon can be explained by loss of secondary structures as Rg values increase and further resulting in protein unfolding at high external stresses (Ashutosh Singh, Orsat & Raghavan, 2013). Mild alteration of Rg values was observed in chignolin when low intensity fields at 0.1 and 0.25 V/nm were applied, which is similar to the results observed in our study (Astrakas, Gousias, & Tzaphlidou, 2011). In the case of aβ-Amyloid Peptide, the protein was found to be more compact without electric field (Toschi, Lugli, Biscarini, & Zerbetto, 2008). The variance of Rg values with the exposure to oscillating electric field can be caused by the protein realigning due to the changed field direction (Akin Budi et al., 2005).

(a)



(b)

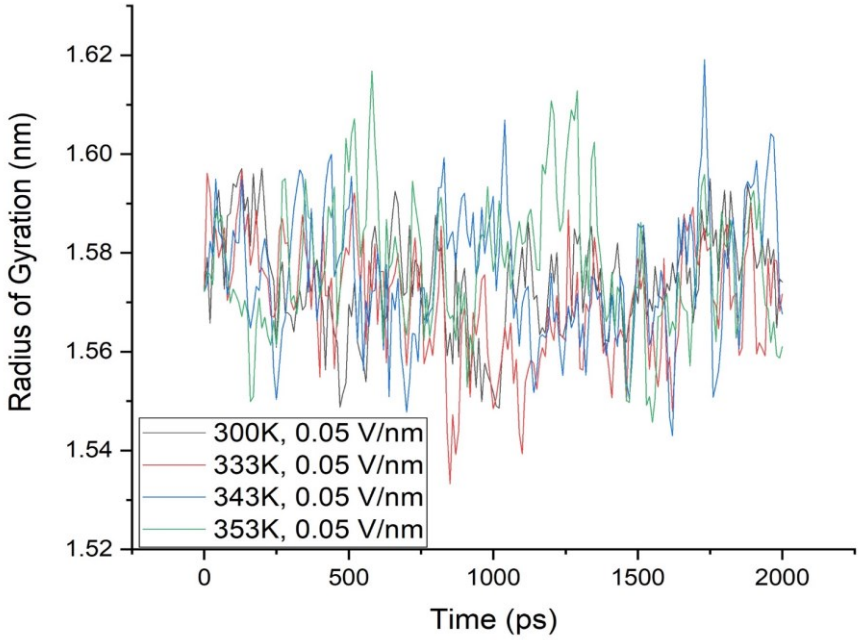


Figure 4.6. Rg variations observed in avidin (a) thermal processing (b) thermal processing + oscillating electric field

Table 4.4. Radius of gyration

Temperature (K)	No electric field (nm)	Oscillating electric field (0.05 V/nm, 2450 MHz) (nm)
300	1.573±0.007	1.574±0.011
333	1.582±0.008	1.570±0.012
343	1.568±0.010	1.576±0.014
353	1.559±0.023	1.578±0.014

4.4.5 Solvent Accessible Surface Area

The functional properties of protein are significantly determined by the surface characteristics. The secondary structures play an important role in surface properties Thus, the alteration to secondary structures will cause changes of surface properties and further lead to functional changes of protein. SASA is defined as the surface area that is available to interact with solvents and other molecules. The solvent accessible surface area can evaluate the effect of structural changes on the protein accessibility to solvent and potentiality to ligands. (Budi et al., 2004; Ashutosh Singh, Munshi, et al., 2013). SASA can be calculated by the following equation (Ashutosh Singh, Orsat & Raghavan, 2013)

$$SASA = A = \sum(R/\sqrt{R^2 - Z_i^2}) \times D \times L_i \qquad (4.3)$$

Where, L_i is the length of the arc drawn on a given section i; Z_i is the perpendicular distance of section i from the center of the sphere.

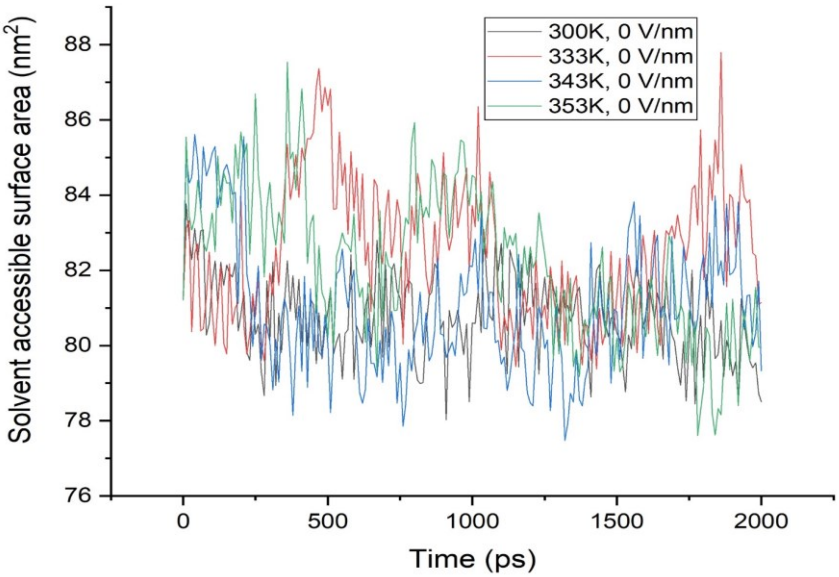
Table 4.5 summarized the average SASA values observed during the simulation of avidin molecule. From Fig. 4.7, an obvious increase in SASA values was noticed when the temperature raised form 300 K to 333K, which indicates that new binding sites occurred on the surface of the molecule. Similar results were observed by Vagadia et al. (2016). The value reduced from 333 K to 343 K and increased again at 353K. Similar fluctuation in SASA values were observed when oscillating electric field was applied. A significant rise in SASA value was noticed only at 353 K with the application of external electric fields. The SASA value with oscillating electric field is higher compared to that of the value with only application of thermal stress at all the temperatures except for 333 K. This finding is consistent with the other previous results. Budi et al. reported that severe disruptions in the secondary structures of protein were caused by oscillating electric field (Akin Budi et al., 2005). The slight decrease of SASA value under thermal stress from 333 K to 343 K

and under oscillating electric field from 300 K to 333 K may attribute to slightly more compactness of unfolding structures with the less accessibility of protein to the solvent (Budi et al., 2004). Another possibility is that there is a small proportion of α-helix in structure of avidin. α-helices have their own dipole moment and tend to orient themselves under external electric field, leading to conformational alteration of protein (Vagadia et al., 2016). The eight-stranded antiparallel β-sheet in the avidin molecule is attributed to its relatively stable structure. A total of 7.7% change in SASA values was reported for insulin chain B under oscillating electric field at the frequency 2.45 GHz (Akin Budi, Legge, Treutlein, & Yarovsky, 2007). In present study, 3.7% of solvent accessible surface area were increased. Compared to thermal stress, oscillating electric field have revealed larger variations in regards to SASA which was similar to insulin chain B (Akin Budi et al., 2005). Legge et al. also studied SASA of tryptic peptide of apoC-II by dividing into four systems: lipid, α-helix, x-strand and β-strand (Legge, Treutlein, Howlett, & Yarovsky, 2007). They found that external stresses resulted in SASA value deviations, especially in the β-strand system. The variance of SASA values in our study with the time evolution can be due to changes in the β-sheets present in the avidin's structure.

Table 4.5. Solvent accessible areas of avidin

Temperature (K)	No electric field (nm^2)	Oscillating electric field (0.05 V/nm, 2450 MHz) (nm^2)
300	80.778±1.118	82.237±1.251
333	82.506±1.826	80.952±1.671
343	81.016±1.762	82.547±1.519
353	82.187±1.940	85.272±2.209

(a)



(b)

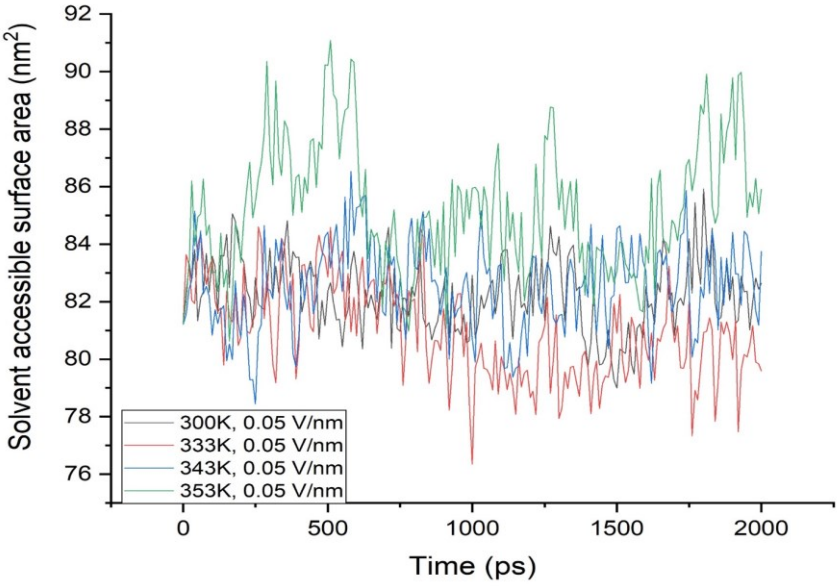
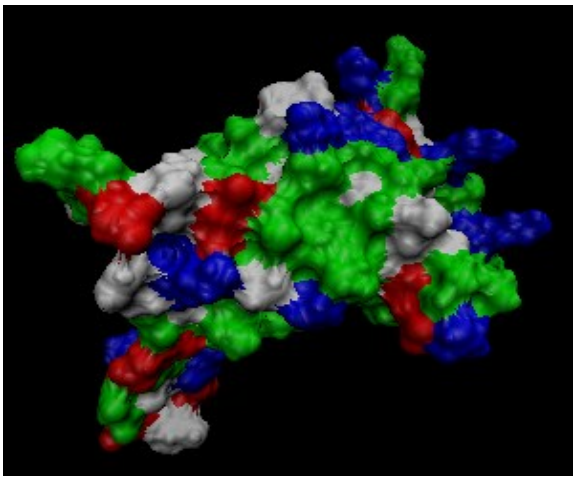
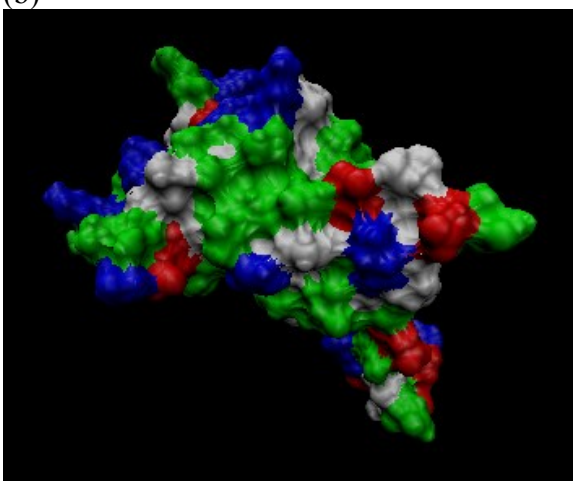


Figure 4.7. SASA variations observed in avidin (a) thermal processing (b) thermal processing + oscillating electric field

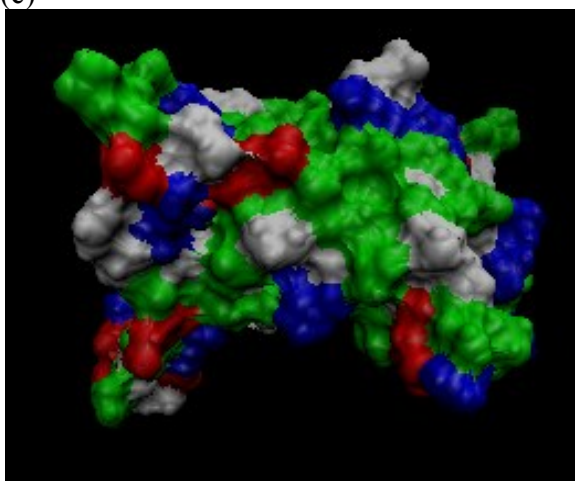
(a)



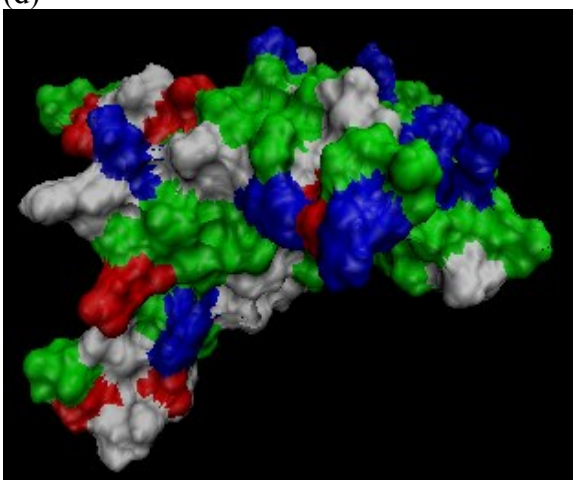
(b)



(c)



(d)



(e)

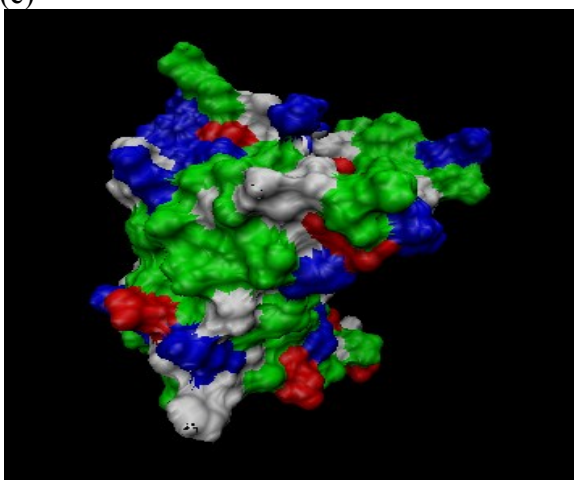
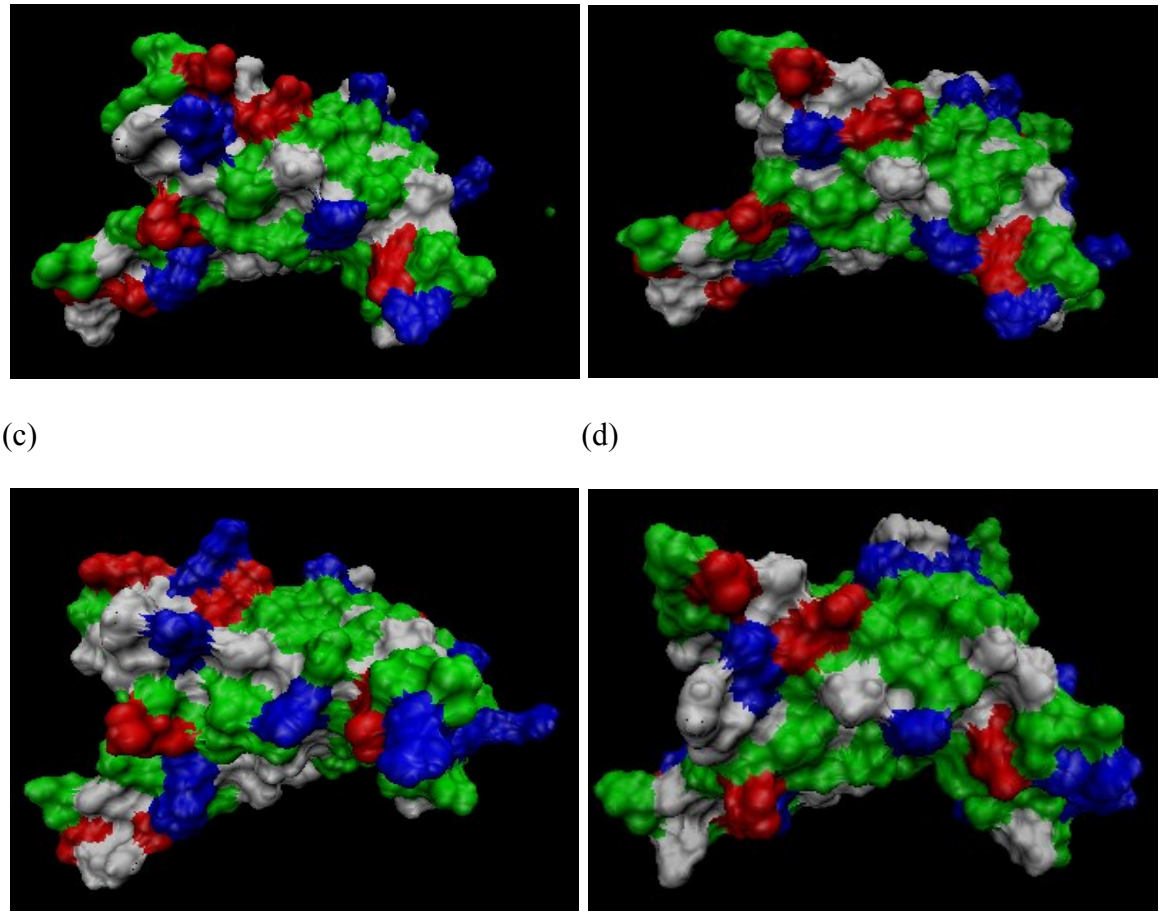


Figure 4.8. Snapshot of surface properties of avidin under thermal stress. (a) molecule with no external stresses (b) molecule at the end of 300 K; (c) molecule at the end of 333 K; (d) molecule at the end of 343 K; (e) molecule at the end of 353 K. Note: polar residues (green), non-polar residues (white), basic (blue) and acidic (red).

(a) (b)



(c) (d)

Figure 4.9. (a) molecule at the end of 300 K and oscillating electric field (0.05 V/nm, 2450 MHz); (b) molecule at the end of 333 K and oscillating electric field (0.05 V/nm, 2450 MHz); (c) molecule at the end of 343 K and oscillating electric field (0.05 V/nm, 2450 MHz); (d) molecule at the end of 353 K and oscillating electric field (0.05 V/nm, 2450 MHz).

Fig. 4.8(a) shows the structure of avidin under non-external stresses. Some residues which are protruded out of the molecule began to revert back and joined in with the core of the molecule as the temperature increased from 300 K to 353 K (Fig. 8(b), (c), (d) & (e), respectively) and in the end of the simulation at 353 K, almost all the protruded parts joined into the core structure of the molecule, leading to the conformational alteration of the protein. Fig. 4.9 presents the structure of avidin under oscillating electric field at four temperatures. With the temperature increased, the obvious unfolding of the molecule can be noticed. The overall increase in the surface area of the avidin molecule corresponds to this observation of unfolding. In general, the hydrophobic residues

of a protein are located inside the molecule to reduce exposure to solvents. However, the protein may change its conformation with the application of external stresses such as thermal and electric field, resulting in more exposure of hydrophobic residues to solvents (Ashutosh Singh, Orsat & Raghavan, 2013). Further, this phenomenon can lead to protein unfolding.

4.5 Conclusion

In the present study, the effect of external stresses including thermal and oscillating electric fields at 2450 MHz and 0.05 V/nm strength on the structure of avidin was determined. It showed that the number of hydrogen bonds in protein can fluctuate with the application of external stresses. With the increase of temperature, the number of hydrogen bonds in helical structure decreased. This alteration in the hydrogen bonds reduces the stability of the molecule influencing the root mean square deviation and radius of gyration values, leading to conformational changes. Our observations indicate that oscillating electric field is more effective than heat on the structure of avidin for denaturation/inactivation resulting in the protein unfolding during simulation. More studies about evaluating the effect of external stresses on the secondary structure and functional properties of avidin need to be done. Study of MD simulation under external electric field provides information about the effect of novel food processing techniques like microwave and pulsed electric field on the behavior of proteins and it can be used for verifying the experimental predictions.

CHAPTER V

SUMMARY AND CONCLUSION

Food allergy has become one of the most important health problems since a great number of people all over the world are suffering from this issue. Proteins are found to trigger allergic reactions in food products. Numerous studies were conducted to alleviate the allergenicity of food. Some studies have shown that changes in the secondary structure may result in alteration of allergenicity. Eggs are the second common cause of allergic reactions. Thus, egg white protein is selected as the center of our project. Avidin present in egg white exhibits anti-nutritional properties, but there are limited studies conducted on it. Therefore, avidin is another focus of our study.

In the review, the structural and functional properties of allergens present in egg white and the anti-nutrient avidin were described in detail. Then, the influences of different processing techniques on the allergenicity and structural properties of several egg white proteins were delineated. The effect of various processing methods on inactivation of avidin was also evaluated.

The first objective of this study was to investigate the effect of microwave and ultrasonic processing on the secondary structure of egg white protein using Fourier transform infrared spectroscopy (FT-IR) technique and Circular dichroism (CD) spectroscopy. FTIR and CD are two common techniques to study changes in secondary structure of the protein. The secondary structures of egg white protein can be changed using these two processing methods. The obvious trend observed in this study is that β-sheet content in secondary structure increased with the decrease of α- helix when increasing the processing time and temperature. However, the secondary structure tended to be almost constant when egg white was subjected to microwave processing at 80°C and processed for 8-12 min using ultrasound.

The second objective was to study the impact of microwave and ultrasonic processing on inactivation of avidin and egg white protein digestibility. The avidin activity declined compared to untreated control when processed using microwave and ultrasound. The maximum inactivation rate of avidin can reach up to 95% for microwave treatment at 80°C for 5 min and 45% for ultrasonic processing of 16 min. It is shown that ultrasonic processing has less effect on inactivation of avidin than microwave treatment, but this work confirmed the effectiveness of microwave and ultrasonic processing on reducing the avidin activity to a certain extent. When egg white was subjected to microwave processing for one minute at three temperatures, protein digestibility increased significantly in comparison to the control. However, the IVPD values decreased as the processing time further increased, suggesting the complete denaturation of protein. For ultrasonic processing, it showed minimal effect on improving protein digestibility.

The third objective was to evaluate the applicability of molecular dynamic simulation for studying the effect of thermal and oscillating field on avidin. The analysis of avidin molecule was carried out using GROMACS software under external stresses including thermal and oscillating electric field. Root mean square deviation (RMSD), radius of gyration (Rg), number of hydrogen bonds and solvent accessible surface area (SASA) were evaluated to study the conformational changes of protein. It can be concluded that avidin undergoes conformational changes in its structure under thermal stress and external electric field. The oscillating electric field is more effective than heat on altering the structure of avidin molecule. With the rise of temperature, number of hydrogen bonds in helical structure reduced with or without electric field. The reorientation of protein was also observed under oscillating electric field. Avidin tended to unfold itself with the application of electric field. It lost ordered structures (especially α- helix) under external stresses including

thermal and electric field. This work has shown that molecular dynamic simulation can be applied to study the protein molecules.

FUTURE WORK

1) Extraction of avidin from egg white and evaluating the effect of electric field on the conformation of avidin secondary structure which can be compared to the simulation results.

2) Isolation of different proteins from egg white and the changes in digestibility of each protein can be evaluated. The functional properties such as foaming ability, emulsifying property of a specific protein can also be studied.

3) Simulation studies can be carried out for extended time like 10 ns- 50 ns and varied intensities for more data.

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